Environmental toxins responsible for the development of Chronic Kidney Disease of unknown etiology (CKDu) in Canacona taluka, Goa

THESIS

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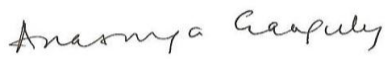
CERTIFICATE

This is to certify that the thesis titled **"Environmental toxins responsible for the development of Chronic kidney disease of unknown etiology (CKDu) in Canacona taluka, Goa"** submitted by **Starlaine Chrizelle Mascarenhas (ID No: 2013PHXF0402G)** for award of Ph.D. of the Institute embodies original work done by her under my supervision.



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Starlaine Chrizelle Mascarenhas

Abstract

This thesis deals with a strange form of Chronic kidney disease, the etiology of which is unlinked to traditional causes like diabetes and hypertension and hence is renamed as Chronic kidney disease of 'unknown etiology' (CKDu). CKDu has been reported since the 1990s in various developing countries of the world like India, Central America, Sri Lanka, and Egypt. CKDu is characterized by progressive, slow, and asymptomatic development affecting younger adults in the age group of 30-50 yrs. This disease mainly targets financially poor rural communities that rely on groundwater for their drinking requirements. Histopathological studies of CKDu have revealed the major pathological manifestation to be chronic tubulointerstitial nephritis (CTN). CTN affects proximal tubular cells (role in toxin elimination) and surrounding interstitial cells of the renal nephron that ultimately manifests in chronic renal failure. This nephropathy is induced by environmental exposure to various nephrotoxins like heavy metals, ochratoxin, aristolochic acid, and more recently silica. Owing to histopathological similarities in CKDu and CTN presentation, environmental nephrotoxins are implicated as potential etiological agents of CKDu.This wave of CKDu has hit an Indian state viz. Goa specifically the Canacona taluka of South Goa district but is least understood as compared to other regional nephropathies reported in developing countries. The electronic media, print and Directorate of Health Services, Goa have constantly reported the rising CKDu incidence for the past 25yrs in Canacona taluka. But this disease's exact prevalence statistics is unavailable till date due to lack of appropriate registries. Troubled by this epidemic, a sole environmental cum biological monitoring study was conducted by a joint team from NIOH (National Institute of Occupational-Health), ICMR (Indian Council of Medical-Research) and Department of Preventive and social medicine of Goa medical hospital, in an attempt to resolve the etiology of CKDu in 2005.This study had analysed the role of 3 environmental nephrotoxins i.e. Cd, As and ochratoxin via exposure through food, water and blood routes in CKDu causation in the taluka, but no conclusive results were obtained (Saiyed et al.,2005). Unfortunately, no follow-up studies were conducted ever since till date to assess the etiological contribution of various other environmental nephrotoxins like heavy metals (lead, cadmium arsenic and mercury), ochratoxin, aristolochic acid and silica in CKDu development in the taluka, resulting in the etiology being unidentified. Hence our group decided to take up the challenge in 2014, of attempting to decipher the etiology of this mysterious form of CKDu noted in the Canacona taluka, hence was the core focus or aim of this thesis.

The sole environmental monitoring study (Saiyed et al., 2005) suffered from major limitations. It failed to establish prevalence statistics, geographical cum demographical distribution of CKDu and potential risk factors or demographic population segments that are highly prone to CKDu development. Moreover, the biochemical based pathological pattern of CKDu presentation which is described for other regional nephropathies in developing countries and is crucial for identification of renal pathology type and manifested disease presentation has not been elucidated for CKDu in Canacona. Hence this thesis attempted to address these limitations, which have been extensively described in Chapter 2. The results from our analyses have shown that CKDu in the taluka was endemic to two villages namely Chaudi and Ponsulem. Demographically, the disease exhibited a lack of traditional causals (diabetes and hypertension) prevalence, no gender or occupational bias and significantly prominent in adults of 30-50yrs of age. Strangely, a high prevalence (55%) of skeletal-disorders and chronic consumption of NSAIDs (a reported nephrotoxin) for pain alleviation was noted among CKDu affected subjects. This finding suggested NSAIDs to possibly contribute in renal damage aggravation noted in the affected population. Additionally various risk factors/predictors of CKDu were identified by statistical analysis of measured demographic, anthropometric and lifestyle characteristics of the study population by determination of significant risk-ratios(R.R). From this

study it was noted that chronic consumption of untreated well-water (R.R-13.78), occurrence of skeletal discomfort (R.R- 7.37),prolonged intake of NSAID's (R.R-7.15), presence of a non-operational mine in the vicinity (R.R-12.48) were identified as significant risk-factors in CKDu development in Canacona. Since the biochemical pattern of disease presentation (i.e. trend of characteristic biomarkers) of CKDu in Canacona was not described till date, this thesis elucidated the same by measurement of biochemical markers specific for two varied origins of renal pathology viz. tubular and glomerular origin in major biological matrices (i.e. blood and urine) of the CKDu affected, general diabetes-hypertensive CKD affected and healthy control study populations, for comparison of the disease presentation pattern in CKDu and general CKD pathology. Tubular damage is mainly characterized by increased urinary excretion of tubular specific protein markers viz. uBCR (urinary b2M to creatinine ratio) and uNCR (urinary NAG to creatinine ratio).Glomerular injury is signified by elevated urinary elimination of glomerular specific markers viz. uPCR (urinary protein to creatinine ratio), uACR (urinary albumin to creatinine ratio), uAPR (urinary albumin to protein ratio) and uAlb/b2M ratio (urinary albumin to b2M ratio).Biochemical cum clinical analyses of CKDu in Canacona reported the occurrence of a tubular proteinuric pattern characterized by significantly increased urinary excretion of tubular nephropathy specific markers viz. uBCR and uNCR along with normal levels of elimination of glomerular injury based markers. This trend was reversed in the general CKD pathology of Canacona. Thus signifying CKDu and CKD in the corresponding endemic and non-endemic regions to be of tubular and glomerular pathological origin respectively. This biochemical pattern of CKDu in Canacona was consistent with the tubular injury based biochemical trend noted in similar CKDu analyses in other affected developing countries. Hence provided confirmatory evidence to the tubular pathological manifestation of CKDu in the taluka, highlighting the potential etiological contribution of environmental nephrotoxins in disease development.

Since environmental nephrotoxins have been reportedly implicated in CKDu causation in similarly affected developing countries, its potential contribution in CKDu etiology of Canacona was comprehensively analysed as well. The environmental nephrotoxins implicated in CKDu development include naturally arising nephrotoxins (from living organisms) like mycotoxin (i.e. ochratoxin) and phytotoxin (i.e. aristolochic acid) along with anthropogenically emerging nephrotoxins(from human activities) like heavy metals (i.e. lead, cadmium, arsenic and mercury).These aforementioned nephrotoxns share a common pathological mechanism of nephrotoxicity i.e. they induce oxidative renal proximal tubular damage which manifests in development of chronic tubulointerstitial nephritis(CTN), the hallmark of CKDu. Recently, growing evidence has emerged for the nephrotoxic potential of a trace geogenic element viz. silica. Silica abundantly constitutes the earth's crust but possesses limited bioavailability hence categorized as a trace geogenic element. It's availability to humans can be rapidly enhanced on anthropogenic disruptions of it's major exposure matrices (i.e. air and groundwater) that can result in increased exposure which ultimately induces severe renal damage. Animal based histopathological analysis of silica induced renal toxicity and a few epidemiological studies in Balkan region (Europe) and Andhra Pradesh (India) have highlighted a causal link between chronic consumption of silica contaminated groundwater and induction of CTN, which is also the pathological presentation of CKDu. These nephrotoxins i.e..heavy-metal, ochratoxin, aristolochic-acid & silica although being ubiquitously present in the environment are restricted in its bioavailability under ideal conditions. However their bioavailability is enhanced on anthropogenic invasion of their major exposure matrices viz. groundwater (exploited through infiltration wells) & food by activities like acid-mine drainage associated with mining, industrial discharge etc. that consequently inflicts severe renal damaging effects on prolonged high exposure levels. Hence these nephrotoxins were extensively analysed in major exposure matrices viz. groundwater and food consumed by the CKDu affected and unaffected regions of Canacona, whose results are stated in Chapter 3. Our results

depicted that in the groundwater, significantly elevated silica levels (i.e.115.5 mg/L) which surpassed the 90 mg/L threshold (established from animal and few epidemiological studies) along with borderline lead levels (i.e. 9.98 µg/L) which approached the WHO permissible limit of10 µg/L were noted in the CKDu affected region of Canacona. Contrarily, remarkably lowered silica levels (i.e.13.5 mg/L) and below WHO permissible lead levels (0.83 µg/L) were noted in healthy control regions. The difference in both of these nephrotoxins levels in CKDu affected and unaffected regions were attributed to the variations in the aquifer's bedrock geological constitution, pH and chemistry of the groundwater. The elevated nephrotoxins levels in the CKDu affected region's groundwater was primarily linked to close proximity to a non-operational granite mine due to which the resulting and inevitable acid mine drainage originating from the abandoned mine could have travelled to the neighboring affected region's aquifer causing increased groundwater acidity that excessively leached out these nephrotoxins (i.e. silica and lead) from the aquifer's bedrock into the groundwater owing to the enhanced nephrotoxin availability at an acidic pH. Moreover the silica (81% by weight) and lead (2.5% by weight) laden granitic composition of the CKDu affected region's aquifer bedrock (Fernandes and Widdowson, 2009); further contributed to the nephrotoxin enrichment owing to acidic groundwater amplified rock-water interactions. The availability was further enhanced by the groundwater's essential metal cation (viz. Ca, Mg, Al etc.) deficiency as these ions restrict the availability by nephrotoxin trapping in inert metal complexes. These confounding factors affecting nephrotoxin availability explains the remarkably reduced nephrotoxins levels in the healthy region's groundwater, attributed to the groundwater's neutral pH and the bedrock's metabasitic constitution (being inherently silica poor- containing <40% silica, lead deficient comprising 0.025% lead and enriched in essential metal cations like Ca, Mg etc.). Therefore, high silica and lead nephrotoxin levels in the CKDu affected region's groundwater resulted in significantly enhanced daily nephrotoxins intake (surpassing WHO established tolerable intake) and a remarkably increased risk of developing severe nephrotoxicity(evident from target hazard quotients exceeding the WHO safe limit of 1) at prevalent exposure levels in the affected subjects. Concurrently, no significant hazardous intake or associated nephrotoxic risk was noted for these nephrotoxins on food exposure in the CKDu affected region due to JCEFA established safe nephrotoxin levels present in the food. Contrarily, tolerable intake and no nephrotoxicity risk were noted on groundwater and food consumption in the healthy region, owing to safe nephrotoxin levels present in these matrices. Therefore these findings highlighted groundwater to be the major exposure source to these nephrotoxins viz. silica and lead in Canacona's CKDu affected region, which was homologous to the trend noted in similarly affected regions such as Andhra Pradesh (India), Balkan region (Europe) and Central America. Additionally, the high silica and lead levels in the groundwater exposure-matrix directly translated into significantly enhanced nephrotoxins level in the blood of Canacona's CKDu affected subjects [silica=100.2 mg/L and Pb=317.8 µg/L] which was supported by high correlations between levels in the groundwater and blood. Contrarily, significantly lowered & WHO safe levels (i.e. below permissible limits of 50 mg/L for silica & 50 µg/L for lead) of these nephrotoxins (silica=30.7 mg/L and Pb=6.3µg/L) were noted in the taluka's healthy individual's blood, attributed to the lowered concentrations of the same in that region's groundwater. These elevated silica and lead exposure levels noted in the blood of CKDu affected subjects highlighted a strong potency of these nephrotoxins in inducing severe renal tubular damage on chronic exposure. This silica and lead induced renal damage was further worsened by the significantly lowered blood levels of essential metals ions (i.e. Zn, Mn, Cu, Fe, Se) in CKDu affected subjects as these metals ions under optimal levels triggers heavy metal detoxification via enhanced synthesis of metal excretion proteins (i.e. metallothionein proteins) and provides anti-oxidative protection from heavy metal induced oxidative renal damage as these ions majorly constitute the structure of anti-oxidant enzymes viz. SOD, GSH as metal cofactors. The anti-oxidative protective effect of these essential cations explains the absence of renal damage in the taluka's healthy study

population owing to the presence of optimum levels of these essential cations and safe levels of silica and lead nephrotoxin in these individuals blood. Moreover, significantly elevated blood silica and lead levels (doses) in the CKDu affected subjects demonstrated a strong and significant ($p<0.05$) dose-effect response relationship with the tubular dysfunction biomarkers viz. uBCR & uNCR wherein the intensities of these markers remarkably enhanced with increasing blood silica and lead nephrotoxin doses. These observations were supported by the complete absence of associations between blood silica or lead internal doses with tubular injury markers in the healthy subjects, signifying the lack of nephrotoxin induced tubular damage in these subjects as potentiated. In totality, the occurrence of significant dose response associations between blood lead and silica levels with tubular injury markers as observed in the Canacona's CKDu-affected subjects were in agreement with the findings of independent dose-tubular damage response analyses of these nephrotoxins that have been implicated in the CKDu causation in Central America and Balkan region (Europe) respectively. Therefore, our results strongly confirmed the etiological role of elevated and chronic exposure to lead and silica nephrotoxins via chronic consumption of untreated nephrotoxin enriched groundwater in the induction of severe tubular dysfunction associated with the pathogenesis of CKDu in the Canacona.

Previous animal based histopathological studies and a few epidemiological founded clinical cum biochemical analysis in the Balkan region (Europe) have established CTN to be the major histopathological manifestation of silica induced renal toxicity. However, these studies failed to establish the cellular and molecular toxicity mechanisms of silica induced nephrotoxicity till date. Hence this thesis aimed to understand the toxicological mechanisms of silica induced nephrotoxicity at the cellular and molecular level, as a function of dose and time using the kidney's nephrotoxin susceptible/targeted cells viz. normal human renal proximal-tubular cells (HK-cells) as an *in-vitro* model. For this, the renal proximal-tubular cytotoxicity outcomes following prolonged exposure (for 7 days) of HK-cells to increasing silica doses (80-120 mg/L) were analyzed via a panel of assessments comprising of cell-viability,mitochondrial-integrity, oxidative-damage, cell-cycle arrest, inflammatory responses, genomic-damage & apoptotic-pathway regulation. Moreover, this study attempted to gather supporting causal evidence for role of silica in CKDu development in Canacona. The results of which are detailed in Chapter 4. From our results, it was evident that silica exhibited grave proximal-tubular-cytotoxicity (indicated by declining HK-cell-density) on long-term dosing (for 7 days) to high-concentrations ($\geq$100 mg/L), that was mediated by silica-toxin triggered mitochondrial-dysfunction. The high-silica-doses ($\geq$100 mg/L) induced incessant mitochondrial-injuries which manifested in generation of continuous oxidative-stress (i.e. ROS > anti-oxidant defense), owing to mitochondrial involvement in ROS-homeostasis. This prolonged oxidative-stress (enhanced ROS) inflicted severe protein, membrane and DNA-damage (genotoxicity) which consequently elicited the DNA-damage responsive p53 mediated damage repair coupled with G2/M cell-cycle arrest (Chk1-dependent).However, these persistently elicited DNA-injuries surpassed the cellular-repair potency, which provoked p53 to unceasingly activate the mitochondrial apoptotic pathway that culminated in incessant proximal-tubular (HK) cell-death. The amplification of renal tubular apoptosis was achieved by a p53 created cross-talk with the inflammatory cascade (i.e. latter being primarily triggered by ROS stimulated activation of inflammatory transcription factor-NF-kβ). This cross-talk arose from silica generated stressors (i.e. ROS and DNA-injuries) stimulating p53-gene which in-turn activated the inflammatory-regulator i.e. NF-kβ, owing to presence of binding-sites in p53 for NF-kβ. This p53-bound NF-kβ complex simultaneously triggered the enhanced activation of principal apoptotic-executioners (i.e. Bax, cytochrome c, caspase-9 and caspase 3) and pro-inflammatory cum fibrogenic-cytokines (IL-1β, IL-2, IL-6, TGF-β and TNF-α) which collectively resulted in persistent tubular apoptotic cell-death and inflammation. This silica triggered incessant tubular cell-death and inflammation eventually culminates in the development of tubular-atrophy and fibrosis, the major

histopathological characteristics of CTN,that is associated with CKDu pathogenesis. These silica induced nephrotoxicity mechanisms were in agreement with those inflicted by well-known renal-toxins (heavy-metals); thereby justifying silica's nephrotoxic potential in CKDu causation. Additionally, our study confirmed ≥100 mg/L to be the nephrotoxic silica-dose, capable of inducing severe proximal-tubular toxicity and CKDu development on chronic exposure. This dosage's CKDu-inducing potential was supported by its similarity with silica-levels in the groundwater and blood of the affected individuals that were responsible for CKDu development in Canacona. Moreover, silica exhibited toxicity reversal on sub-toxic dosing, justified from the absence of CKDu-incidence in non-endemic regions of Canacona containing sub-toxic silica levels (<100 mg/L) i.e. 13.5 mg/L and 30.7 mg/L in the groundwater and blood of the healthy individuals respectively. This non-toxicity could be attributed to faster toxin-elimination (possibly by metallothioneins) and uninhibited cellular-repair of these tubular cells at lower-doses which could have averted nephrotoxicity induction at these doses. Thus, these findings provided supporting evidence to the causal role of this nephrotoxic silica-dose (100 mg/L) prevalent in the affected individuals blood in significant induction of renal tubular damage and associated CKDu development on chronic exposure in the Canacona taluka.

A few epidemiological studies in Balkan have established this silica nephrotoxicity to be mediated by the water soluble, monomeric and highly reactive form of silica viz. orthosilicic acid. Orthosilicic acid generates H^+ ions that are regulated under low silica exposure conditions by the intrinsic cellular-repair. However it can excessively accumulate on anthropogenically induced increased silica availability due to it's bioaccumulative propensity which surpasses the saturation point of polymerized silica formation causing retention of its unpolymerised status. This unpolymerised form on chronic higher exposure incessantly generates toxic H^+ ion & free radicals which on exceeding intrinsic cellular oxidative repair capacity manifests in nephrotoxicity development. Despite silica's nephrotoxic potency, sensors detecting its toxic form viz. orthosilicic acid accumulation in targeted human proximal tubular cells, are unavailable. Hence, we devised a water-dispersible and biocompatible 'turn-on' fluorimetric-sensor (Rh1@TiO_2) comprising of rhodamine-based chemodosimeter (i.e. rhodamine hydrazide, Rh1) adsorbed onto biocompatible TiO_2 nanoparticles for detection and imaging of toxic silica sp. (viz. orthosilicic acid) bioaccumulation in an ideal in-vitro nephrotoxicity model viz. human proximal tubular HK-cells.Rh1 was chosen as the chemodosimeter based on the ability to detect orthosilicic acid by interaction with incessantly generated H^+ ions in the intrinsically neutral proximal tubular cells that ultimately triggers significant ring opening and associated transduction of a strong orange fluorescence output. This sensor demonstrated merits such as good water dispersibility, lack of organic solvents usage during fluorimetric studies, rapid turn-on type signal transduction and imaging of silica accumulation even at lower orthosilicic acid levels(i.e. 0.1 mg/L for 8 days).The probe proved its biocompatibility by penetrating the cell-membrane and detecting orthosilicic acid accumulation as a function of dose and time, wherein significantly higher fluorescence intensities were noted on chronic exposure to 100 mg/L silica for 8 days, stemming from enhanced accumulation under such conditions. This sensor showed promising potency for identification of silica accumulation related nephrotoxicity linked to CKDu development in the taluka. From this PhD research, we have managed to establish prolonged exposure to high levels of silica and lead routed through untreated contaminated groundwater consumption to be the major environmental nephrotoxins responsible for the etiological development of CKDu in Canacona. Moreover, through our *in-vitro* toxicity studies, we listed mitochondria and nucleus to be sub-cellular targets of silica nephrotoxicity. Thus in lieu with our findings, preventive, remediative and therapeutic measures can be appropriately adopted to reduce exposure of susceptible populations to these nephrotoxins and protect the aforementioned cellular targets from silica induced toxic effects, thereby could assist in averting the future rise in CKDu incidences in this taluka.

DEDICATED TO MY BELOVED FAMILY

who valued education above all....

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Abbreviations

1˚Ab	Primary antibody
A498	Renal proximal tubular carcinoma
AA	Aristolochic acid
AAS	Atomic absorption spectrometry
AER	Albumin excretion rate
AMD	Acid mine drainage
ANOVA	One–way analysis of variance
Apaf-1	Apoptotic protease-activating factor-1
APHA	American Public Health Association.
ATM	Ataxia telangiectasia-mutated kinase
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related protein kinase
B2M	Beta-2-microglobulin
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BDL	Below detectable levels
BEN	Balkan endemic nephropathy
BIS	Bureau of Indian Standards
BMI	Body mass index
BP	Blood pressure
BSA	Bovine Serum Albumin
Bw	Body weight
CA	California
CDC	Centre for Disease Control
Cdc25	Cell-division control protein 25(Dual-Specificity Phosphatase)
Cdk1	Cyclin dependent kinase 1
CdSe	Cadmium selenide
CdTe	Cadmium telluride
CH_3CN-H_2O	Acetonitrile-water
CH_3CN-HEPES	Acetonitrile-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic-acid)buffer
CH_3OH-phosphate buffer	Methanol phosphate buffer
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CKD	Chronic kidney disease
CKD-EPI	CKD-EPI Chronic Kidney Disease Epidemiology Collaboration
CKDu	Chronic kidney disease of unknown etiology
COMISCA	Commission of National Ministries of Health of Central America
CrCl	Creatinine clearance rate
CRF	Chronic renal failure
CTN	CTN-chronic tubulointerstitial nephritis
CuO	Copper(II) oxide
CV-AAS	Cold vapor atomic absorption spectrometer
CVD	Cardiovascular disorders

DCF	Dichlorofluorescein
DCFH-DA	2'-7'-Dichlorofluorescin Diacetate
DEVD-pNA	N-Acetyl-Asp-Glu-Val-Asp-p-Nitroanilide(Caspase-3 Substrate)
DMEM	Dulbecco's Modified Eagle's Medium
DMT	Divalent metal transporter
EC	Electrical conductivity
ECM	Extracellular matrix
EDI	Estimated daily intake
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EP	The Eastern Province of Sri Lanka
ESKD	End stage kidney disease
ESRD	End stage renal disease
FAAS	Flame atomic absorption spectrometer
FBS	Fetal bovine serum
FESEM	Field Emission Scanning Electron Microscopy
FITC	Fluorescein Isothiocyanate
FTIR	Fourier-transform infrared spectroscopy
GFAAS	Graphite furnace atomic absorption spectrometer
GFR	Glomerular filtration rate
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H_4SiO_4	Orthosilicic acid;
HbA1c	Glycosylated hemoglobin
HCl	Hydrochloric acid
HG-AAS	Hydride generation atomic absorption spectrometer
HI	Hazard index
HK-cells	Human renal proximal-tubular cells
HMV	High molecular weight proteins
HNO_3	Nitric acid
HPLC	High-performance liquid chromatography
HRP	Horse raddish peroxidase
HVG	Hydride vapour generator (HVG-1 system,
HVG	Hydride vapor generator
ICMR	Indian Council of Medical-Research
ICP-AES	Inductively coupled plasma-atomic emission spectrometer
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectrometer
ICP-MS	Inductively coupled plasma mass spectrometer
IDMS	Isotope dilution mass spectrometry
IETD	pNA- N- Acetyl-Ile-Glu-Thr-Asp-p-Nitroanilide/ caspase-8 substrate
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IR	Infra-Red
IRET	Institute for Studies on Toxic Substances
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3'-Tetraethylbenzimi-Dazo-Lylcarbocyanide Iodine
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JNK	c-Jun-NH(2)-terminal-kinase

K/DOQI	Kidney Disease Outcome Quality Initiative
KDIGO	Kidney Disease: Improving Global Outcomes
LC-MS	Liquid chromatography–mass spectrometry
LDH	Lactate Dehydrogenase
LEHD	pNA- N-Acety-Leu-Glu-His-Asp-p-Nitroanilide (caspase-9 substrate)
LMV	Low molecular weight proteins
LOD	Limit of detection
LPO	Lipid Peroxidation
MA	Massachusetts
MDA	Malonidialdehyde
MDRD	MDRD-Modification of diet in renal disease
MeN	Mesoamerican nephropathy
MMP	Mitochondrial Membrane Potential
MO	Missouri
MOMP	Mitochondrial Outer Membrane Permeabilisation
MON	Monsoon period
MRP-2	Multidrug resistance-associated *protein* 2
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
Na_2EDTA	Disodium Ethylenediamineteracetate
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAG	N-acetyl-glucosaminidase
NCCS	National Centre for Cell-Sciences
NCP	The North Central Province of Sri Lanka
NFK	NFK-National Kidney Foundation
NF-kβ	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NHANES	National Health and Nutrition Examination Survey *(*NHANES*)*
NIH	National Institute of Health (NIH)
NIOH	National Institute of Occupational-Health
NIST	National Institute of Standards and Technology
NIST-CRM	National Institute of Standards and Technology Certified Reference Material
NJ	New Jersey
NLRP3	NLR Pyrin Domain-Containing Protein
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
NTU	Nephelometric Turbidity Unit
NY	New york
OAT1	Organic anion transporter
OSHA	Occupational Safety and Health Administration
OTA	Ochratoxin A
PAHO	Pan American Health Organization
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PMSF	Phenylmethane Sulfonyl Fluoride
POM	Post-monsoon period
PRM	Pre-monsoon period
PS	Phosphatidylserine

PT	Proximal tubular cells
PVC	Polyvinyl chloride
P-XRF	Portable X-ray fluorescence spectrometer;
R.R	Risk-ratio
RA	Rheumatoid arthiritis;
RfD	Oral reference dose
RF-IC	Reagent-free ion chromatography
Rh1	Rhodamine B hydrazide;
Rh1@TiO_2	Rhodamine hydrazide adsorbed onto TiO2 nanoparticles;
ROS	Reactive oxygen species
SALTRA	Program in Health, Work, and Environment in Central America
SEM	Scanning electron microscopy
SiO_2	Silica
SLE	Systemic lupus erythematosus;
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TBARS	Thiobarbituric Acid Reactive Substance
TDI	Tolerable daily intake
TDS	Total dissolve solids
TGA	Thermogravimetric analysis
TGF-β	Transforming Growth Factor beta
The DNA	The Daily news action
THQ	Target hazard quotient
$TiCl_3$	Titanium trichloride
TiO_2	Titanium dioxide.
TMAH	Tetramethylammonium hydroxide
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
TRITC	Tetramethylrhodamine
TTHQ	Total target hazard quotient
uACR	Urinary Albumin to creatinine ratio
uAlb/b2M	Urinary Albumin to B2M ratio
uAPR	Urinary Albumin to protein ratio
uBCR	Urinary β2-microglobulin to creatinine ratio
UNA	Universidad Nacional
uNCR	Urinary N-acetyl-glucosaminidase to creatinine ratio
UP	The Uva Province of Sri Lanka
uPCR	Urinary Protein to creatinine ratio
UV-Vis	Ultraviolet-visible
WHO	World health organization
XRD	X-ray diffraction
ZnO	Zinc oxide
ZnS	Zinc sulfide

Chapter 1

Introduction

and

Review of literature

1.1 Chronic Kidney Disease: a worldwide health problem

1.1.1 Definition of chronic kidney disease (CKD)

Defining and staging of chronic kidney disease **(**CKD) based on severity helps in providing a communication tool among patients, health care practitioners, policy-makers and researchers for assisting in health care management by providing therapy to improve CKD outcomes (Vassalotti et al., 2016). The National Kidney Foundation (NFK) Kidney Disease Outcome Quality Initiative (K/DOQI) defined CKD in 2002 as "the presence of kidney damage or decreased level of kidney function maintained for 3 months or more, irrespective of a primary diagnosis" (Levey et al., 2005).This definition covers conditions causing advanced kidney function loss or problems from reduced renal function (Inker et al., 2014; Norris et al., 2017). Kidney function is estimated through glomerular filtration rate (GFR) that defines flow rate of filtered fluid through the kidney, or creatinine clearance rate (CrCl) that is blood plasma volume clarified of creatinine per unit time, serving as a GFR measuring tool. Both GFR and CCrl are calculated by formulas based on blood test results (eGFR and eCCr). eGFR is computed using formulas like Modification of diet in renal disease (MDRD), Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI), etc. that take into consideration serum creatinine, weight, age and corrective factors like sex and race (which effect muscular and serum creatinine (Fernandez-Prado et al., 2016; Inker et al., 2014). CKD definition is founded on NHANES III study, USA (1988-1994) comprising of 5 stages elaborated in **Table 1.1**.

Table 1.1. Description of stages, prevalence, and clinical plan of action for chronic kidney disease (CKD) and individuals who are at enhanced risk of CKD (Courtesy: Laws, 2015)

Stage	Description	GFR (*mL/min per 1.73 m²*)	US Prevalence N [1,000s]	Clinical Action Plan
---	Increased risk for CKD • Age ≥60 years • Hypertension • Diabetes • Cardiovascular disease • Family history of CKD	Not applicable	Risk factor prevalence • Age >60: 50,600 (23.2%) • Hypertension: 65,000 (32.3%) • Diabetes: 20,600 (9.6%) • CVD: 71,300 (34.2%) • Family History: unknown	Screening Primary prevention and CKD risk reduction, including blood pressure and glycemic control
1	Kidney damage with normal or increased GFR	≥90	3,600 (1.8%)	Diagnosis of CKD cause Education about CKD Treatment of comorbid conditions Evaluation of risk for and assessment of rate of progression Treatment to slow progression CVD risk reduction Referral to nephrologist for rapid kidney disease progression
2	Kidney damage with mild decrease in GFR	60-89	6,500 (3.2%)	
3	Moderately decreased GFR	30-59	15,500 (7.7%)	Evaluate and treat complications, including bone and mineral disorder, anemia, and dyslipidemia Consider discussion of kidney replacement therapy options, particularly in late stage 3 or rapid progressors
4	Severely decreased GFR	15-29	700 (0.4%)	Prepare for kidney replacement therapy Place vascular access or develop plan for peritoneal access or pre-emptive transplant Referral to nephrologist
5	Kidney failure	<15 or dialysis	~400 (0.2%)	Kidney replacement therapy

Early stages of CKD (1-2) develops under marginally reduced GFR conditions, demarcated by pathological aberrations or damage markers like proteinuria evident from renal-biopsies and biochemical analyses. Stages 3-5 signifies GFR diminution well beneath the "normal" limit (60ml/min/1.73m^2) which rapidly progresses to complete renal function disruption manifesting in chronic renal failure called as end stage renal disease (ESRD) (K/DOQI, 2002, Levey and Coresh, 2012; Robinson et al., 2016).

A new and improved initiative for staging of CKD was developed in 2004 called the "Kidney Disease: Improving Global Outcomes" (KDIGO), modified in 2012, for universal integration of various clinical strategies (Eknoyan et al, 2004; Levin et al., 2013). The CKD guidelines preserve the CKD definition (2002) but provide a detailed CKD classification, diagnosis, and global marker for kidney injury viz.albuminuria. It comprises of "heat maps" founded on an amalgamated ranking of comparative risks of all causal agents. Colors from green to red define patients at growing risks for developing CKD, helping clinicians for effort prioritization to improve health-outcomes(Levey et al.,2011; Levin et al.,2013) **(Figure 1.1).**

Prognosis of CKD by GFR and Albuminuria Categories

GFR categories (ml/min/1.73 m^2) Description and range				Albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-299 mg/g 3-29 mg/mmol	≥300 mg/g ≥30 mg/mmol
	G1	Normal or high	≥90			
	G2	Mildly decreased	60-90			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.
KDIGO 2012

Figure 1.1 Prognosis of chronic kidney disease by glomerular filtration rate and albuminuria categories (Source: Levey et al., 2011)

1.1.2 Causation of CKD

There are various causes of CKD which are grouped into four broad categories **(Table 1.2)**:- glomerular, tubulointerstitial, vascular and obstructive etiologies (Brooks, 2009, Laws, 2015). Vascular and obstructive causes are restricted in number and easily diagnosed, while glomerular and tubulointerstitial diseases have a huge spectrum of causals (Brooks, 2009; Webster et al., 2017). Glomerular diseases are the most prevalent form of CKD witnessed globally and displays typical urine findings like proteinuria and red blood cells. Proteinuria, comprises of protein excretion exceeding 2g/day consisting majorly of albumin (Brooks, 2009; Levey et al., 2015). Tubulointerstitial diseases are the 2nd highest reported CKD form

presenting proteinuria that includes tubular cell origin proteins like beta-2-microglobulin (b2M); N-acetyl-glucosaminidase (NAG), whose excretion is lower than glomerular disease (Brooks, 2009; Venkatachalam et al.,2015). As CKD advances specific causation is clinically unapparent, manifesting in complete renal function disruption (Vassalotti et al., 2016).

Table 1.2 Etiopathology of Chronic kidney disease (Courtesy: - Laws, 2015)

Classification	Condition	Associated Disease States		Manifestations
VASCULAR	Main Renal Arteries	Renal artery stenosis Fibromuscular dysplasia		Often accompanied by hypertension
	Intrarenal vasculature	Thrombotic microangiopathies Benign and malignant nephrosclerosis Atheroembolic disease		Minimal albuminuria, often with bland urine sediment
	Renal vein	Renal vein thrombosis		May be a cause of or be caused by nephrotic syndrome
GLOMERULAR	Focal GN	Age <15 yo	Post-infectious, IgA, TMBD, Hereditary nephritis, HSP, mesangial proliferative GN	Active sediment without reduced GFR or nephrotic syndrome
		Age 15-40 yo	IgA, TBMD, SLE, Hereditary nephritis, mesangial proliferative GN	
		Age >40 yo	IgA	
	Diffuse GN	Age <15 yo	Post-infectious, MPGN	Active sediment with reduced GFR and variable proteinuria
		Age 15-40 yo	Post-infectious, SLE, RPGNs, Fibrillary GN, MPGN	
		Age >40 yo	RPGNs, vasculitides, fibrillary GN, Post-infectious	
	Nephrotic Syndromes	Age <15 yo	MCD, FGS, mesangial proliferative GN	Heavy proteinuria, usually bland sediment
		Age 15-40 yo	FGS, MCD, Membranous, Diabetes, Pre-eclampsia, Late stage post-infectious	
		Age >40 yo	FGS, Membranous, diabetes, MCD, IgA, Amyloid/LCDD, HTN/nephrosclerosis, Late stage post-infectious	
TUBULOINTERSTITIAL	Drugs and toxins	Analgesic nephropathy (non-steroidal anti-inflammatory drugs) Aminoglycoside associated nephrotoxicity Aristilochic Acid Calcineurin inhibitors Chemotherapeutic agents (Cisplatin, nitrosureas) Contrast induced nephropathy Lithium Metals (Lead, cadmium, arsenic, uranium, mercury) Ochratoxin Pigment nephropathy (rhabdomyolysis)		Minimal albuminuria, usually bland sediment, even in presence of reduced GFR, tubular proteinuria
	Hereditary	Fabry Disease Hereditary nephritis (Alport syndrome) Medullary Cystic Disease Polycystic Kidney Disease		Fabry Disease is rare and may have glomerular or tubular manifestations Hereditary nephritis is often accompanied by hearing impairment; Medullary cystic disease may be indolent and is difficult to diagnose PKD manifests with massively enlarged cystic kidneys

Diabetes (contributing to 44.4% of renal failure cases) and hypertension (28.6%) are major causals for CKD in developed countries, and to some extent in developing countries also that majorly manifests in glomerular pathology (Hu and Coresh, 2017). Whereas, the burden of CKD in developing countries is mainly attributed to tubulo-interstitial disorders which is majorly contributed from exposure to environmental toxins (Stanifer et al., 2016).

1.1.3 Worldwide prevalence and burden of CKD

CKD has turned into a major worldwide public health problem (Webster et al., 2017). In developed countries, 30 million adults (2016) suffered from CKD, depicting a 30% rise as compared to last decade. This is reflective of increasing obesity and its related outcomes like hypertension, diabetes, and cardiovascular disease (Murphy et al., 2016; Saran et al., 2017). CKD was initially believed to be a problem in developed countries only owing to prevalence of major causals viz. diabetes and hypertension. Recently it's emerging as a problem in developing countries like southern China, Congo, Australia which have obtained a general CKD incidence of 10-20% due to the increasing frequency of occurrence of diabetes and hypertension in these countries (Hill et al., 2016; Stanifer et al., 2016).

In another developing nation viz. India, the CKD prevalence in different regions have been approximated to be 17-20% wherein the majorly affected cohorts are in the age group of 50-70 yrs as reported by the International Society of Nephrology's Kidney Disease Data Center Study. However the true CKD burden in India is unknown till date due to a lack of access to renal replacement or dialysis and an absence of a registry (Abraham and Varughese, 2016; Ene-Iordache et al., 2016; Varughese and Abraham, 2018). From published data, it was concluded that since life expectancy in India has risen from 41.68 yrs (in 1960) to 66.9 yrs (2013) with prevalence of hypertension and diabetes rapidly increasing, inevitably CKD prevalence will continue to rise. Hence drastic changes in lifestyle habits needs to be adopted and frequent monitoring needs to conducted to avert the exposure to causal risk factors and impending rise in CKD incidence and mortality in India respectively (Anupams and Uma, 2015; Lobo et al., 2017; Rajapurkar and Dabhi, 2010; Varma, 2015).

1.2 Chronic kidney disease of unknown etiology (CKDu) - a rapidly progressing global health problem in developing countries

Since the 1990s, a new form of CKD, with no obvious identifiable cause and unlinked to risk factors like diabetes, hypertension has been reported in some developing countries, which has been renamed as chronic kidney disease of unknown etiology (CKDu) (Lunyera et al., 2016).

This epidemic has mainly affected developing countries like Sri Lanka, (Rajapakse et al., 2016; Wanigasuriya, 2014), India (Ganguli, 2016; Tatapudi, 2018); Egypt (El Minshawy, 2011a) and some central American countries i.e. Costa Rica, Nicaragua, Panama and El Salvador (Campese, 2016; Correa-Rotter et al., 2014; Lusco et al., 2017) with common trends of disease presentation displayed in these countries (Gifford et al., 2017; Weaver et al., 2015).The disease develops progressively, slowly, and asymptomatically initiating commonly in younger adults in their third to fifth decade. The disease affects economically poor rural communities (Gifford et al., 2017; Weaver et al., 2015; Wijkstrom et al., 2018).

The rising severity of the problem has caused International health regulatory bodies like WHO and the Ministry of Health of developing countries like Sri Lanka, India, and Egypt to conduct research on deciphering the etiology, epidemiology, clinical features, risk factors, and early detection of CKDu which are elaborated in subsequent sections of this chapter. This will assist in the appropriate design of public health interventions (Jha et al., 2013) for addressal of this problem in order to prevent the future rise in incidence of CKDu.

1.2.1 CKDu definition

For appropriate CKDu detection, defining disease characteristics is crucial. The Ministry of Healthcare and Nutrition (Sri Lanka) in collaboration with WHO have defined CKDu wherein CKD is considered to be of unknown origin in the "absence of a past history of long-term or severe hypertension, diabetes mellitus, glomerulonephritis or urological diseases, blood-pressure <160/100mmHg untreated or <140/90 mmHg on not more than two anti-hypertensive medications and normal HBA1C (<6.5%)" .Other defining CKDu characteristic includes residence in an endemic area for greater than fifteen years (Jayatilake et al., 2013).

1.2.2 Clinical features of CKDu for early screening and detection of the disease

The primary requirement for screening of CKDu globally is the illumination of its disease pattern. The pattern of disease presentation can be interpreted by screening of various biochemical markers in biological matrices like blood and urine (Sayanthooran et al., 2017).

Biochemical markers are crucial for exact diagnosis, risk evaluation, and implementation of therapy for improvement of clinical outcome. Routine renal function biomarkers like uric acid, urea will not suffice detection of CKDu disease pattern (Gowda et al., 2010).

Nephrologists involved in CKDu screening have observed that minimal-proteinuria (i.e. microalbuminuria) demonstrating a sub-nephrotic range of high molecular weight proteins in the urine i.e. albumin in the range of 30-300 mg/g creatinine, typical of tubulointerstitial

diseases are the major abnormalities associated with CKDu (Wijkström et al., 2018).Hence, screening tools like urine dipstick proteinuria regularly used for detection of albuminuria in hypertensive/ diabetic CKD cases are not optimal for CKDu detection. CKDu analysis studies in Sri Lanka and Central America have observed CKDu manifestation to be related to nhanced levels of narrowly distributed low-molecular weight proteins like β2-microglobulin (b2M), proximal tubular specific enzymes i.e. N-acetyl glucosaminidase (NAG) and electrolytes like bicarbonates and phosphates in the urine. Hence these analytes serve as biomarkers for early screening and CKDu detection (Ratnayake et al., 2017).

1.2.3 Histopathological discoveries in CKDu

Tubulointerstitial dysfunction specifically chronic tubulointerstitial nephritis is a prominent pathological manifestation noted in CKDu patients (Wijetunge et al., 2015). Hence, CKDu is mainly proved to cause pathological changes in proximal tubular cells (mainly involved in reabsorption and toxin elimination) and interstitial cells surrounding the tubules (Badurdeen et al., 2016). Histopathological studies in asymptomatic CKDu patients with proteinuria depicted that major proportion of subjects (90%) had varying magnitudes of tubular atrophy, interstitial inflammation, with or without non-specific mononuclear cell infiltration and interstitial fibrosis, as predominant characteristics. The tubular histopathological dysfunction was supported from enhanced excretion of tubular damage urinary biomarkers viz.b2M and NAG (Gifford et al., 2017; López-Marín et al., 2014; Wijkström et al., 2018).

1.2.4 Risk factors associated with CKDu

Over the last decades extensive research has been carried out on the prevalence and demographic distribution of CKDu in affected developing countries especially Central America and Sri Lanka. This is crucial for the identification of susceptible populations or risk groups that are most likely to develop the disease permitting us to take necessary preventive measures to avert future rise in CKDu incidence (Lunyera et al., 2016).

Community based screening studies conducted in Central America (Correa-Rotter et al., 2014; Laws et al., 2015) and Sri Lanka (Jayasekara et al., 2015; Redmon et al., 2014; Senevirathna et al., 2012) depicted a significant variation in CKDu occurrence with occupation. Higher risk of developing CKDu was noted in male agricultural workers of younger age-groups as compared to the other occupations. Sugarcane farmers (in Central America) and chena(vegetables) cultivators (in Sri Lanka) displayed greatest prevalence in terms of occupation although considerable prevalence was also noted in construction

workers, and miners. This was backed by increased risk ratios of 5.6 and 6.7 respectively for the risk of CKDu development noted for male agricultural workers and younger adults as compared to lowered risk-ratio in unexposed groups (i.e. older adults and other occupations). This observation was supported by the increased incidence of tubular injury urinary biomarkers viz. b2M and NAG of younger agricultural male workers as compared to other occupational groups. Additionally, drinking well water ($p<0.01$) [risk ratio=3.3, $p<0.05$), and past history of ayurvedic treatment ($p<0.01$) [risk ratio=2.7, $p<0.05$] were noted as significant risk factors for CKDu in these studies. Family history of CKDu and previous history of snake bites were ruled out as significant predictors of CKDu in these CKDu risk factor analyses.

1.2.5 Epidemiology of CKDu

1.2.5.1 Worldwide prevalence of CKDu

High incidence of CKDu have been described in various developing countries of the world viz. Central America, Sri Lanka, Tunisia, Egypt and India **(Figure 1.2).** In order to assess for a common trend in the pattern of disease presentation in these developing countries, it is important to understand the epidemiology of the disease in these areas. Hence, a brief overview on the common clinical features/findings of CKDu among the aforementioned countries, epidemiological distribution, disease prevalence, demographic features, risk factors, clinical features/findings and histopathological characteristics of CKDu disease pattern witnessed in all of these countries have been summarized in tabular forms **(Tables 1.3 and 1.4).** CKDu scenario in Sri Lanka and Central America has been widely studied but in Egypt and India analysis is limited signifying need for urgent attention (Gifford et al., 2017).

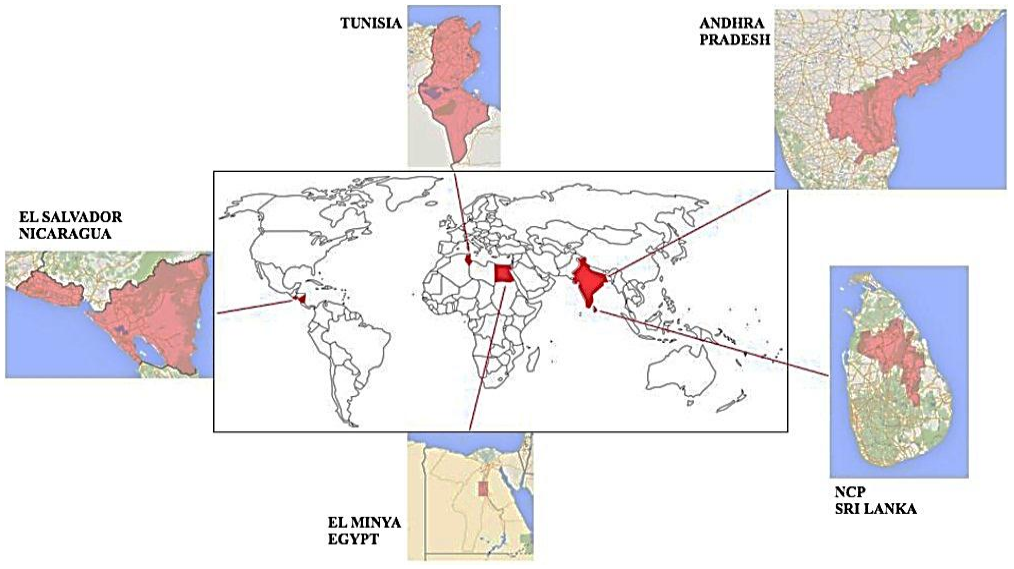


Figure 1.2. Global distribution of the prevalence of chronic kidney disease of unknown etiology(CKD*u*) (Courtesy:Giffiord et al., 2017)

Table 1.3: Comparison of the scenario of chronic kidney disease of unknown etiology (CKDu) in various affected developing countries (Courtesy:-Gifford et al., 2017)

	BEN	MeN	SL CKD*u*	Indian CKD*u*
Date first described	1956	2002	Early 1990s	2010
Endemic areas	Danube region: Serbia, Bulgaria Croatia Romania Bosnia	Nicaragua El Salvador Costa Rica **Low-altitude Agricultural areas**	"Dry Zone" of Sri Lanka First noticed in NCP **Low altitude Agricultural areas Geographical foci of disease Low socio-economic status**	Uddanam coastal region, Andhra Pradesh **Foci of disease Low socio-economic status**
Etiology confirmed?	Yes: 1993 *Aristolochia sp.*	Unexplained	Unexplained	Unexplained
Characteristic clinica features	**Presents: fifth through sixth decade** M:F = 1:1 Tubular proteinuria Impaired concentrating capacity Tubular acidosis	**Presents: fourth through fifth decade** M:F = 5:1 **Asymptomatic until ESRF Recurrent "*Chistata*": dysuria, frequency, sterile urine**	**Presents: fourth through fifth decade** M:F = 1:1.3 Severe disease more common in men **Asymptomatic until ESRF Recurrent dysuria, loin/back pain, sterile urine**	**Presents: fifth through sixth decade** M:F = 1:1 **Asymptomatic until ESRF**
Associated findings	Urothelial carcinoma in 50%	**Normotensive at presentation Absent/mild proteinuria Elevated tubular biomarkers** Hyperuricemia Hypokalemia **Small kidneys on US**	**Normotensive at presentation Absent/mild proteinuria Elevated tubular biomarkers** Peripheral edema with late disease **Small kidneys on US**	**Normotensive at presentation Absent or mild proteinuria**
Renal histology	**Interstitial fibrosis Tubular atrophy** Aristolactam (AL)-DNA adducts in renal cortex	**Interstitial fibrosis Tubular atrophy**	**Interstitial fibrosis Tubular atrophy**	**Interstitial fibrosis Tubular atrophy** Normal glomeruli
Frequently reported risk factors	Consumption of wheat contaminated by Aristolochia sp.	**Occupation: sugarcane Heat stress Alcohol "Lija" consumption Heavy metal exposure**	**Agricultural workers** Resident in dry zone ≥5 yrs **Heat stress Heavy metal exposure Alcohol/betel/tobacco**	**Agricultural workers Heat stress Heavy metals Genetic predisposition**

Highlighted in bold are the features common across different endemic nephropathies. BEN, Balkan endemic nephropathy; BMI, body mass index; BP, blood pressure; Cd, cadmium; CKD*u*, chronic kidney disease of unknown etiology; ESRF, end-stage renal failure; F, females; GFR, glomerular filtration rate; M, males; MeN, Mesoamerican nephropathy; NCP, North Central Province (Sri Lanka); SL, Sri Lankan; US, ultrasound.

Table 1.4 Comparison of the clinical features, risk factors and pathological presentation among developing countries reported to be gravely affected by CKDu (Courtesy: Weaver et al., 2015).

Risk Factor/Characteristic	Sri Lanka	Central America	India	Egypt
Reported Areas	North Central Province [3] Present, although to a lesser extent, in Uva and North Western Provinces [36]	Most reports from El Salvador and Nicaragua but appears to extend across Pacific coast areas of Central America [37]	In state of Andhra Pradesh: coastal in Uddanam area and 30–40 km inland in Chimakurthy mandal [52] In India overall, highest in south which included Andhra Pradesh [44]	Reported in El-Minia Governorate [53]
Age	Wide age range; increased prevalence of eGFR ≤ 60 ml/min per 1.73 m^2 in fourth and fifth decades [12]	Third to fifth decade [37]	In India overall, younger than patients with diabetic nephropathy [44]	Mean age of 46 (n = 800 patients on renal replacement therapy) [53]
Sex	Female > male overall but male > female for CKD stage III –IV [3]	Male > female [37]	Male=female in Uddanam area, [6] and in India overall [44]	Male=female [53]
Geographical Characteristics	Rural [12] Dry weather except for two monsoon periods [36]	Rural, especially the lowlands along the Pacific coast [37] Coastal communities at lower elevations (<500 m) [45]	Rural - coastal and inland [52]	Rural [53]
Occupations	Chena (vegetable and other crops) farmers; rice farming had a lower risk compared to chena farming [3]	Risk in coastal agricultural workers but not in agricultural workers employed at elevations > 500 m; sugarcane workers studied in both locations [45] Compared to coastal agricultural workers, risk lower in service sector and agricultural workers at higher elevations [55] Intense heat noted in working conditions in Central America [49]		
Socio-economic Status	Low	Low	In India overall, lower than those with diabetic nephropathy [44]	Not reported
Pathology	In biopsies from 211 CKDu patients, the main pathological features were interstitial fibrosis, interstitial inflammation and tubular atrophy of varying degrees [10]. Authors concluded that interstitial fibrosis was the earliest detectable pathological change. Interstitial fibrosis and tubular atrophy, sometimes with nonspecific interstitial mononuclear cell infiltration, predominated	A study of 57 CKDu patients observed chronic tubulointerstitial nephropathy [56]. The authors considered the glomerular and vascular damage also observed to be secondary to the tubulointerstitial damage.	Chronic tubulointerstitial nephritis (no details as reported in abstract from conference proceedings) [6]	Not reported, biopsies rarely performed [53]
Presentation	Slow progression; minimal proteinuria (mean 24 h urine protein = 612.8 mg in 109 participants) without active sediment; bilateral small echogenic kidneys [12] Urinary excretion of alpha-1-microglobulin elevated in CKDu patients, even in the earliest CKD stage, compared with first-generation related controls residing in the same community and Japanese controls, suggesting early renal tubular damage in CKDu [57]	Minor or no proteinuria or albuminuria [6, 55] Small echogenic kidneys on ultrasound [37] Urinary symptoms, when present, are positive for pyuria and leukocyte esterase but urine culture negative [37]	In India overall, advanced CKD, few initial symptoms, absent or mild hypertension and little or no proteinuria [44] In Uddanam area, proteinuria prevalence of 20 % in males and 12 % in females [6]	Not reported
Magnitude	Age-standardized prevalence (95 % CI) of albumin–creatinine ratio ≥30 mg/g on two separate tests [3]: 15.1 % in Anuradhapura 20.6 % in Polonnaruwa 22.9 % in Badulla 16.9 % (15.5 %–18.3 %) in women 12.9 % (11.5 %–14.4 %) in men Stage 3 and 4, respectively: 23.2 % and 22 % in men 7.4 % and 7.3 % in women	Mortality from chronic renal failure (2007) [58] El Salvador Men: 85.5/100,000 Women: 34.1/100,000 Nicaragua Men: 66.2/100,000 Women: 22.3/100,000 USA Men: 9.5/100,000 Women: 7.0/100/000 Cuba Men: 3.0/100,000	CKDu is second most common cause of CKD in India (16.0 %) after diabetic nephropathy (31.3 %) [44]	Unknown etiology, at 27 %, was leading cause of end-stage renal disease (ESRD) followed by hypertension at 20 % and glomerulonephritis at 11 % [53]

1.2.5.2 Prevalence of CKDu in India

The findings of the Indian Society of nephrology reported 16% of the adult CKD population to comprise of CKDu conferring CKDu to be the 2nd largest causal factor for CKD development after diabetes (that comprised of 32% of the cases) (Singh et al., 2013), with Indian CKDu cases contributing 21% to the global burden. The geographical foci of CKDu were identified in coastal regions of the states of Andhra Pradesh and Goa (Jha et al., 2013).

Reports of endemic renal disorders started emerging in 1990s from a Southern Indian state viz. Andhra Pradesh in a coastal belt denoted as Uddanam region which comprises of Srikakulam and Prakasham districts (The Hindu, 2009; Reddy and Gunasekar, 2013). Unpublished cross-sectional analysis from these endemic foci have proposed a significantly high prevalence of CKDu in these regions (50%) that is approximately 3 times greater than the national prevalence of CKD (i.e. 17.2%) (Ganguli, 2016). This disease was renamed as Uddanam nephropathy at the World Congress of Nephrology (China, 2013) (The Hindu, 2015; The Times of India, 2013). Although a study of CKDu funded by the International Society of Nephrology is presently ongoing, few published results indicate the disease onset to be in the 2nddecade peaking at 5th and unlinked to diabetes and hypertension. The disease also depicted an asymptomatic onset, and minimal proteinuria (i.e. tubular proteinuria) that suggested a tubulointerstitial pathological manifestation (Khandare et al., 2015).

Many scientific groups have analysed biological samples, water, food and charted the affected region's populace for deciphering causal factors. Results have stated prolonged use of non-steroidal anti-inflammatory drug, high levels of heavy metals (arsenic) and silica in the commonly consumed groundwater have been recommended as potential causes. However, none can conformingly explain the rampant nature of this disease (Ganguli, 2016; Khandare et al., 2015; Reddy and Gunasekar, 2013).Hence is still under active investigation.

1.2.5.3 Prevalence of CKDu in Goa specifically Canacona taluka.

Homologous to CKDu noted in Andhra Pradesh, a rising incidence of an endemic form of CKDu was noted since 1990 in the famous tourist destination that is the state of western India viz. Goa specifically Canacona taluka of South Goa district (The Hindu, 2007). Canacona taluka is the most famous tourist attraction of south Goa endowed with a luscious green terrain and tropical climate and is known for cultivation of leafy vegetables (The Hindu, 2007). Although this CKDu wave has managed to touch Canacona taluka, this nephropathy in comparison to other regional nephropathies is least publicized and understood. The electronic media, print and the Directorate of Health Services, Goa have constantly reported the rising

incidence of CKDu for the past two decades in a few villages of Canacona taluka named Ponsulem and Chaudi (DNA, 2010; The Hindu, 2016). But the disease's exact prevalence statistics and geographical distribution is unavailable till date due to lack of appropriate registries. Despite a deficiency of official statistics, gravity of the problem was determined from personal communications with epidemiologist, Mr. Anil Kumar of Directorate of Health services (Panjim, Goa) who stated as of 2015, approximately 18,000 people display renal diseases in Canacona (in the villages of Ponsulem and Chaudi) and nearly 3,100 people have succumbed to it over last ten years (Personal communication, Mr. Anil Kumar, March 2017). The remarkably higher prevalence of CKDu in the Canacona taluka has always been a concerning matter for the administrators, health practitioners and local people (The Hindu, 2016). Troubled by this epidemic curse, the residents of Canacona and medical personnel have made numerous pleas on several occasions to DHS (Government of Goa) highlighting that CKDu incidence in this taluka are higher and needs urgent relief (The Hindu, 2007).

In a quest of complying to the numerous representations made by the residents and health practitioners, the state Government appointed a joint team officials from NIOH (National Institute of Occupational-Health), ICMR (Indian Council of Medical-Research) and Department of Preventive and social medicine of Goa medical hospital to conduct an environmental cum biological monitoring study in February 2005 to identify probable causative agents viz. environmental risk factors that are responsible for CKDu development in this region (Saiyed et al., 2005). This research group had examined various exposure matrices like food, water and the biological matrix viz. blood for the presence of nephrotoxins like heavy metals (i.e. Cd and As) and ochratoxin and associated exposure to these toxins in affected individuals and healthy controls. The results from their study depicted the nephrotoxins levels in the exposure sources (i.e. food and water) and the blood of the CKDu patients and healthy controls were comparable and significantly below WHO permissible limits. Thus highlighted lack of these nephrotoxins in CKDu development in the taluka (Saiyed et al., 2005).Unfortunately no follow up study was conducted by this research group to assess for emergence of variations in the exposure (if any) to potential environmental nephrotoxins. This study was the sole environmental monitoring study conducted on the CKDu scenario of Canacona, until our group decided to take up the challenge in 2014 in attempting to decipher the etiology of CKDu in Canacona.

Another preliminary medical screening of the pattern of genitourinary problems of 298 patients residing in Canacona was conducted at a two day free medical camp organised in 2009 by Department of Urology, KLES Kidney foundation headed by Dr.Nerli (Nerli et al.,

2010). In this screening, laboratory tests like blood glucose, hypertension, serum creatinine, serum urea and urinary uric acid were conducted. Results indicated elevated levels of blood and urine parameters with normal blood pressure and glucose levels in 75% of the patients. This study merely assessed the prevalence of CKD in Canacona and concluded that a high incidence of CKD cases were noted in this taluka out of which 75% were not associated with an identifiable causal like diabetes and hypertension further supporting the disease to be of unknown etiology. They also highlighted males and females to be affected at a comparable extent with no gender bias depicted, with younger age groups (4^{th}-5^{th} decade) being impacted. These aforementioned limited studies that focused on attempting to decipher the etiology of CKDu in the Canacona taluka (Saiyed et al., 2005) and merely confirming the occurrence of a high number of CKDu cases (Nerli et al., 2010) in the taluka suffered from major limitations. Firstly, they failed to establish detailed prevalence statistics, geographical cum demographical distribution of CKDu in the taluka. Secondly, prospective risk factors and demographically stratified population groups that were most susceptible towards disease development were not identified. Thirdly, biochemical based pathological disease presentation pattern that has been appropriately described in other regional nephropathies and are crucial for identification of the type of renal pathology manifested has not been described for CKDu in Canacona. Hence, preliminarily this PhD work focused on addressing the aforementioned limitations which have been elaborated in detail in Chapter 2 of this thesis.

1.2.6 Potential causative or etiological factors for CKDu

The major histopathological characteristics of CKDu include tubular atrophy, interstitial inflammation, and interstitial fibrosis which are typical of the tubular pathological manifestation viz. chronic tubulointerstitial nephritis (CTN). CTN is major pathological condition known to affect the nephron's proximal tubular cells that are involved in toxin elimination and also interstitial cells surrounding proximal tubules which on incessant progression manifests in Chronic renal failure development (Lopez-Marín et al., 2014; Lusco et al., 2017). CTN is mainly caused by exposure to environmental nephrotoxins like heavy metals (lead, arsenic, cadmium, mercury), mycotoxin (i.e. ochratoxin), phytotoxin (i.e. aristolochic acid) and geogenic element-silica (Jayasumana et al., 2016; Levine et al., 2016)
Owing to the similarities in histopathological presentation of CKDu with CTN that is caused by exposure to environmental nephrotoxins, strong contribution of environmental toxins related to human activities has been advocated in the pathogenesis of CKDu. Hence CKDu is

suggested to be an environmentally acquired disease, possibly linked to prolonged exposure to nephrotoxins through various sources like drinking water or food (Lusco et al., 2017).

Therefore in order to assess for the potential exposure to nephrotoxins and associated manifestation of CKDu, it is essential to evaluate nephrotoxin levels in its chief exposure matrices viz. water and food (environmental monitoring). This provides the magnitude of nephrotoxin exposure in the targeted population that helps predict renal tubular damaging effects that can be inflicted (Wimalawansa, 2016). Hence levels of potential nephrotoxins implicated in CKDu etiology were determined in its exposure matrices viz. water and food in Canacona taluka. The results have been described in Chapter 3 (section 1 and 2) of this thesis.

It has been well reported that despite the presence of high nephrotoxins levels in exposure sources, the targeted population need not necessarily will be exposed to such nephrotoxins. Therefore in order to gauge the nephrotoxin exposure level, it becomes mandatory to monitor various biomarkers of exposure (i.e. the nephrotoxin itself) in the human biological matrix viz. blood and toxin induced biomarkers of renal tubular damaging effects(viz. tubular injury markers in the urine) and this process is called biomonitoring (Nanayakkara et al., 2014; Wimalawansa, 2016). Hence, in the current study, the nephrotoxin levels in blood [described in section 3 of chapter 3 in this thesis] and tubular dysfunction biomarkers in the urine of CKDu patients and controls [described in Chapter 2 and section 3 of chapter 3 in this thesis] were measured and their dose-effect association was established for evaluation of the role of the environmental nephrotoxins at the prevalent exposure levels in inducing renal tubular damage associated with CKDu development in the taluka [described in Chapter 3].

The potential nephrotoxins which have been implicated in CKDu causation in various developing countries and could possibly be implicated in Canacona are detailed below.

1.2.6.1 Nephrotoxic Heavy metals

Heavy metals are persistent, resistant, bio accumulative and not easily metabolized, hence perish for several years gradually accumulating in the food chain causing various health perils on prolonged exposure (Orr and Bridges, 2017). They possess long half-lives and high chemical stability which confers bioccumulative tendencies that allow accumulation in major target cells i.e. proximal tubule cells of renal nephron causing structural and functional damage resulting in secretory and reabsorptive defects which ultimately manifests in chronic tubulointerstitial nephritis. This tubular toxicity stems from the tendency of proximal tubular cells to reconcentrate divalent metals (heavy metals) which on accumulation consequently induces damaging effects (Jayasumana et al., 2016). The mechanism underlying the heavy

metals induced tubular damage is similar and is linked to oxidative damage manifested by oxidative stress associated with lipid peroxidation induced membrane damage, apoptosis, and inflammation that serve as mediators of metal induced nephrotoxicity (Wu et al., 2016).

1.2.6.1.1 Lead nephrotoxin

Lead (Pb) intoxication has been well reported to cause impairment of the proximal tubular architecture and histological changes, such as eosinophilic intranuclear inclusions in the tubular cells consisting of lead-protein complexes (Gwaltney-Brant, 2018; Lentini et al., 2017). These lead protein complexes possesses tendency to trigger the inflammatory cascade which in-turn simultaneously triggers apoptotic cell death via p53 gene activation resulting in development of interstitial fibrosis and tubular cell death, which manifests in the induction of grave proximal tubular toxicity (Cao et al., 2015;Jan et al., 2015; Tchounwou et al., 2012).

Another route of lead induced nephrotoxicity is mediated via formation of lead deposits in proximal tubular cells. On uptake in the human body, lead generally binds to low molecular weight proteins like b2M present in the blood which is freely filtered at the glomerulus but is reabsorbed by the proximal tubules (PT) cells owing to the intrinsic potency of the latter to reabsorb low-molecular weight proteins from circulation (Sabath and Robles-Osorio, 2015; Xu et al, 2018). This proximal tubular reabsorption is primarily mediated by endocytosis by binding to proximal tubular specific transporter i.e. ATP binding cassette-multidrug resistance protein (MRP-2) that results in increased uptake of lead in these cells. The longer half-life, higher chemical stability and bioaccumulative tendencies of lead, confers the tendency of lead to further form larger deposits in these cells causing swelling of tubular mitochondria (Jan et al., 2015; Lentini et al., 2017). This swollen mitochondria ultimately disturbs cellular respiration which is further intensified by intrinsic potency of lead to bind to sulphydryl groups founding phosphorylating enzymes forming inert metal complexes resulting in uncoupling of oxidative phosphorylation that is crucial for effective functioning of mitochondrial respiration. Additionally this accumulated lead disrupts antioxidant levels by forming inactive metal complexes with the thiol groups of anti-oxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase, compromising the anti-oxidant protection of these cells as well. Therefore this disturbed cellular respiration manifests in development of amplified oxidative stress (increased induction of reactive oxygen species (ROS) and depleted anti-oxidant levels by lead). The lead induced enhanced oxidative stress further inflicts oxidative damage to the DNA, proteins, and membrane that subsequently induces the activation of the apoptotic cell demise pathway via continual induction of DNA

damage sensitive p53 gene. Concomitantly, increased ROS triggers incessant induction of inflammation in the interstitial cells by activation of ROS sensitive inflammatory transcription factor viz. NF-kβ (nuclear factor kappa-light-chain-enhancer of activated B-cells). Therefore, prolonged exposure to high lead levels causes incessant induction of apoptosis and inflammation that consequently exaggerates into advancement of tubular atrophy and interstitial fibrosis respectively disrupting structural and functional integrity of proximal tubular-interstitial cells which ultimately manifests in CTN development, the major CKDu pathology (Lentini et al.,2017; Matović et al.,2015; Orr and Bridges, 2017).

Lead induced nephropathy has distinct histopathological findings on renal biopsy examination of the exposed subjects which includes acid-fast intranuclear inclusions of proximal tubule cells, interstitial fibrosis, interstitial inflammation with mononuclear cell infiltrates and focal tubular atrophy. Laboratory and clinical features include increased levels of urinary tubular dysfunction biomarkers like b2M and NAG and serum creatinine, decreased levels of serum bicarbonates and phosphates and progressive decline of GFR. Lead induced nephropathy can be detected by measurement of lead levels in the blood which is an ideal indicator of body burden of lead (Lentini et al., 2017; Sabath and Robles-Osorio, 2012).

The major exposure pathway to lead in humans is by ingestion and absorption in the gastrointestinal tract via consumption of groundwater or food contaminated by various anthropogenic activities like discharge of effluents enriched in lead into the land from smelting, batteries, paints, petroleum extraction industries. The released lead can consequently seep into the groundwater table or can be absorbed by plants entering humans due to its bioaccumulative tendencies through entry into the food-chain. Moreover, mining can also contribute to the contamination of the exposure sources viz. groundwater with lead which arises as a result of excessive leaching of lead from the ubiquitous lead deposits present in the aquifer's bedrock via acid mine drainage. It has been well-established by the WHO that 63% of the human exposure to lead primarily comes from consumption of groundwater contaminated with lead over a long-term followed by consumption of lead contaminated foodstuffs like cereals (Flora et al., 2012; Sankhla,et al., 2016).

The initial evidence supporting the causative role of lead in the development of CTN (major CKDu pathological manifestation) emerged from three epidemiological studies in Nicaragua, Central America. In these studies, exceedingly high blood lead levels were noted in an average of 92% of the CKDu affected subjects working in petroleum refineries as compared to below detectable levels in healthy controls (Abiola, 2017; Brooks, 2009; Laws, 2015). Epidemiological studies in Sri Lanka and Nicaragua in Central America have strongly

reported a remarkable dose-response relationship between lead levels in groundwater consumed and increased incidence of tubular dysfunction associated with CKDu manifestation in endemic areas. Herein, lead content in drinking water and blood in the endemic region were remarkably higher than healthy controls. Moreover, a prolonged exposure to lead levels $\geq$10 µg L^{-1} through long-term contaminated groundwater intake and related increase in blood lead levels of $\geq$50 µg L^{-1} was found to be associated with higher induction of tubular proteinuria (i.e. high levels of tubular specific proteins-b2M and NAG), significantly higher levels of serum creatinine, lower serum bicarbonates and phosphate levels, all of which are characteristic of tubular nephropathies (Johnson et al., 2013; Orantes et al., 2014; Ramirez-Rubio et al., 2013).

Few epidemiological studies in El-Salvador and Nicaragua of Central America have depicted that even low-level environmental lead exposure (6-8 µg L^{-1}) over time via consumption of contaminated drinking water causes development of lead inclusion bodies in proximal tubular cells which results in the development of interstitial fibrosis and tubular dysfunction (typical features of CTN), evident from increased serum creatinine, low levels of plasma β2-microglobulin, lowered GFR and hyperuricemia. The levels of these tubular dysfunction biomarkers can be subsequently reversed on adoption of chelation therapy. This nephrotoxicity displayed at chronic low exposure levels is attributed to the inherent potency of proximal tubules to reconcentrate multivalent metals and intrinsic bioaccumulative potency of lead that causes increased lead bioaccumulation in proximal tubules with time. This bioaccumulated toxin induces tubular damaging effects that consequently aggravate with time which results in the manifestation of CTN disease, which is the major pathological presentation of CKDu (Fadrowski et al., 2013; Lin et al., 2006; Rastogi, 2008).

1.2.6.1.2 Cadmium nephrotoxin

Prolonged cadmium exposure is mainly associated with the induction of progressive renal tubular dysfunction in humans with effects being dose dependent (Thévenod et al., 2016).On uptake in the human body, it binds to LMV proteins like b2M and metallothioneins which permits it to escape glomerular filtration that helps in complete reabsorption in PT cells by endocytosis through receptors viz. megalin and cubilin. On endocytosis, Cd dissociates from metallothioneins and is transported into the cytoplasm via divalent metal transporter (DMT1), localized in the luminal plasma membrane of the epithelial cells that allows it to induces severe renal damaging effects (Orr and Bridges, 2017; Yang and Shu, 2015). Cd deposits and directly attacks the mitochondria on prolonged higher exposure level by disrupting the

respiratory chain complex III of the same resulting in enhanced ROS production that manifests in oxidative stress induction which subsequently triggers DNA, protein and membrane damage signaling the incessant apoptotic cell death activation (Sabath and Robles-Osorio et al., 2012; Lentini et al., 2017) and simultaneous induction of inflammation by ROS mediated inflammatory factor viz. NF-kβ activation. This continual excessive apoptotic cell death and inflammation manifests in the development of tubular atrophy and fibrosis, the primary hallmarks of tubular injury characteristic of CTN, the major CKDu pathological manifestation (Friberg, 2017; Johri et al., 2010; Matovic et al., 2015; Orr and Bridges, 2017).

The major exposure pathway to cadmium in humans is by consumption and absorption in the gastrointestinal tract via consumption of food or groundwater contaminated by various anthropogenic activities like discharge of untreated industrial effluents from steel electroplating, battery manufacturing, tanning, plastic industries etc. It has been established by WHO that 61% of the human exposure to cadmium comes from Cd contaminated food like cereals (rice) and pulses (Paranagama et al., 2018; Swaddiwudhipong et al., 2015).

The preliminary evidence of the causal role of cadmium in CKDu came from a case-control analysis in Nicaragua wherein measured Cd levels were significantly higher in CKDu cases v/s the controls i.e. 0.78 vs. 0.22 μg/day with difference being attributed to heavily Cd contaminated rice-consumption in the CKDu group owing to proximity to a battery industry (Lunyera et al., 2016). Similarly, additional evidence supporting the contribution of cadmium in CKDu causation emerged from several studies conducted in North Central Province (NCP) of Sri Lanka. These studies determined levels of Cd in soil, well-water (used for drinking), freshwater fish viz. Tilapia and plants (like rice, lotus rhizomes) from CKDu prevalent regions and highlighted a strong association between increased Cd concentrations in the environment and CKDu development. Higher Cd levels significantly surpassing WHO permissible levels were noted in all sources which resulted in a higher total intake of cadmium through rice, water and fish consumption. The higher Cd levels in affected areas was attributed to overuse of super phosphate fertilizers enriched in Cd (70 mg/kg) as opposed to negligible use in healthy regions; which could have resulted in greater uptake in food crops and thus higher human exposure to Cd in the endemic region (Bandara et al., 2008; Bandara et al., 2010 and 2011; Jayatilake et al., 2013; Paranagama et al., 2018; Rajapakse et al., 2016; Wanasinghe et al., 2018; Wanigasuriya, 2014; Wasana et al., 2016; Wimalawansa, 2014). Other biochemical based community cross-sectional studies in Sri-Lanka obtained a strong dose-response relation ($p<0.05$) between blood Cd levels and CKDu stage (evident from

tubular dysfunction marker levels) in CKDu patients as compared to healthy controls .Herein, the mean blood Cd levels significantly increased in CKDu patients as compared to controls (i.e. 1.039 μg/g v/s 0.021 μg/g) which proportionally resulted in increased levels of tubular dysfunction biomarkers in CKDu patients signifying enhanced CKDu induction on Cd exposure (Nanayakkara et al., 2014; Jayasumana et al., 2015; Rajapakse et al., 2016).

1.2.6.1.3 Arsenic nephrotoxin

Arsenic (As) has been well reported to exist in inorganic and organic forms with the inorganic form (i.e. As metal ion) being most toxic. The major toxicological effect inflicted by As is CTN that ultimately manifests in renal failure development of (Fowler, 2015).

Homologous to the other heavy metals, As targets proximal tubular cells. Arsenic gains entry into PT cells via binding to proximal tubular specific transporter i.e. ATP binding cassette-multidrug resistance protein (MRP-2) and owing to its bioaccumulative tendency significantly deposits in these cells. The arsenic deposits in the cells directly attack mitochondria by disrupting the respiratory enzymes structure and function by binding to constituting sulfhydryl groups causing uncoupling of oxidative phosphorylation involved in respiration resulting in the excessive ROS generation. Simultaneously As depletes antioxidant anti-oxidant enzymes viz. SOD and glutathione peroxidase by forming inert metal complexes with its constituting thiol groups that disrupts the enzymes functional integrity. Overall, resulting in enhanced ROS production which can simultaneously trigger DNA damage and inflammatory factor (i.e. NF-Kβ) activation that consequently triggers the incessant induction of apoptotic cellular death and inflammation which manifests in tubular atrophy and fibrosis development, hallmarks of CTN, distinctive of CKDu development (Orr and Bridges et al., 2017; Robles-Osorio et al., 2015; Sabath and Robles-Osorio, 2012).

Human exposure to As can occur through oral and inhalation routes with oral route being the significant route. As can enter the human body via the oral route by consumption of groundwater or food contaminated with arsenic by various anthropogenic activities like discharge of untreated effluents from metal smelting, fossil fuel combustion industries and runoff from agricultural lands utilizing As enriched (10-30 mg/kg) superphosphate agrochemicals. WHO has established As concentrations in drinking groundwater to contribute to 72% of exposure and infliction of health perils (Bhattacharya et al. 2002; Hsu et al., 2017).

Limited epidemiological evidence is available from the NCP of Sri Lanka that supports the role of arsenic in CKDu causation. These studies evaluated the levels of As in the well-water,

fish, plants, and blood of the subjects residing in CKDu prevalent regions. The results demonstrated a strong involvement of high As content in the well-water (i.e. 15.3 ppb) that significantly surpassed the WHO permissible limit of 10 ppb which resulted in elevated blood As concentrations in CKDu affected individuals (i.e. 25.6 µg/L) as compared to healthy controls that significantly exceeded the WHO permissible blood levels of 15 µg/L which highlighted a strong association with CKDu development in the region. This difference in As levels in CKDu prevalent and healthy control region was attributed to excessive use of arsenic enriched superphosphate agrochemicals for paddy cultivation that is widely conducted in CKDu endemic regions as compared to control regions. No contribution to As exposure in CKDu affected individuals were noted from freshwater fish-tilapia and rhizomes consumption (Dharma-Wardana et al., 2015; Jayasumana et al., 2013).

Another study exploring the role of arsenic in the groundwater in the causation of CKDu in the NCP of Sri-Lanka found that the levels of arsenic in the blood of the CKDu affected cases (i.e. 40.9 µg/L) were found to be well above the levels known to cause oxidative injury in kidneys (i.e. 30 µg/L) with significantly lowered levels of blood arsenic noted in the healthy unaffected controls (i.e. 5.6 µg/L).This difference in blood levels of the CKDu cases v/s the controls was attributed to the variations in groundwater contamination of both of these regions with the groundwater of the CKDu prevalent region being heavily contaminated with WHO limit surpassing levels of arsenic (Jayasumana et al., 2014; Rajapakse et al, 2016)

An epidemiological cross-sectional study of CKDu cases in Uddanam region of Andhra Pradesh (India), demonstrated a significant ($p<0.05$) association between groundwater containing toxic As levels (i.e. 35 ppb) which resulted in elevated blood As levels (i.e. 42.1 µg/L) and associated increased induction of tubular proteinuria (i.e. elevated b2M and NAG) associated with CKDu manifestation in this region (Reddy and Gunasekar, 2013).

1.2.6.1.4 Mercury nephrotoxin

Mercury (Hg) is known to inflict severe renal injuries specifically proximal tubular toxicity on high exposure. The major exposure source (i.e. 62%) is via consumption of food contaminated with Hg by anthropogenic activities like discharge of untreated effluents from industries (like batteries, smelting) or municipal or domestic sewage enriched in medical waste like Hg from thermometers, dental amalgam etc. in the aquatic ecosystems or run off of Hg enriched fertilizers form the agricultural soils into the water bodies (Kim et al., 2016). Upon ingestion Hg is readily absorbed by the gastrointestinal tract into systemic circulation

which is then delivered to the target organ-the kidney specifically proximal tubular cells owing to involvement of the latter in metal detoxification (Bridges and Zalups, 2017).

Information on the exact Hg induced renal toxicity mechanism is scarce. However some studies indicate that the Hg ions gain access into the proximal tubular cells by binding to amino acid transporters viz. Cysteine S (Cys-S) located in the luminal plasma membrane of the cells due to the strong affinity of Hg to bind to thiol containing molecules like Cys-S. Electronic microscopic studies have revealed that Hg on gaining entry into tubular cells excessively deposits within which ultimately triggers incessant tubular cell injuries via induction of oxidative membrane damage. These damaged tubular cells releases excessive proximal tubular specific brush-border lysosomal enzyme viz. NAG and proteins viz.b2M in the urine that indicates enhanced induction of tubular proteinuria owing to compromised structural and functional integrity signifying severe tubular dysfunction development, typical of CKDu manifestation (Bridges and Zalups, 2017; Lentini et al., 2017; Orr and Bridges, 2017).

Limited reports are available from Sri Lanka and Central America that highlight the role of Hg in CKDu causation. In these studies a significant dose-effect relationship was noted between the high levels of Hg present in the blood of CKDu affected subjects in the endemic region and increased levels of excretion of tubular dysfunction biomarkers (viz. b2M and NAG) into the urine. This was backed by the lack of significant association between safe blood Hg levels noted in unaffected controls and absence of tubular injury induction. Herein the difference in blood Hg levels in endemic and unaffected regions arose from the variations in Hg poisoning of the fish consumed in these regions with high levels of contamination noted in endemic regions which was attributed to the discharge of untreated effluents from the smelting industry into the regions' sea water. These results highlighted the role of chronic high level of Hg exposure in CKDu manifestation (Akerstrom et al., 2017; Franko et al., 2005; Jayasumana et al., 2015; Levine et al., 2016).

1.2.6.2 Nephrotoxic mycotoxin viz. Ochratoxin

Ochartoxin A is a mycotoxin produced by the fungal species viz. *Aspergillus ochraceus* in tropical climatic regions of India, Sri Lanka, and Central America. Ochartoxin A is a common contaminant of food grains, like cereals (rice and wheat) and pulses (chickpea), which is produced during inadequate storage conditions like high humidity, increased moisture content and low temperatures (Duarte et al., 2010).

Humans are solely exposed to ochratoxin through the dietary route via consumption of food grain cereals and pulses. Owing to the thermal and chemical stability of ocharatoxin that causes it to be unaffected by normal food processing temperatures, permits it to bioaccumulate, conferring a long half-life of 35 days in the human body. On ingestion, it is highly absorbed by the gastrointestinal tract into systemic circulation wherein it acts as an etiological agent of chronic tubulo interstitial nephritis and urinary tract tumors (Bui-Klimke and Wu, 2015, Heussner and Bingle, 2015; Malir et al., 2016). Like other nephrotoxins, ochratoxin mainly targets renal proximal tubular cells. It possesses a high binding potential to LMV plasma proteins like b2M which precludes glomerular filtration and permits subsequent reabsorption in these cells (Asrani et al., 2015). In the proximal tubular cells, OTA induces defects in organic anion transporters located on the cell's brush border and basolateral membranes which are crucial for tubular filtration, reabsorption, and secretion thereby triggering disruption of structural and functional integrity which manifests in tubular nephropathy development. On entry into tubular cells, OTA forms large deposits due to its inherent bioaccumulative potential which causes direct attack on mitochondria by inducing swelling (Burckhardt and Burckhardt, 2011). The swollen mitochondria disturb cellular respiration by uncoupling oxidative phosphorylation resulting in excessive ROS generation that surpasses the antioxidant defense manifesting in oxidative stress. This excessive oxidative stress simultaneously induces DNA damage that triggers p53 mediated apoptotic pathway activation and concomitant induction of inflammation via ROS sensitive inflammatory transcription factor (viz. NF-kβ) activation. This continually induced apoptosis and inflammation exaggerates into the development of tubular atrophy and interstitial fibrosis, the unique hallmarks of CTN, that is typical of CKDu pathological manifestation (Bui-Klimke and Wu, 2015; El-Haleem et al., 2016; Enyiukwu et al., 2018; Heussner and Bingle, 2015; Tao et al., 2018; Vettorazzi, et al., 2013; Zhao et al., 2017).

OTA was also found to be majorly involved in the pathogenesis of Balkan endemic nephropathy (BEN) as well. BEN is a form of CTN that was first described in 1956 and was endemic to the Balkan region comprising of Croatia, Bosnia, Serbia, Romania and Bulgaria distributed along Danube River. It majorly affects younger adults (3^{rd}-5^{th} decade), equally in males and females. The major clinical features include tubular proteinuria (increased urinary secretion of b2M and NAG), enhanced serum creatinine, reduced serum bicarbonate, or phosphate and severely decreased GFR. Biopsy histopathological analysis displays tubular atrophy and interstitial fibrosis with 20% patients also demonstrating a concomitant urothelial carcinoma (Pavlovic, 2014; Stefanović et al., 2011). Thus the disease's major pathological

presentation encompasses sudden onset of asymptomatic chronic renal failure followed by transitional incidence of urothelial tumours. The primary cause of BEN was believed to be long-term exposure to OTA via regular consumption of contaminated bread (staple diet) which was produced from improperly stored wheat grains enriched in contaminating *Aspergillus* sp. (Gifford et al., 2017; Pfohl-Leszkowicz et al., 2002; Stiborova et al., 2016). Detection of high ochratoxin levels in association with CKDu incidence is reported in developing countries like Tunisia, Central America and Sri Lanka (Domijan et al. 2009). Cross-sectional CKDu analysis in Tunisia demonstrated that the concentrations of urinary tubular dysfunction marker viz. b2M enhanced in strong association with the elevation of blood ochratoxin levels noted in CKDu patients, therefore confirming the high occurrence of tubular structural and function alterations that are typical of CTN and involvement of this mycotoxin in these tubular alterations. Moreover, the high blood ochratoxin poisoning (i.e. mean= 42.3µg/L) in CKDu patients positively correlated with high ochratoxin contamination in the food (i.e. mean=35.6 µg/kg) of the region as compared to lowered WHO safe levels in unaffected regions that suggested diet (through food grains-rice and wheat consumption) to be the major exposure route in the CKDu endemic region. This difference in ochratoxin food contamination in CKDu affected and non-affected region proportionally translated into significant variations in blood ochratoxin levels that consequently induced grave induction of proximal tubular toxicity in CKDu endemic region (Khlifa et al., 2012; Hassen et al., 2004).

Epidemiological studies of CKDu cases in Sri Lanka and Central America demonstrated a strong and significant ($p<0.05$) dose-effect relationship between increased daily ochratoxin intake levels and enhanced incidence of tubular dysfunction associated with CKDu development in the affected region. In these studies, significantly higher levels of ochratoxin were detected in food grains (i.e. rice) consumed in the CKDu prevalent region as compared to the non-affected regions (i.e. 39.3 µg/kg in cases v/s 1.1 µg/kg in controls) which resulted in intake levels of the endemic region (i.e. 35 ng/kg bw) to surpass WHO established tolerable daily intake levels (i.e. 14 ng/kg bw) and intake levels (i.e. 20 ng/kg bw) known to induce severe proximal tubular nephrotoxicity. The enhanced intake translated into higher blood ochratoxin levels in CKDu patients as opposed to controls (i.e. 45.6 µg/L in cases v/s 2.6 µg/L in controls) that exceeded the WHO permissible range of 5 µg/L and CTN inducing range of 6 µg/L.This elevated blood ochratoxin contamination in CKDu patients positively correlated with enhanced urinary excretion of tubular damage specific proteins (viz. b2M and NAG) which signified the increased prevalence of tubular dysfunction associated with CKDu

manifestation in this region (Badurdeen et al., 2016; Castegnaro et al. 2005; Correa-Rotter et al., 2014; Desalegn et al., 2011; Rajapakse et al., 2016; Seneff and Orlando, 2018).

1.2.6.3 Nephrotoxic phytotoxin viz. Aristolochic acid

Aristolochic acid (AA) is a natural alkaloid produced by weed-*Aristolochia indica* (birthwort), which is endemic to cereals (rice and wheat) and pulses (chickpea) fields. Hence the major exposure route to AA is dietary i.e. by consumption of cereals (rice and wheat) contaminated with AA (Debelle et al., 2008; Michl et al., 2013).AA displays bioaccumulative tendency and thermal cum chemical stability at high food processing temperatures that confers a long-half-life of 33 days in the human body. On intake it is absorbed by the gastrointestinal tract into systemic circulation, which inflicts nephrotoxicity, genotoxicity and carcinogenicity on chronic exposure (Gökmen et al., 2013). Homologous to other nephrotoxins, AA mainly targets renal proximal tubular cells (Luciano and Perazella, 2015). AA being anionic abolishes the homeostatic tubular organic anion transport mechanism which is crucial for xenobiotic efflux and reabsorption of essential molecules. This AA induced disruption of organic anion transporter (OAT1) causes AA accumulation within these cells inducing tubular toxicity. This tubular toxicity is mediated by AA deposits directly attacking the mitochondria by inciting swelling that causes disruption of cellular respiration and enhanced ROS generation. This excessive ROS simultaneously triggers incessant induction of DNA damage and inflammation that results in the development of tubular atrophy and fibrosis respectively manifesting in CTN, the major hallmark of CKDu (De Broe, 2012; Gokmen et al., 2013; Jadot et al., 2017; Luciano and Perazella, 2015). Moreover, these AA deposits at remarkably higher levels (exceeding the nephrotoxicity inducing range can rapidly bind to DNA to form aristolactam DNA adducts which induces hallmark A:T → T:A base transversion in the proximal tubular p53 gene that manifests in the development of urothelial malignancies. The involvement of AA in inducing nephrotoxicity was first confirmed by detection of AA-DNA adducts in renal tubular cells of biopsies from AA-intoxicated patients. Their involvement was further confirmed by positive correlation between cumulative ingested AA dose and progression of renal function deterioration especially CTN induction (Arlt et al., 2007; Grollman, 2013; Jadot et al., 2017; Stiborova et al., 2017; Wu et al., 2015). Moreover owing to the carcinogenicity of AA, chronic renal failure is sometimes succeeded by transitional induction of urothelial malignancies which occurs when blood AA level surpasses the nephrotoxicity inducing range (10-20 µg/L) (Jelakovic et al.,2012; Lemy et al., 2008; Gokmen et al.,2013; Yun et al., 2015).

Furthermore, these studies have strongly suggested the contribution of AA in BEN causation owing to similarities in renal pathological presentation of CTN being induced in both forms of nephrotoxicity viz. aristolochic acid induced nephropathy and BEN (Gruia et al., 2018; Stiborova et al., 2016).The exact implication of AA in BEN development was confirmed by Ivic in 1969. He proposed etiology of BEN to be related to chronic *Aristolochia* poisoning in which plant seeds encountered in wheat fields, intermingled with wheat grains during the harvesting process. This resulted in human exposure to AA by ingestion of bread (staple diet) prepared from flour derived from contaminated grains. He also demonstrated in rabbit models that flour prepared from *A.indica* seeds induced a nephropathy, resembling BEN (Grollman and Jelakovic, 2007; Jadot et al., 2017; Jelakovic et al., 2012; Luciano and Perazella, 2015).

There is limited evidence from Sri Lanka and India that support causation of AA in CKDu development.An environmental study of CKDu cases in Uddanam region of Andhra Pradesh,found intake levels of AA in the endemic region to significantly exceed those that induce proximal tubulotoxicity (i.e. 5 ng/day kg bw) as opposed to negligible and well below JCEFA established tolerable intake levels of AA (i.e.1.1 ng/day kg bw) noted in non-affected region (Jha and Chugh, 2003;Vanherweghem, 1997).CKDu assessments in Sri Lanka demonstrated mean blood AA levels in CKDu patients(i.e. 16.5 µg/L) to significantly exceed proximal tubular dysfunction inducing levels(i.e.10.3 µg/L) as opposed to WHO safe levels noted in healthy controls (i.e.1.1 µg/L). This difference in blood AA levels between CKDu prevalent and non-endemic region rested on variations in AA contamination of wheat consumed by these regions with higher levels noted in the endemic region that surpassed WHO permissible levels (2 µg/kg). Moreover, rise in blood AA levels in CKDu patients significantly correlated with rising urinary excretion of tubular proteins(NAG),supporting tubular nephropathy induction in this region, characteristic of CKDu manifestation (Jha, 2010; Rajapakse et al.,2016; Stanifer et al., 2016; Wanigasuriya et al.,2007 and 2011).

1.2.6.4 Emerging nephrotoxic potency of a trace geogenic element-Silica

Silica abundantly constitutes the earth's crust occurring in rock, sand and soil owing to high reactivity of silicon for oxygen. Despite its innate abundance, it lacks bioavailability under ideal environmental conditions categorizing it as a trace geogenic-element (geogenic owing to emergence from the earth's crust) (Cornelis and Delvaux, 2016). Recently, silica is claiming emerging contaminant status by the USEPA and EPA owing to its ability to induce health effects when made excessively bioavailable for human exposure by anthropogenic activities like mining. Drilling/blasting and acid mine drainage (AMD) associated with

mining can respectively contaminates silica's major exposure routes viz. air and groundwater. Air is the most widely studied exposure route during on-going mining activities owing to direct release of silica particles in the air by drilling, but this route does not contribute to exposure on termination of mining (OSHA, 2016). Recently, few epidemiological studies highlighted groundwater to be a potential exposure route that sustains chronic exposure even after closure of mining by outcomes of mining viz. AMD (Fantong et al., 2009; Khan et al., 2015; Khandare et al., 2015). AMD is a persistent environmental problem in tropical countries that causes acidification of the groundwater in immediate contact and of the neighbouring aquifers via drainage that consequently decreases the quality of the groundwater by leaching out various nephrotoxins like heavy metals and trace geogenic elements viz. silica from ubiquitously enriched aquifer's bedrock into the water, whose availability is reported to be enhanced at an acidic pH. Silica availability is well noted to rise at an acidic pH due to absence of reactive silicate anion formation (that ideally forms at an alkaline pH) thereby preventing inert metal silicate complex formation of silica with essential metal ions (like calcium and magnesium) which under ideal conditions restrict its availability for human exposure by silica trapping ,thus conferring protection from silica's toxic effects (Amjad and Zuhl, 2010; Fantong,et al., 2009; Icopini et al., 2005; Kumari et al., 2010; Pradeep et al., 2016; Zuhl and Amjad, 2013). Silica on exposure can be absorbed to the maximum (i.e. approx. 95%) by the gastrointestinal tract into systemic circulation and inflicts disorders like silicosis, bronchitis etc. (Pollard, 2016). Epidemiological studies have primarily focused on induction of respiratory diseases on silica exposure neglecting possibility of extra-pulmonary toxic-effects. Recently, studies shifted attention to development of proximal tubular toxicity and associated CKD induction in conjunction with silica exposure (Mohner et al., 2017; Sponholtz et al., 2016). Animal renal toxicity studies have suggested that absorbed silica binds to LMV blood proteins (i.e. b2M) just like heavy-metals allowing escape from glomerular filtration and is reabsorbed by proximal tubules by endocytosis owing to inherent tendency of the latter for LMV protein reabsorption. On reabsorption, it dissociates from b2M and binds to tubular transporters like metallothioneins and metal transporter-2 (MT2) enriched in these cell's luminal plasma membrane, forming silica deposits owing to its proven chemical stability, long half-life and bioaccumulative potency. These deposits can inflict proximal tubular toxicity manifesting in kidney damage but the exact molecular toxicity mechanism is unknown till date (Dobbie and Smith, 1982; Ghahramani, 2010; Howse and Bell, 2011; Markovic and Arambasic, 1971).

Preliminary evidence for silica nephrotoxicity came from studies in animal models like guinea pigs (Dobbie and Smith, 1982; Markovic and Arambasic, 1971), mice (Kawanabe et al., 1992), dogs (Newberne and Wilson, 1970). Herein the animal models were administered with silica (in drinking water) at increasing concentrations of 10-150 mg/L for period of 10 months with suitable untreated controls also maintained. The levels of urinary tubular dysfunction markers (viz. b2M and NAG) significantly increased in direct correlation with the silica levels administered through drinking water with remarkable increase noted from 50 mg/L onwards and no significant rise noted on lower dosing following 4 months of exposure. The elevation in tubular injury markers were also remarkably associated with blood silica levels with highest marker concentrations noted at elevated blood silica levels of 100 mg/L which shows a direct translation of silica levels in the water to the blood signifying high gastrointestinal silica absorption and consequent induction of tubular damage. Significant elevated numbers of silica deposits were noted on immunohistochemical staining in the renal cortical peritubular parenchyma with rising concentration and duration of exposure which further confirmed silica's bioaccumulative potency that can amplify renal damaging effects on prolonged exposure. Renal histopathological changes on animal sacrifice and biopsy analysis following 10 months exposure comprised of tubular atrophic and dystrophic changes along with fibrotic lesions noted in the cortical parenchyma. These alterations consisted of interstitial inflammatory proliferative lesions with mononuclear and plasma cell infiltrates coupled with tubular cell death which led to hyalinization and fibrotic changes in the tubular cortex resulting in tubular atrophy and scarring that manifested in kidney contraction and shrinkage inflicting renal failure in these animals. These silica induced renal histopathological changes displayed a dose-dependency wherein despite emergence of significant changes at 50 mg/L of silica, it remarkably increased with rising silica exposure duration being more prominent at higher doses (i.e. 100 mg/L) and above with no characteristic changes noted on lower dosing. Overall, these animal based silica toxicity studies highlighted the nephrotoxic potency of silica on chronic exposure to doses(55-150 mg/L) in triggering tubular atrophy along with proliferative interstitial inflammatory lesions and fibrotic alterations that were typical of CTN, the major CKDu pathological manifestation. Following animal studies, few epidemiological studies (Markovic and Lebedev,1965; Markovic, 1968 and 1974) explored silica's nephrotoxic potency in inducing CTN, wherein chronic intoxication with silica in drinking well water emerging from excess erosion of a silicate mineral (i.e. granite) were strongly suggested to be involved in BEN. These results were concluded from the observance of increased urinary excretion of tubular dysfunction

markers viz. b2M with enhancing silica exposure to high doses (102.5 mg/L and above) that induced the manifestation of CTN. Silica nephrotoxicity was believed to be exhibited by its water soluble form viz. silicic acid which was proved to bind to LMV proteins (b2M), and reabsorbed into nephron's proximal tubule by endocytosis. This endocytosed silicic acid consequently accumulated forming silica deposits in proximal tubular cells that triggered tubular toxicity, thereby allowed those authors to strongly highlight silica's nephrotoxic tendency (Markovic and Lebedev, 1965 and Markovic, 1974).

Initially silica nephrotoxicity was reported from occupational silica dust exposure and renal pathology was proved to be a tubulointerstitial disease that was incited as silicotic complication. However studies reported occurrence of renal damage in non-silicotic subjects (not occupationally exposed) but exposed through other routes-drinking water. In studies of 50 long-term silicotic patients (exposed to silica for $\geq$ 25 yrs), tubular proteinuria was prevalent in 69% and renal insufficiency in 25% (Saita and Zavagilia, 1951). Tubular histopathological analysis revealed alterations of tubular atrophy and fibrosis and silicon content in 66.3% who died of progressed silicosis (Kolev et al.,1970; Saldanha et al., 1975).

Various epidemiological studies further highlighted silica nephrotoxicity to be dependent on the duration of exposure as well apart from the dosage. In studies of granite mine or sand blasters or ceramic workers exposed to silica (mean level of more than 75 $\mu g/m^3$ for an average duration of 25 yrs) and related sex and age matched unexposed controls with no renal disorder history were examined for urinary tubular dysfunction biomarkers levels viz.b2M and NAG. The results demonstrated b2M and NAG excretion in silica exposed subjects significantly rose in direct correlation with exposure duration and dose with highest biomarker levels noted on exposure for more than 20yrs at levels above 60 $\mu g/m^3$. Results indicated that subjects chronically exposed to silica were highly likely to develop chronic renal failure (risk ratio=3.8, $p<0.05$) as opposed to those who were not exposed and risk of renal failure elevated in a dose and time dependent manner with higher risk noted in those exposed at higher exposure levels i.e. >70 $\mu g/m^3$ for 25 yrs of employment (Risk ratio=7.7, $p<0.05$). These studies highlighted severe induction of irreversible tubular dysfunction specifically CTN on prolonged exposure to higher silica levels (Calvert et al., 1997; Ng et al., 1993; Rosenman, 2000; Steenland et al., 1990). Additionally, WHO and US Centre for Disease Control (CDC) have stated that latest epidemiological silica dust exposure analyses have described enhanced prevalence of death from extra-pulmonary disorders like renal dysfunction on chronic exposure to high silica doses i.e. greater than 50$\mu g/m^3$. The mode of silica's toxic action was assumed to be by direct attack on proximal tubular cells but the exact

molecular mechanism has not been described (Mcdonald et al., 2005; Steenland et al., 2001; Steenland, 2005). Cross-sectional analyses of silica exposed workers and matched controls for a duration of 35 yrs illustrated that any type of silica exposure (as opposed to none) was directly related with 70.2 % enhanced risk of CKD (risk ratio= 1.70, $p<0.05$) in multivariate adjusted models. The risk ratios demonstrated a linear association with exposure duration with increased risk noted at higher periods of 32 yrs (Mohner et al., 2017; Vupputuri et al., 2012).

Recently few epidemiological studies have highlighted silica's ability inducing nephrotoxicity via another exposure route i.e. consumption of contaminated drinking groundwater. Various other studies have scrutinised relationship between silica levels in drinking water and induction of kidney disorders in different CKDu endemic regions. In cross-sectional studies in El Salvador (Central America), kidney damage was examined following chronic silica exposure (mean level of 96.3 mg/L for 20 years) through contaminated groundwater consumption in subjects who had no silicosis history. In these subjects, a remarkably enhanced ($p<0.05$) excretion of tubular dysfunction markers viz. b2M and NAG was noted which advocated that silica can trigger subclinical renal tubular disorder on chronic high levels of silica exposure, even in the absence of silicosis (Hotz et al., 1995). Cross sectional studies of CKD cases in Yugoslavia, Europe determined levels of urinary tubular dysfunction marker viz. b2M and blood silica in CKD patients and matched controls. The results revealed that urinary b2M concentrations enhanced in strong association with the elevation in blood silica levels noted in CKD patients, thereby highlighted involvement of silica in induction of high incidence of tubular damage, typical of CTN. Moreover high blood silica levels (89.3 mg/L) in CKD patients positively correlated with elevated silica levels in well-water (i.e. mean= 94.9 mg/L) consumed in this region, suggesting alimentary contamination to be the major silica exposure route in this region. This variation in silica levels in well-water of CKD affected and non-affected regions proportionately translated into noteworthy differences in the blood silica in these groups which induced severe proximal tubular toxicity associated with CKD (Goldsmith and Goldsmith, 1993; Markovic, B., 1968; Markovic, 1971; Markovic et al., 1976; Radovanovic et al., 1991 Stiborova et al., 2016).In a another epidemiological and biochemical analysis of endemic CKDu in Egypt, significantly higher silica levels were noted in blood of CKDu patients (88.3 mg/L) that surpassed levels proved to induce severe renal histopathological alterations of tubular damage viz. tubular atrophy and fibrosis in animal-models (i.e. 65 mg/L), thus highlighted significant induction of tubular dysfunction at prevalent exposure levels in this region. This was backed by the

presence of lowered blood silica levels (i.e. 3.6 mg/L) in non-endemic regions. This variations in blood silica levels in CKDu affected and non-affected regions rested on differences in silica groundwater contamination in these regions that manifested in differential silica exposure levels and associated tubular injuries induction (El-Safty et al., 2003; Ibrahim et al., 2011). In a separate multivariate regression evaluation of CKDu case-control cases in Sri Lanka, demonstrated significantly higher levels of silica (90.1 mg/L) in 89.6% of groundwater samples collected from CKDu endemic region as compared to lowered levels (12.3 mg/L) noted in 100% of samples from non-affected region with former affected region's silica levels demonstrating a positive relationship with magnitude of CKDu development. Additionally this study indicated an increased risk of 65.3% (risk ratio=1.65, $p<0.05$) of CKDu development and 23.1% risk of mortality (risk ratio=1.23, $p<0.05$) from renal failure on exposure to prevalent levels in the endemic region (Gunatilake et al., 2015; Valcke et al., 2017). In a study of 372 silica exposed individuals through drinking water in Central America, there was an increased prevalence of tubular dysfunction associated with CKDu manifestation in 71.1 % exposed individuals and 16.3% succumbed to renal failure. The higher CKDu prevalence in silica exposed subjects displayed a mean age of 42.3 yrs. and significant association with exposure duration (i.e. $\geq$ 27.4 yrs) with enhanced induction of tubular proteinuria noted at longest exposure period (i.e. 36.3 yrs), signifying remarkable induction of nephrotoxicity on chronic silica exposure (Millerick-May et al., 2015). Further evidence for silica role in CKDu causation emerged from an epidemiological cum biochemical cross-sectional analysis of CKDu in Uddanam (Andhra Pradesh). In this study, significantly positive dose-response relationship was noted between high blood silica levels in CKDu patients in endemic region and enhanced urinary excretion of tubular injury biomarkers (viz. b2M and NAG). The elevated blood silica levels in CKDu patients as compared to controls (89.6 mg/L in cases v/s 7.9 mg/L in controls)emerged from comparatively higher silica groundwater contamination in the endemic region as opposed to lowered contamination in non-endemic region (i.e. 92.2 mg/L in cases v/s 13.3 mg/L in controls). These enhanced blood silica levels in CKDu patients positively associated with elevated urinary excretion of tubular injury proteins (viz. b2M and NAG) that indicated enhanced induction of tubular damage directly linked with CKDu pathological manifestation in this region (Khandare et al., 2015).

The best way to assess nephrotoxic potency of a toxicant is by evaluating renal pathological changes induced on nephrotoxin exposure. Limited human epidemiological studies have reported renal histopathological alterations manifested on silica exposure. Initial evidence

emerged from studies wherein silica in drinking water (≥ 90mg/L) proved to be nephrotoxic on prolonged exposure that inflicted chronic inflammation in the interstitial tissue typical of CTN (Policard and Collet, 1954; Policard et al., 1960 a and b; Policard et al., 1961). Renal histopathological analysis during autopsy of 45 granite mine workers who died of advanced silicosis (after 31 yrs employment) revealed intraluminal sloughing of proximal tubule, cytoplasmic granularity, vacuolization of tubules filled with dense osmiphilic particles along with focal tubulointerstitial nephritis with significant occurrence of silica deposits in the renal corticular tissue comprising of proximal tubules (Saldanha et al.,1975). An independent renal biopsy examination of the 21 endemic nephropathy cases noted in Yugoslavia caused by chronic intoxication with silica (96.3 mg/L) via groundwater consumption (for 33.3 years) revealed primary tubular lesions with diffused dystrophic cum atrophic processes in the parenchyma and remarkable silica deposition in renal cortico-tubular portion. Moreover, proliferative inflammatory lesions in the interstitial tissue (surrounding proximal tubules) with mononuclear and lymphocytic cellular infiltrates were noted followed by hyalinisation and tissue fibrosis that resulted in kidney contraction. These pathological changes were typical of CTN (Markovic and Lebedev, 1965; Markovic, 1972and1974; Radovanovic et al., 1991). In autopsy based renal histopathological analysis of 43 sand blasting workers who developed severe tubular proteinuria and lethal renal failure after 29.3 yrs of high silica exposure (greater than 75.3 $\mu g/m^3$) revealed tubular hypercellularity cum fibrosis with characteristic dense microtubules and lysosomes in the proximal tubules. Additionally, distinctive interstitial inflammation with mononuclear cell infiltrates was also noted that culminated in fibrotic manifestation and kidneys shrinkage (Giles et al., 1978). In a clinical and histopathological analysis of 39 silica exposed subjects (for 34.7 yrs) through drinking water (levels of 98.6 mg/L) in Bulgaria revealed clinical representation to be characterized by normotension and tubular proteinuria (i.e. increased urinary excretion of tubular injury protein viz. b2M) which was associated with tubular lesions. Pathologically, kidneys appeared contracted and histologically, characterized by tubular damage, luminal dilation and atrophy coupled with interstitial fibrosis (Bolton et al., 1981). In a similar renal biopsy examination of 15 silica exposed individuals through chronic consumption of silica contaminated groundwater (105.6 mg/L for 40.2 yrs) in Yugoslavia, depicted silica deposition in the nephron's proximal tubular portion in the cortex. Pathological changes comprised of degenerative alterations in tubular epithelium, tubular scarring and interstitial inflammatory and fibrotic lesions in silica exposed subjects (Markovic and Lebedev, 1965b). Therefore, evidence gathered from the renal histopathological analysis of aforementioned

silica exposed case-control studies revealed that the silica on prolonged exposure to high levels induced renal pathological alterations comprising of tubular atrophy and interstitial inflammatory lesions coupled with fibrosis. These histopathological changes are typical of CTN which is reported to be the CKDu pathological manifestation, thus suggesting silica's nephrotoxic potential in CKDu causation.

However, *in-vitro or in-vivo* studies explaining cellular and molecular mechanisms underlying silica-induced nephrotoxicity were lacking till date. Hence in this thesis we elucidated the cellular and molecular toxicological mechanisms of silica induced nephrotoxicity as a function of dose and time using nephrotoxin's primarily targeted renal cells viz. normal human renal proximal-tubular cells (HK-cells) as an *in-vitro* model (Li et al., 2017); which has been described in Chapter 4.

1.2.7 Treatment and Prevention of CKDu

No precise recommendations/guidelines are present for CKDu treatment. The strategies used for CKD treatment specifically tubular dysfunction are utilized in majority of CKDu settings. For heavy-metals induced nephropathy, chelation therapy may assist in reduction of renal damage. Disease progression can be retarded by aversion of future exposure to toxins which requires correct identification of causal agents (Lunyera et al.,2016; Rajapakse et al., 2016).

Since the affected subjects are majorly exposed to the etiological agents' viz. nephrotoxins through two environmental exposure routes viz. groundwater and food, remediation of these exposure matrices could help in reducing exposure to renal toxicants. For proper remediation of exposure matrices disturbed by human invasion-i.e. anthropogenic activities need to be regulated. For example closing of open pits in abandoned mines could reduce susceptibility to AMD and associated groundwater contamination with nephrotoxins. Prevention of heavy metal enriched industrial effluents discharge in terrestrial and aquatic ecosystem and reduction in usage of heavy metal laden agrochemical could further improve groundwater quality (Gifford et al., 2017; Wimalawansa, 2016). In order to further avoid increased risk of developing CKDu from direct untreated groundwater consumption, an alternative water source viz. municipality pretreated surface water further subjected to home filtration could be used for drinking (Siriwardhana et al., 2018). This alternative source was suggested for two reasons: Firstly, it inherently contains lower nephrotoxin levels due to decreased rock-water interaction because of decreased residence-time with river's bedrock that result in lower toxin exposure. Secondly, nephrotoxins (i.e. heavy metals & silica) can be further reduced via water-treatment techniques like membrane-filtration (reverse-osmosis or ultrafiltration) or

chemical-coagulation (addition of alum to co-precipitate silica by insoluble-silicate complex-formation) or chelation (to precipitate out heavy metals by formation of inert metal complexes) by municipality before distribution, which can be further facilitated by home water-purifiers equipped with RO/ultrafiltration technology; as these techniques reportedly reduce nephrotoxins (Greenlee et al., 2009; Najm and Trussell, 1999; Oner et al., 2011).On the other hand, adoption of proper food grains storage conditions (maintenance of reduced moisture content and higher temperatures of 40˚C) and usage of proper harvesting techniques to avoid undesired mixing of weeds with food grains could assist in reduction of exposure to nephrotoxins- ochratoxin and aristolochic acid. Moreover, stringent intervention of government regulation and monitoring of industrial effluents and agrochemical usage could avert heavy metals discharge into terrestrial ecosystem preventing human nephrotoxin exposure via food chains (plants and animals) (Wimalawansa and Wimalawansa, 2016).Therefore, by adoption of these remedial and treatment measures of nephrotoxin exposure sources (i.e. water and food) could help in reducing exposure to aforementioned nephrotoxins and assist in decreasing manifestation of tubular dysfunction and associated future CKDu incidences witnessed in developing countries like Sri Lanka, India and Central America and in the Indian taluka under investigation viz. Canacona taluka of south Goa (Wimalawansa, 2014).

1.3 Gaps in existing research

Chronic kidney disease of unknown etiology (CKDu) is a strange form of an endemic nephropathy that is unlinked to traditional causals of CKD like diabetes and hypertension and is reported in various developing countries like India, Sri Lanka and Central America. Owing to similarities in the renal histopathological presentation of CKDu and environmental toxin induced CTN, CKDu is believed to be triggered by exposure to environmental nephrotoxins (Gifford et al., 2017). The wave of this newly emerged global CKDu has also managed to hit the world famous tourist destination, the state of Goa specifically Canacona taluka located in the state's southern district. Although this CKDu epidemic has managed to touch the Canacona taluka of south Goa, this endemic nephropathy in comparison to other regional nephropathies in developing countries is least publicized and understood. The electronic media, print and the Directorate of Health Services (Goa) have constantly reported the rising incidence of CKDu in the Canacona taluka for the past two decades (from 1990-till date) (DNA, 2010; The Hindu, 2007; The Hindu, 2016). However, exact prevalence statistics and demographic distribution of CKDu in Canacona are unknown till date due to lack of registries

maintenance. Troubled by this epidemic curse, the Government of Goa appointed a joint team of officials from NIOH (National Institute of Occupational-Health), ICMR (Indian Council of Medical-Research) and Department of Preventive and social medicine of Goa medical hospital to conduct an environmental cum biological monitoring study in 2005 to determine probable environmental causals responsible for CKDu development in this region. This study analyzed the nephrotoxins major exposure matrices (i.e. food and water) in CKDu affected and healthy regions of Canacona for the presence of a few environmental renal-toxins like heavy metals (i.e. cadmium and arsenic) and ochratoxin. Moreover, the level of nephrotoxin exposure in affected and healthy individuals were analyzed by measurements of their levels in biological matrix (blood) of study subjects. The results demonstrated that the analyzed nephrotoxin levels in exposure sources (i.e. groundwater and food) and blood of CKDu and healthy subjects were comparable and significantly below their respective WHO permissible limits. Thus, this study highlighted absence of the role of assessed nephrotoxins in etiological development of high CKDu incidence in Canacona (Saiyed et al., 2005). Unfortunately, no follow up analysis was conducted to assess for emergence of possible variations in exposure (if any) to the potential nephrotoxins and role of other nephrotoxins in CKDu causation. This study was reported to be the sole environmental monitoring study that analyzed CKDu cases in the taluka, until our group took the challenge in 2014 in attempting to decipher the etiology of this strange form of CKDu noted in Canacona.

Following the epidemiological study conducted by Saiyed et al in 2005, basic screening of genitourinary problems of 298 patients of Canacona was conducted in 2009, at medical camp headed by Dr. Nerli This study through basic laboratory tests highlighted that 75% of prevalent CKD cases in the taluka were unlinked to traditional causals like diabetes and hypertension, supporting unknown etiology of this endemic nephropathy in Canacona.The study did not evaluate the role of various nephrotoxins in disease causality (Nerli et al., 2010).

Despite, rising severity of CKDu prevalence in Canacona for past 20yrs, negligible importance has been given to this, by state government and health research institutes due to which limited research (i.e. two aforementioned studies in 2005 and 2009) have been conducted till date with no conclusive results obtained resulting in the etiology being undeciphered. These two aforementioned studies that focused on attempting to decipher the CKDu etiology in Canacona (Saiyed et al., 2005) and merely confirming occurrence of a high CKDu incidence (Nerli et al., 2010) in the taluka suffered from major limitations:-

Firstly, these studies failed to establish detailed prevalence statistics of CKDu and geographical coverage cum demographical distribution of the same in the taluka. The studies also did not identify potential risk factors and demographically classified population segments that are most prone to disease development. Moreover these analyses did not describe biochemical based pathological pattern of CKDu disease presentation observed in Canacona which are crucial for identifying the type of renal pathology manifested in this disease (i.e. affecting tubular or glomerular regions) for taking necessary therapeutic measures.

Secondly, etiological factors or causal agents of CKDu in the taluka were not successfully deciphered through these previous studies. These studies were limited to the analysis of the role of only 3 nephrotoxins i.e. Cd, As and ochratoxin in CKDu causation and utilized a limited sampling-size (30 food, 30 water samples and 40 blood samples) for assessments which prevented establishment of significant conclusions. These studies failed to explore the contribution of various well-reported nephrotoxins like heavy metals (such as Pb, Cd, As and Hg), ochratoxin (mycotoxin), aristolochic acid (phytotoxin) and emerging renal toxicant viz. silica in CKDu development in the taluka. Recently, the nephrotoxic potency of trace geogenic element viz. silica is emerging apart from its known pulmonary toxic potential. The evidence for silica's nephrotoxic potency initially emerged from animal toxicity studies which reported the clinical/biochemical pathological pattern to be of tubular type and induced renal histopathological alterations to be typical of CTN (Dobbie and Smith, 1982; Kawanabe et al., 1992; Markovic and Arambasic, 1971; Newberne and Wilson, 1970).This was supported by epidemiological studies that proved the biochemical pattern of nephropathy triggered on silica exposure to be of tubular pathological manifestation (Mohner et al., 2017; Sponholtz et al., 2016; Vupputuri et al., 2012).Following which, several epidemiological studies of CKDu causation in Andhra Pradesh (India), Central America and Balkan region had explored role of silica in CKDu causation and strongly suggested contribution of this emerging nephrotoxin in disease etiology (Ibrahim et al., 2011; Khandare et al., 2015; Millerick-May et al., 2015; Stiborova et al., 2016). However contribution of this geogenic nephrotoxin was not explored in the etiological study of CKDu causation in Canacona taluka by Saiyed et al (2005).

Lastly, several animal and epidemiological studies exploring silica's nephrotoxic potency have suggested the clinical pattern of induced renal toxicity manifestation to be of tubular pathological type, which was evident from the trend in various urinary and serum biochemical markers characteristic of tubular injuries (Dobbie and Smith, 1982; Kawanabe et

al., 1992; Markovic, 1972; Markovic, 1974; Markovic and Lebedev, 1965; Markovic and Arambasic, 1971; Mohner et al., 2017; Khandare et al., 2015;Millerick-May et al., 2015; Newberne and Wilson, 1970; Radovanovic et al., 1991; Sponholtz et al., 2016). Moreover, these studies (mainly animal models and few epidemiological analyses) have also highlighted tubular atrophy and interstitial inflammatory fibrosis to be major renal histopathological alterations induced on chronic higher silica exposure which are typical of CTN. Thus have strongly advocated role of silica in CKDu causation. However, these studies suffer from limitation of not elucidating the cellular & molecular toxicological-mechanisms underlying silica induced renal-pathological manifestations.Thus preventing a clear understanding of silica's mode of toxic action that is crucial for nephrotoxicity assessments.

1.4 Objectives

Taking into consideration the existing gaps in the limited research conducted till date on deciphering the etiology of this endemic form of CKDu in the Canacona taluka, this PhD research study aimed to address the aforementioned limitations by conversion of the same into objectives of this work as listed below:

1) To study the prevalence and demographic distribution of Chronic kidney disease of unknown etiology (CKDu) in the Canacona taluka based on the demographic survey and medical records and identify the prospective risk factors and the biochemical pattern of CKDu disease in this taluka.

2) To study the presence of emerging nephrotoxin viz. silica and various other well reported nephrotoxins (such as heavy metals) in the environment and biological exposure matrices viz. water and blood samples collected from affected and non-affected areas of the Canacona taluka.

3) To study the presence of ochratoxin, aristolochic acid and nephrotoxic heavy metals in other environmental and biological exposure matrices viz. food and blood samples collected from affected and non-affected areas of the Canacona taluka.

4) *In-vitro* cytotoxic studies of recently emerging nephrotoxin viz. silica on kidney cell lines with respect to cellular morphology, cell viability (MTT assay), oxidative stress, DNA damage, inflammatory pathway, cell-cycle arrest and cellular death pathway.

In objective 1:-

Owing to the lack of presence of a defined registry and official prevalence statistics of CKDu cases in Canacona , the 1st objective aimed to determine the geographical coverage of CKDu

(i.e. endemic villages that are affected) and the demographic prevalence of CKDu in this taluka. This was achieved by an elaborate demographic survey of CKDu cases and the age & sex matched controls in the taluka at the 2 major hospitals of the state which conduct dialysis for the affected patients. The extensive statistical analysis of variously studied clinical & demographic parameters helped us identify potential risk factors and susceptible populations or demographic groups that were at higher risk of CKDu development in Canacona. Furthermore, the pathological pattern of CKDu presentation (viz. tubular or glomerular) in the taluka was identified by detailed analysis of the trend in various urinary & serum based biochemical markers & their association which are characteristic of tubular or glomerular injuries. Identification of biochemical based pattern of disease presentation assists in quick detection of CKDu in the taluka, which could help improve safety of general population & affected patients by adopting timely treatment measures that could prevent future rise in CKDu incidence.

The work carried out for resolving 1st objective has been described in Chapter 2. A major part of this objective's work is published as a research article whose citation is:- *Mascarenhas, S., Mutnuri, S., Ganguly, A., 2017. Deleterious role of trace elements–Silica and lead in the development of chronic kidney disease. Chemosphere (IF-5.1) 177, pp.239-249.*

In objectives 2 and 3:-

Since environmental nephrotoxins are potentiated to be responsible for CKDu etiology, a comprehensive analysis of all possible environmental nephrotoxins (like heavy metals, ochratoxin, aristolochic acid & silica) in its major exposure matrices viz. groundwater & food that are commonly consumed by CKDu affected and non-affected regions of Canacona was conducted & compared with their corresponding WHO permissible levels. Moreover factors that affect bioavailability of nephrotoxins in these matrices were evaluated. This detailed analysis was carried out to measure the magnitude of possible exposure of the susceptible population of the taluka to nephrotoxins via 2 major routes viz. groundwater and food. However, accurate level of nephrotoxin exposure in CKDu affected & non-affected populations was confirmed by measurement of biomarkers of nephrotoxin exposure i.e. levels of nephrotoxin itself in the biological matrix viz. blood in these study subjects followed by comparison with their respective WHO permissible limits. Various factors (like trace metal content in blood) that affect bioavailability of nephrotoxins for absorption cum uptake by kidney and its consequent nephrotoxicity were also evaluated. Moreover, association between nephrotoxin exposure levels in blood (i.e. biomarkers of nephrotoxin exposure) & biomarkers

of renal damage (i.e. tubular or glomerular) was assessed to evaluate the etiological role of investigated nephrotoxins in inducing renal tubular damage associated with CKDu development, at prevalent exposure levels. The work carried out for resolving 2nd & 3rd objective (i.e. environmental monitoring of nephrotoxins in exposure sources viz. water & food followed by biological monitoring of the same in blood) has been described in sections 1, 2 & 3 of Chapter 3 respectively. The monitoring of toxins in water (section 1) & blood (section 3) is published as a research article whose citation is:- "*Mascarenhas, S., Mutnuri, S., Ganguly, A., 2017. Deleterious role of trace elements–Silica and lead in the development of chronic kidney disease. Chemosphere (IF-5.1) 177, pp.239-249.*

In objective 4:-

The cellular & molecular toxicological mechanisms underlying silica induced nephrotoxicity were analyzed as a function of dose & time (i.e. on prolonged exposure to silica doses that were prevalent in the CKDu region) using normal human proximal tubular cells viz. human kidney cell (HK cells) as an *in-vitro* model. Proximal tubular cells serve as nephrotoxicity assessments tools and hence were used for evaluation of silica nephrotoxicity mechanisms as these cells are primary targets of toxins owing to its intrinsic role in re-absorption, filtration & toxin-clearance (Li et al., 2017). The proximal-tubular cytotoxicity outcomes following chronic exposure of HK cells to increasing silica doses (prevalent in CKDu region) were analyzed via a panel of assessments comprising of cell-viability, mitochondrial-integrity, oxidative-damage, cell-cycle arrest, inflammatory responses, genomic-damage and apoptotic-pathway regulation. Also, ability of silica to trigger incessant tubular inflammation & apoptotic cell death was assessed as these toxicological mechanisms when incessantly induced results in tubular atrophy & fibrosis development on extrapolation at organ level which are proved to be pathological manifestations of CKDu. Hence, exploration of silica's ability at prevalent exposure doses (in CKDu region) in induction of tubular inflammation & apoptotic cellular death would assist in providing causal evidence for silica's role in CKDu manifestation of Canacona.

The work carried out in fulfillment of 4th objective has been described in Chapter 4 of this thesis and published as a research article whose citation is:- *Mascarenhas, S., Mutnuri, S., Ganguly, A., 2018. Silica- a trace geogenic element with emerging nephrotoxic potential. Science of the Total environment (IF-5.7) 645, pp.297-317.*

Since this PhD research work has established silica to be one of the toxins responsible for CKDu development in Canacona, suitable detection of silica deposits in targeted proximal tubular cells on chronic exposure to prevalent high silica levels in the CKDu region could provide supporting evidence to its role in CKDu manifestation. Previous animal based silica toxicity analyses have depicted silica deposits in tubular cells on renal biopsy analysis by immunohistochemical staining, that were responsible for induction of renal toxicity. But immunohistochemical staining procedure for silica deposits detection is highly tedious & time consuming (Dobbie and Smith, 1982; Markovic and Arambasic, 1971; Mohner et al., 2017; Millerick-May et al., 2015; Newberne and Wilson, 1970).Sensors for quick detection of intracellular silica accumulation in tubular cells are unavailable till date. Hence there is a need for the same

Moreover our *in-vitro* silica toxicity study established mitochondrial apoptosis to be the major toxicological mechanism of silica induced tubular toxicity. It is well reported that mitochondrial apoptosis is mediated by intracellular stressors like ROS. which is generated from intra-cellularly accumulated toxin (in this case-silica) (Gamboa et al., 2016) as evidenced in our study (Chapter 4) as well. Thus in order to provide supporting evidence to our discovery of mitochondrial apoptosis to be the major toxicity mechanism of silica induced tubular nephrotoxicity, we decided to assess for intracellular silica deposition on prolonged exposure to prevalent levels in the CKDu region (Lentini et al., 2017). Hence, we attempted to develop a biocompatible chemosensor for quick detection of silica accumulation within targeted human renal proximal tubular cells (HK cells) on chronic exposure to high silica doses.

The work carried out for this purpose has been described in Chapter 5 and published as a research article whose citation is:- *Mascarenhas, S., Gawas, R., Ghosh, B.K., Banerjee,M., Ganguly, A., Chatterjee, A., Ghosh. N.N., 2018. Water-Dispersible Rhodamine B Hydrazide Loaded TiO_2 Nanoparticles for "Turn On" Fluorimetric Detection and Imaging of Orthosilicic Acid Accumulation In-Vitro in Nephrotoxic Kidney Cells. Journal of Nanoscience and Nanotechnology (IF-1.35) 18(12), pp. 8142-8154.*

Chapter 2

Demographic, epidemiological and biochemical analysis of the CKDu scenario in the Canacona taluka of Goa, India.

2.1 Introduction

Recently, a growing concern has aroused about a new form of CKD that is unlinked to traditional causals of diabetes and hypertension, hence renamed as 'CKD of unknown etiology (CKDu)'. It is prominent in developing countries like Sri Lanka, India, Egypt and Central America. CKDu is asymptomatic, slowly progressive and mainly affects adults in their 3^{rd}-5^{th} decade. CKDu's main histopathological features include tubular atrophy and interstitial fibrosis whereas glomerular lesions are distinctive of diabetic/hypertensive CKD. CKDu's histological pattern is typical of chronic tubulo-interstitial nephritis (CTN), pathological condition affecting nephron's proximal tubules which on prolonged manifestation results in chronic renal failure (CRF). CTN is caused by chronic exposure to environmental-toxins like heavy-metals, ochratoxin, aristolochic acid, trace geogenic elements-silica. Owing to the histopathological resemblance between CKDu and CTN, studies advocate environmental factor(s) to be major causals of CKDu, possibly related to chronic exposure through matrices like food or drinking water (Gifford et al., 2017; Lunyera et al., 2016; Wijkstrom et al., 2018).

The wave of this newly emerged CKDu has also managed to touch the Canacona taluka of south Goa (India).Electronic media and print have reported a remarkable rise in CKDu cases of Canacona for the past two decades (DNA, 2010; The Hindu, 2007 and 2016).For resolution of this epidemic, a monitoring study was conducted by NIOH,ICMR and GMC in 2005 to identify environmental risk factors responsible for CKDu development in this taluka. Presence of nephrotoxins like heavy metals (Cd, As) and ochratoxin were analysed in exposure sources viz. food, water and blood but no significant and conclusive results were obtained (Saiyed et al., 2005). In another study, basic medical screening of genitourinary problems of 298 patients residing in Canacona, was conducted wherein etiology was not deciphered but a mere validation of high prevalence of CKD cases unliked to traditional causals of diabetes and hypertension was established supporting unknown etiology in this taluka (Nerli et.al, 2010). These aforesaid studies suffered from major limitations in establishing prevalence geographical cum demographical distribution of CKDu in the taluka; deciphering the etiology; identification of prospective clinical risk-factors for CKDu development and defining pathological patterns for CKDu by analyzing its resemblance with CTN pathophysiology. Owing to lack of official statistics & defined registry of CKDu cases in Canacona, primary aim of the current study was to determine geographical (endemic villages affected) and demographic prevalence of CKDu in this taluka. This was achieved by

descriptive demographic cum epidemiological analysis of CKDu cases in endemic region, CKD cases from non-endemic regions & age-sex matched healthy controls of the taluka. Statistical analysis of studied demographic and clinical parameters permitted us to identify potential risk factors in CKDu development in this taluka. Due to unknown etiology, intervention and modification of recognized risk factors in early stages is useful in prevention of progression to CRF (Senevirathna et al., 2012; Wimalawansa and Sunil, 2014).

The rising incidence of CKDu in Canacona highlights the need for quick diagnosis and screening for effective management of the disease (Jha et al., 2017). The primary prerequisite in developing a screening tool for CKDu in Canacona is the elucidation of its disease pattern which has not been described till date, hence was aimed at in the current study. The pattern of disease presentation can be interpreted by screening of various biochemical markers in biological matrices-blood and urine. Biomarkers are crucial for precise diagnosis, risk assessment and therapy adoption for clinical outcome improvement (Gowda et al., 2013). Since CKDu and CTN share histopathological similarities, CKDu displays a similar trend of biomarkers as CTN. CTN Manifestation is mainly associated with elevated levels of LMV proteins like β2-microglobulin,tubular specific enzyme i.e. N-acetyl glucosaminidase (NAG), in the urine along with raised levels of metabolism by-products viz. creatinine in the serum. Furthermore, CKDu is reported as a minimally-proteinuric disease demonstrating sub-nephrotic range of HMV proteins (albumin) in urine, typical of tubulointerstitial diseases. Hence, dipstick-proteinuria used for albuminuria detection in diabetic CKD cases is not optimal for CKDu diagnosis (Al-Saleh et al., 2017; Rajapakse et al., 2016).Thus, indicating need for sensitive screening markers for early CKDu detection to attain patient safety and alleviate morbidity. In the current study, efficiency of reported biomarkers like serum creatinine, phosphates and bicarbonates as renal function markers along with albumin to creatinine ratio (ACR), b2M to creatinine ratio (BCR), protein to creatinine ratio (PCR), NAG to creatinine ratio (NCR) and albumin to protein ratio (APR) as renal damage markers were tested on environmentally induced CKDu patients, diabetic/ hypertensive CKD cases and healthy controls for proper differentiation of environmental toxin induced tubular proteinuria cases (wherein BCR, NCR urinary excretions are elevated) and diabetes/ hypertension triggered glomerular proteinuria cases(wherein ACR, PCR, APR urinary excretions are increased) in the taluka (Azar, 2013; Saucedo et al.,2018; Siriwardhana et al., 2014; Wanigasuriya et al., 2017). This study is the 1st to analyse substitute screening biomarkers for CKDu diagnosis for effective prediction, prevention & therapeutic modalities of CKDu in the taluka.

2.2 Materials and Methods

2.2.1 Ethical approval and consideration

The entire study was approved by the Government of Goa by obtaining the necessary permission from the primary government aided health safety regulatory body i.e. The Directorate of Health-Services, Panaji, Goa (Reference No:-DHS/Sp.Cell/Sect/8/1119).It was conducted in agreement with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Furthermore the ethical approval for this study was granted by two Institutional Ethics regulatory bodies of the host research-institute and the state head government hospital of Goa viz. Institutional Human-Ethics Committee of BITS-Pilani, India (Reference No:-IHEC/11M/2) and Institutional Ethics Committee of Goa Medical Hospital (Dated 20th July 2015) respectively.

Chronic kidney disease of unknown etiology (CKDu) was preliminary diagnosed in agreement with the existing diagnostic criteria established by numerous research groups extensively involved in the study of the CKDu scenario globally and in amalgamation with the laboratory and clinical findings (Epidemiology Unit-Colombo, 2009; Jayatilake et al., 2013). Participants having a confirmed diagnosis of CKD (i.e. possessing an estimated glomerular filtration rate (eGFR) of <60 ml/min per 1.73 m^2 for more than 3 months) and consistently had minimal proteinuria i.e.<150 g/g and albuminuria, i.e. albumin:creatinine Ratio (ACR) <30mg/g in the urinalysis reports for a period of 3 months (with a minimal interval of 3 weeks) to chronic renal failure development and subsequent administration of dialysis were deliberated to have "CKDu" if they fulfilled all the subsequent criteria:

- Absence of a previous history of glomerulonephritis, ureteric calculi snake bite or pyelonephritis.
- Not on therapeutic management for diabetes and absence of random blood sugar value of ≥200 mg/dL.
- Possesses Standard HbA1c levels (<6.5%).
- Not on therapeutic management for blood pressure or presence of a blood pressure <130/90 mm Hg.
- Abnormal kidney function (raised serum creatinine> 2mg/dL) with absence of a noticeable underlying cause.
- unusual urinalysis results (higher urinary β2M microglobulin levels > 30 µg/dL) with absence of a noticeable underlying cause (Epidemiology Unit-Colombo, 2009; Jayatilake et al., 2013; Ratnayake et al., 2017).

Patients exhibiting acute kidney injury, urinary tract infection and renal malignancies were excluded. Those CKD patients who were not residents of Canacona taluka and did not satisfy CKDu case definition were excluded from the study. Moreover, CKD patients that were native to non-endemic villages of the taluka & did not comply with CKDu case definition but possessed an obvious etiological factor for CRF like diabetes & hypertension were considered as 'non-endemic cases'.

2.2.2 Study design and patient selection

Electronic media and print reports have stated that the CKDu cases are endemic to Canacona taluka of south Goa but lack specifics such as which particular villages this endemicity is restricted to (DNA, 2010; The Hindu 2007 and 2016). Moreover, there are no pre-existing or defined registries that maintain records of the prevalence of CKDu in the taluka specifically its geographical and demographic coverage. Hence in order to gain an initial insight into the prevalence of CKDu in the Canacona taluka, we approached two main hospitals in Goa which regularly conducts dialysis for the chronic renal failure patients of Canacona taluka, namely Apollo Victor hospital and Canacona Health Centre, to gain access to their medical records for appropriate selection of the study population. Permission to gain access to hospital registries were obtained from the major state government health regulatory body i.e. The Directorate of Health Services followed by the Medical Superintendents/Directors of the selected hospitals. The study encompassed no added risk to patients and the study's objectives were described to them before data collection. Following a thorough review by the ethical committee of both the hospitals and patient consent, an exhaustive list of the taluka's CKD affected patients along with their clinical and biochemical characteristics were obtained from both the hospitals. Appropriate technical, administrative, physical, & procedural safeguards were maintained to safeguard data confidentiality & avert unofficial access to it.

Detailed analysis of the hospital records displayed a combined total of 180 patients (from both the selected hospitals) were undergoing dialysis owing to CRF. Out of the 180 CKD patients, 36 patients were excluded from the study because of non-residency in Canacona taluka and did not satisfy the CKDu criteria. 142 patients out of the total of 180 were hailing from Canacona taluka and thus were recruited for the current study. Another interesting observation was that among the 142 patients ,around 80% (i.e. 114 patients) fulfilled the CKDu inclusion criteria and were residents of two villages namely Ponsulem and Chaudi signifying endemicity of the disease to these villages. The remaining 28 patients that did not meet the CKDu criteria, hailed from scattered non-endemic villages of the taluka namely

Cola, Poinguinim and Anvali and exhibited a known cause for CKD i.e. diabetes or hypertension, hence were included in the study as non-endemic cases. CKD etiologies among the non-endemic cases were such that 9 and 19 patients were from hypertensive nephropathy and diabetic nephropathy respectively. Thus for convenience, the CKD affected patients of the taluka were grouped under two broad categories. Patients (n=114) belonging to the endemic villages-Ponsulem and Chaudi were grouped as 'CKDu endemic cases' collectively annotated as 'study group 1'.The remaining 28 patients that are residents of the non-endemic villages-Cola, Poinguinim and Anvali were grouped as 'non-endemic cases' annotated as study group 2. Every epidemiological study requires the presence of true controls. Thus to serve this purpose, electoral list were utilized to randomly select age and sex matched control subjects (true controls) from two healthy villages of the Canacona taluka- Molorem and Endrem to attain a similar representation of the CKDu cases. A total of 62 volunteers were selected from each of the two healthy villages to achieve a combined total of 124 true controls, bearing similarities to the CKDu case frequency. These selected true controls showed no significant medical history, presence of normal blood pressure, fasting-glucose levels or HbA1c levels & absence of CKD. All true controls underwent extensive screening such as detailed analysis of medical history to eliminate subjects with kidney diseases. All true controls were annotated as study group 3. The details of 3 study groups are presented in **Table 2.1.** All participants were recruited following attainment of written informed consent.

Table 2.1: The number of patients /subjects in the three study groups–CKDu endemic cases, non-endemic CKD controls and true control groups.

Study group/Study area	Number of patients/subjects residing in the village
I.Endemic CKDu villages in Canacona[study area 1](n=114)	
1) Chaudi	51
2) Ponsulem	63
II. Non-endemic CKDu villages in Canacona [study area 2](n=28)	
3)Cola	8
4)Poinguinim	11
5)Anvali	9
III. Non CKDu villages in Canacona[study area 3) (n=124)	
6)Molorem	62
7)Endrem	62

This analysis simultaneously attempted to differentiate CKDu cases in Canacona from hypertensive and diabetic CKD patients in the same region characterized as non-endemic cases for differences in disease presentation. This was followed by sequential comparison of CKDu cases with healthy controls exhibiting insignificant medical history and normal blood

pressure and glucose levels categorized as true controls for accurate representation of the disease pathology.

2.2.3 Anthropometric analysis

The major anthropometric parameters like weight (in kg) and height (in meters) were recorded of the study subjects of all the three groups. Body mass index (BMI) was calculated as the ratio weight / height^2 (kg/m^2). Blood pressure was measured using a calibrated mercury sphygmomanometer after 5 minutes of resting. The reliable and most convenient method of assessing the extent of functioning of the kidneys and gaining an insight into the degree of renal damage is via the analysis of the Glomerular filtration rate (GFR). In the current study, the glomerular filtration rate (eGRF) of all the study subjects was estimated by the formula derived from the MDRD (Modification of Diet in Renal Disease) analysis and their average was reported. All the parameters were measured under the supervision of the hospital's medical nurses (Nanayakkara et al., 2014; Jayasekara et al., 2015).

2.2.4 Demographic study

The patients and the healthy subjects (controls) were interviewed at the selected hospitals using a structured questionnaire to acquire information on various demographic parameters like age, gender, type of occupation (mining/farming/fishing etc.), pesticide handling, education, mines in the vicinity, family history of CKD, history of musculoskeletal disorders, history of NSAID's consumption, duration of NSAID's consumption, duration of residence in the study area, source of drinking water, percentage of diabetes and hypertension affected, use of medications like ayurveda/ alternate medicine, type of CKD(i.e. prevalence of known etiologies:-interstitial or glomerulonephritis). The study subjects were also assessed for the signs of dental fluorosis with the assistance from the doctors. The detailed structured interview was conducted to gain an insight into the personal information, medical history, lifestyle, habits, and occupational history of the study population. The demographic analysis enabled us to assess the plausible risk factors or exposure variables involved in the development of CKDu in the taluka. The risk factors that were assessed were gender, age, family history of CKDu, pesticide handling, occupation, history of continuous ayurvedic treatment for more than a month prior to development of CRF, source of drinking water, prevalence of hypertension or diabetes etc. The association between the risk factors and the absence or presence of CKDu was examined by suitable analysis of risk-ratios (with 95% CI) (Herrera et al., 2014; Jayasekara et al., 2015; Jayatilake et al., 2013; Ratnayake et al., 2017).

2.2.5 Biochemical measurements

It is obvious that the biochemical examination will show abnormal levels in the study groups 1 (CKDu endemic cases) and 2 (typical CKD non-endemic cases) since these patients have already progressed towards End Stage Renal Disease (ESRD), the final stage of CKD ultimately resulting in Chronic Renal Failure (CRF), hence undergoing dialysis. However, it was performed to assess significant difference in the abnormal values of the biochemical parameters measured between the two study groups in order to establish the pattern of CKD disease presentation (i.e. glomerular or tubular nephropathy) manifested in the same. Another reason for carrying out the examination was to make sure that the study group 3 (which showed no prevalence of CKD) served as true controls and to assess their potential if any to develop CKD in the future.

To analyze the degree of renal damage in a study population, various biochemical parameters that serve as biomarkers of kidney function are ideally measured in both the biological samples i.e. urine and blood (Herrera et al., 2014). Since dialysis patients (i.e. subjects of study group 1 and 2) do not pass urine owing to renal failure, spot urinalysis of these patients could not be conducted during the current study. Hence in order to maintain homogeneity in urine and blood sample analysis, avoidance of selection bias and neglecting the assessment of urinary biomarkers of renal function, study of various biochemical parameters was conducted via an exhaustive analysis of the detailed medical records of these study subjects. As stated earlier prior permission and written informed consent were obtained from the hospital directors and the study subjects to access the medical records.

The medical files were thoroughly reviewed for previous urinary and blood (serum) renal biochemical markers measurements conducted over a 6 months period (with an interval of 4 weeks) prior to manifestation of CRF and administration of dialysis. On detailed analysis, the average and range (median, minimum and maximum) of each of the previously measured parameters were compared between the study groups 1 (CKDu endemic cases) and 2 (CKD non-endemic cases) and reported.

In order to assess the extent of kidney function in the true healthy controls (i.e. study group 3), biochemical markers were measured and analyzed during the course of the current study (for a period of 6 months with a 4 week interval) in spot urine and blood samples collected from each of these subjects. Blood samples (5 ml) were collected from the peripheral vein of these study subjects into heparin-EDTA coated blood vacutainer tubes. The early morning first void urine samples were collected in tightly capped polypropylene tubes. Both the biological samples were preserved at -20˚C following acquisition until biochemical analysis.

Herein also the average and range (median, minimum, and maximum) of each of the measured parameters were noted (Wanigasuriya et al., 2017; Wesseling et al., 2016). All the laboratory analyses of urinary and blood biochemical parameters (including the previous measurements of biological markers in study groups 1 and 2) were performed at clinically certified biochemistry laboratories of the selected hospitals in accordance with their standard procedures and guidelines.

The parameters measured in spot urine and blood samples of all the study participants were creatinine, urea, uric acid, bicarbonates, phosphates, proteins, albumin, β2-microglobulin, and N-acetyl-glucosaminidase (NAG). Creatinine was estimated colorimetrically utilizing picric acid based kinetic rate Jaffe's response method (non IDMS-traceable) (Selvarajah et al., 2016). Urea levels were determined colorimetrically by the reaction with diacetyl monoxime-glucuronolactone reagent that has an absorption maximum of 475 nm (Wesseling et al., 2016). Uric acid was determined enzymatically by the Folin-Marenzi method as previously described, wherein specific oxidation of uric acid by uricase was followed by spectrophotometric quantification at 293 nm (García-Trabanino et al., 2015; Wesseling et al., 2016). Bicarbonate and phosphate were assayed in a Hitachi Model 917 automated multi-channel analyser (Roche Diagnostics, Indianapolis, USA) present at the on-site biochemistry laboratory (Kupferman et al., 2016). Total protein levels were estimated by a colorimetric Pyrogallol red-molybdate protein dye-binding assay kit according to the manufacturer's instructions that possesses an absorption maxima of 600 nm (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quantitative analysis of albumin was achieved by Hitachi Model 912 auto-chemistry analyzers (Roche Diagnostics, Indianapolis, USA) (Athuraliya et al., 2011; Herrera et al., 2014; Ratnayake et al., 2017). β2-microglobulin levels were quantitatively measured using a BN ProSpec immunonephelometer (Siemens Healthineers, Erlangen, Germany) (Khandare et al., 2015; Siriwardhana et al., 2014; Wanigasuriya et al., 2017). NAG activity was estimated spectrophotometrically in a Hitachi Model 912 auto-chemistry analyser utilizing commercially available reagents i.e. 3-cresolsulfonphthaleinyl-*N*-acetyl-β-D-glucosaminide (Roche Diagnostics India Pvt. Ltd, Mumbai, Maharshtra, India). This method is centered around the hydrolysis of 3-cresolsulfonphthaleinyl-*N*-acetyl-β-D-glucosaminide by NAG, releasing 3-cresolsulfonphthalein that can be optically quantified at 580 nm (Khandare et al., 2015; Laws et al., 2016; Nanayakkara et al., 2012a and b).

Accuracy of urinary analysis of proteins, albumin, β2-microglobulin and N-acetyl-glucosaminidase (NAG) were confirmed by expression of these parameters as a ratio to urinary creatinine since the rate of creatinine synthesis and excretion in the human body is

ideally constant. Therefore alterations in creatinine excretion in the urine will highlight significant disturbances in the renal function, which in turn will indicate precise variations in these measured parameters. The albumin to creatinine ratio (ACR) was calculated by dividing spot urinary albumin concentration by spot urinary creatinine concentration. Urinary protein to creatinine ratio (PCR), β2-microglobulin to creatinine ratio (BCR) and N-acetyl-glucosaminidase to creatinine ratio (NCR) were calculated in lieu with the trend followed for ACR. PCR, ACR, BCR and NCR are sensitive biomarkers capable of detecting low levels of albuminuria (albumin in the urine) and proteinuria (protein in the urine) along with higher levels of β2-microglobulin and N-acetyl-glucosaminidase in the urine reflective of tubulointerstitial pathology, hence were utilized for biochemical analysis (Herrera et al. 2014; Lawa et al., 2016; Ratnayake et al., 2017; Wanigasuriya et al., 2017).

Moreover, we used albumin to protein ratio (APR) and Alb/b2M ratio calculated from the ratio of urinary APR to urinary PCR (i.e. ACR/PCR) and urinary ACR to urinary BCR (i.e. ACR/BCR) respectively to clarify the contribution of albumin in the total protein and assess the relationship between albumin and β2-microglobulin with total protein excretion which crucial for further analysis of the albumin and β2-microglobulin content of the excreted urinary proteins. By assessment of the albumin and β2-microglobulin content in the eliminated protein we can assess the type of proteinuria manifested that is tubular (dominated by low molecular weight protein-b2M) or glomerular(dominated by high molecular weight protein-albumin) proteinuria which is typical of the tubular and glomerular pathological manifestation respectively. These ratios facilitated in the diagnosing the type of CKD (i.e. tubular or glomerular) prevalent in the study groups 1 and 2 (Laws et al., 2016; Nanayakkara et al., 2012a and b; Ratnayake et al., 2017; Saucedo et al., 2018).

The parameter solely measured in the blood was fasting-glucose and glycosylated haemoglobin (HbA1c) to check the occurrence of diabetes as a causative factor in study group 1 (CKDu endemic cases) and 2 (CKD non-endemic cases) and moreover to rule out the risk of developing diabetic nephropathy in healthy true controls (study group 3). Glucose was also determined by the Hitachi Model 917 automated multi-channel analyzer (Roche Diagnostics, Indianapolis, USA) present on-site at the biochemistry laboratory. HbA1c was assayed using a Bio-Rad D-10 Hemoglobin analyzer (Bio-Rad, Hercules, California, USA) (Jayasumana et al., 2015a). For cross verification of any variations in the serum biochemical parameters of study groups 1 and 2 in the current time, blood samples were collected as described above in the current study and subjected to analysis for the same set of previously measured serum biomarkers. The currently measured serum biomarkers were compared with

the past medical records of the study subjects to observe for remarkable variations (if any) in the levels of the previously measured biochemical parameters with time. On statistical analysis it was observed that the levels of the previously and currently measured serum biomarkers were comparable with no significant differences noted in their presentation patterns, hence the currently measured biomarkers were not described in this study. The stated biochemical analysis in this chapter is restricted to the descriptive study of the biochemical parameters reported from these patients' previous medical records.

The prevalence & distribution of all measured biochemical parameters in each of the study groups across various demographic variables (risk-factors) like age-groups, gender & occupation were computed and represented to analyze predisposition of CKDu development for any of the demographic variables (Jayasekara et al., 2016; Lebov et al., 2015).

2.2.6 Statistical analysis

The characteristics of each study group subjects were represented using descriptive statistics wherein continuous data are depicted as mean±SD and categorical data are indicated as frequency with proportion percentages. One way ANOVA was used to examine differences in continuous variables & Dunnett's test was applied post hoc to analyze differences between biochemical parameters distributed among sub-groups of demographic variables like age, gender, & occupation. Categorical variables were compared by a chi-square test.

The association between the demographic variables that serve as risk factors and the absence or presence of CKDu were examined by univariate risk-ratio analysis(with 95% CI).This assessment was conducted to analyse the role of various risk factors that enhances the susceptibility of the study population to develop CKDu. (Jayasekara et al., 2016; Lebov et al., 2015; Selvarajah et al., 2016).The interrelationship between biochemical / clinical variables were evaluated by univariate correlation assessments.This univariate correlation assessments were conducted to evaluate the association between the biochemical parameters and diagnosis of tubulointerstitial CKD (characteristic of CKDu histopathology). For this, each measured biomarker was analysed for correlation with the complete set of biochemical characteristics of the respective study group population. Thereby attempting to differentiate between tubular nephropathy (caused by environmental toxins) and glomerular nephropathy (typical of diabetes or hypertensive CKD) in study groups 1and 2 respectively in order to confirm the type of CKD pathology that is distinctive for these study cohorts (Herrera et al.,2014; Nanayakkara et al., 2012a and b; Wijkström et al., 2018).Univariate analysis of the categorical and continuous variables was achieved by linear regression assessments. This

analysis was conducted to evaluate relationship between demographic and clinical or biochemical characteristics of the 3 study group's population & provide supporting evidence to contribution of previously established demographic risk factors in CKDu development. This analysis was achieved by individual univariate regression analyses for each measured biochemical parameter as dependent variables on demographic & clinical features of study population belonging to the three study groups (Orantes-Navarro et al., 2016; Ramírez-Rubio et al., 2015). Differences at P-value < 0.05 was considered to be statistically significant. The statistical analyses were conducted using SPSS Statistics software (Version 20.0).

2.3 Results and Discussion

2.3.1 Selection of the study participants

Chronic Kidney disease is a universal health problem, which when untreated manifests in irreversible renal failure that confers fatality on unaffordable treatment (dialysis or transplant) and improper and untimely management (Robinson et al., 2016). It has been reported to be 12^{th} leading cause of death worldwide with risk-factors as hypertension & diabetes. The prevalence of hypertensive & diabetic CKD worldwide is approximated to be 8–16% (Hill et al., 2016). However, recently a mysterious form of CKD unlinked to typical causals has emerged that is retitled as chronic kidney disease of unknown etiology (CKDu). CKDu is becoming a global endemic and has engulfed a number of developing countries like India, Central America & Sri-Lanka (Gifford et al., 2017; Lunyera et al., 2016). The current study is centred around one such CKDu scenario that has been on the rise for past 25 years which is noted in largest taluka of south Goa i.e. Canacona taluka (DNA, 2010; Hindu, 2007 & 2016).

The crucial step in analysis of any disease epidemic in an area is the appropriate selection of the study participants' i.e. accurate recruitment of cases and controls (Ratnayake et al., 2017). Canacona taluka being geographically wide makes screening of the entire taluka for CKDu unfeasible. Moreover there was no reported evidence or a starting point on the exact prevalence (i.e. geographical distribution or coverage) of CKDu in the taluka. Hence, in order to commence the prevalence analysis we decided to screen for hospitals that majorly conduct dialysis for the Chronic renal failure patients of this taluka and derived two main hospitals i.e. Apollo Victor hospital & Canacona health centre involved in this purpose. On detailed analysis of the selected hospitals medical records following attainment of necessary government permission and informed patients consent, it was surprisingly observed that the highest number of reported CKDu cases in the Canacona taluka of south Goa, India which

is not caused by known typical agents (i.e. diabetes and hypertension) were endemic to two villages of the taluka i.e. Ponsulem and Chaudi. These cases were further confirmed on satisfactory fulfilment of the established CKDu criteria as described earlier. For reliability, the total number of CKDu cases from both these endemic villages were categorized under study group 1. Additionally a lesser number of patients suffering from hypertensive or diabetic CKD that do not meet the CKDu criteria were noted in this taluka as well and they were restricted to the non-endemic villages of Cola, Poinguinim and Anvali. These few CKD cases were also included in the study as 'typical CKD non-endemic cases' and this study population were clustered as study group 2. The purpose of including these CKD non-endemic cases was to analyse for differences in the disease presentation between the study group 1 (CKDu cases) and study group 2 (typical CKD cases). Healthy subjects were recruited from unaffected villages as 'true controls' following exclusion of CKDu or CKD via means of evaluation of the recent medical examination records and previous clinical history. For proper management of this disease, early detection and intervention is crucial.

2.3.2 Anthropometric analysis describing the case/control stratified general characteristics of the study population

The initial step in an epidemiological or case-control study is screening of the general characteristics of two cohorts (i.e. cases & controls) for confirming accurate selection of study participants. The general characteristics of 3 evaluated study groups are presented in **Table 2.2**.

As indicated in **Table 2.2,** the mean age compositions of the CKDu endemic cases and the true controls were comparable to obtain a similar representation of the CKDu cases, whereas the CKD group of study subjects (study group 2) possessed a higher mean age constitution. The prevalence of higher age groups in the study group 2 could be attributed to the larger risk of susceptible populations like diabetic and hypertensive adults to commonly develop CKD later in life (DeFina et al., 2016). The weight, height and the index of metabolic functioning i.e. BMI were similar among all the study groups. The mean systolic and diastolic blood pressures among the CKDu endemic cases (study group 1) and controls (study group 3) were normal and comparable. Conversely, the study group 2 participants depicted mean blood pressure values above the ideal reference range of 120/80 mm of Hg highlighting the presence of hypertension among this study cohort. Moreover the mean fasting blood glucose levels and the glycosylated hemoglobin (HbA1c) in the CKDu cases (study group 1) and the controls (study group 3) were normal being in the range of 70-110 mg/dL with no significant

differences among these groups as well. However, study group 2 comprising of CKD cases in non-endemic villages of the taluka showed significantly higher mean values of fasting glucose, thereby confirming occurrence of diabetes in that study group. Therefore, presence of high blood glucose levels & blood pressure among subjects of study group 2 as compared to CKDu endemic cases & true controls highlighted that diabetes & hypertension are major causal factors for CKD development with eventual renal failure in this cohort (Hill et al., 2016; Hu and Coresh, 2017).

Table 2.2.Case/control stratified general characteristics of the study population

Variable (parameter)	Sex	Study group 1 (Endemic CKDu cases)	Study group 2 (Non-endemic CKD cases)	Study group 3 (True Controls)	Reference range
Age (yrs)	Total	43.5±10.2	61.4 ±8.4	40.6±13.9	-
	Male	45.2±8.6	62.3±4.8	41.2±6.3	-
	Female	41.8±5.1	60.5±5.7	40.0±7.8	-
Height (cm)	Total	115.9±6.3	157.3±5.1	151.3±6.2	-
	Male	163.8±8.4	168.0±4.5	158.1±6.2	-
	Female	148.1±4.2	146.2±5.6	144.5±6.3	-
Weight(kg)	Total	48.25±11.5	52.05±8.75	69±6.9	-
	Male	50.9±14.5	54.7±11.2	72.6±9.3	-
	Female	45.6±8.6	49.4±6.3	65.4±4.6	-
BMI(kg/m^2)	Total	21.3±4.0	21.9±2.9	20.4±2.6	between 20-25 kg/m^2
	Male	22.3±3.4	21.4±2.1	20.8±1.8	
	Female	20.3±4.6	22.4±3.8	20.1±3.4	
Systolic BP(mm of Hg)	Total	120.3±2.0	131.4±12.9	123.5±4.2	120 mm of Hg
	Male	119.4±2.4	132.2±12.4*	125.2±3.8	
	Female	121.2±1.6	130.6±13.4*	121.8±4.6	
Diastolic BP(mm of Hg)	Total	80.3±4.7	94.0±6.2	80.4±4.6	80 mm of Hg
	Male	82.1±4.5	94.6±6.8*	78.3±5.7	
	Female	78.6±4.9	93.4±5.6*	82.6±3.6	
Fasting blood glucose (mg/dL)	Total	84.6±1.7	141±4.4	70.3±4.0	70-110 mg/dL
	Male	88.8±1.1	142.4±6.1*	71.2±4.6	
	Female	80.5±2.4	139.1±2.8*	69.4±3.4	
Glycosylated Hb (Hb1Ac)(%)	Total	4.4±1.1	8.8±2.3	4.3±1.0	<6.5 %
	Male	4.6±1.3	8.9±2.5*	4.2±0.9	
	Female	4.3±0.9	8.8±2.1*	4.3±1.1	
eGFR (ml/min/1.73 m^2)	Total	10.2±2.0	11.1±2.9	96.5±7.8	>90 ml/min.1.73 m^2
	Male	10.1±1.5*	11.5±2.4*	98.5±6.9	
	Female	10.3±2.5*	10.7±3.4*	94.6±8.8	

The values are represented as mean±SD wherever applicable. The number of subjects in each study population are as follows:-Study group 1 possesses a total number(n) of 114 subjects with 58 males and 56 females; Study group 2 possesses a total number(n) of 28 subjects with 17 males and 11 females and Study group 3 possesses a total number(n) of 124 subjects with 62 males and 62 females. *Difference at $p<0.05$ were considered as significant.

Moreover, the absence of a history of diabetes or hypertension in the study group 1 suggests that the CKD in this area is of a different kind whose etiology is not related to traditional risk factors like diabetes and hypertension, hence is renamed as Chronic kidney disease of unknown etiology (CKDu) (Lunyera et al., 2016).Similar studies on the CKDu scenario in other developing countries like Sri-Lanka and Central America suggest that an environmental factor(s)specifically environmental nephrotoxins (viz. heavy metals, fungal toxins like ochratoxin, trace geogenic element like strontium, NSAID's etc) plausibly linked to food or drinking water might be responsible for its causation (Gifford et al., 2017; Jayasumana et al.,

2016; Levine et al., 2016; Lusco et al., 2017; Weaver et al.,2015; Wijkstrom et al., 2018). Therefore these reports enabled us to hypothesize that the etiology of the CKDu disease in the Canacona taluka of south Goa, India could be accredited to the chronic exposure to environmental toxins. Hence, needed further exploration as described in Chapter 3. Stage of chronic kidney disease as calculated by the MDRD (Modified Diet for Renal Diseases) formula depicted the possession of an estimated GFR (eGFR) of <15ml/min/1.73 m^2 among the participants of study group 1 and 2.This confirmed that the study population of group 1 and 2 were in the final and irreversible stage of CKD i.e. Chronic Renal failure hence were undergoing dialysis (Correa-Rotter, 2017; Wijkström et al., 2018). However, the subjects of study group 3 possessed an eGFR of more than 90 ml/min/1.73 m^2, which highlighted normal renal functioning among these true controls and no impending risk of developing CKD in the future (Glassock et al., 2017). Moreover, the absence of significant kidney damage among the study group 3 provided supporting evidence to the accurate inclusion/selection of participants in this control group **(Table 2.2).**

2.3.3 Demographic analysis of the CKDu case/control stratified study population

2.3.3.1 Demographic features and its prevalence among the subjects of the three study cohorts

The demographic characteristics and its prevalence of CKDu case/control stratified study population are presented in **Table 2.3.** As indicated that even though the 3 study cohorts were from comparable socioeconomic backgrounds, significant variations in demographic features were noted. The prevalence of CKDu was higher in study group 1 as compared to study group 2 displaying a common histopathological presentation of tubulointerstitial nephritis. However prevalence of CKD of known etiology was more frequent in the study group 2 bearing a general renal pathological injury to glomerulus manifesting in glomerulonephritis. Glomerulonephritis is a typical histopathological diagnosis of CKD induced by the traditional risk factors of diabetes and hypertension as depicted in many landmark studies (Brooks, 2009; Hu and Coresh, 2017; Levey et al.,2015) whereas tubulointerstitial nephritis is majorly induced on prolonged injury to the renal proximal tubules by environmental nephrotoxins (Brooks, 2009; Lopez-Marín et al., 2014; Lusco et al., 2017). Keeping in line with existing evidence, prevalence of tubulointerstitial nephritis and glomerulonephritis in study groups 1 and 2 respectively highlights environmental nephrotoxins (Gifford et al.,2017;Weaver et al., 2015) & diabetes or hypertension (Hill et al.,2016) to be predominant causal agents in the corresponding development of CKDu and CKD in these regions.These observations were

consistent with absence of occurrence of diabetes/hypertension among subjects of study group 1 and prevalence of diabetes (67%) & hypertension (33%) in study group 2,providing supporting evidence to role of diabetes and hypertension in CKD development among group 2 subjects and no causal role in CKDu development in group 1.Thus this observation eliminates diabetes or hypertension as causal factors in CKDu development of study region 1 signifying role of chronic exposure to environmental nephrotoxins in etiological development of CKDu. Moreover, the age constitution of study group 1 comprising of CKDu endemic cases was lower with a mean of 43.5 years (ranging from 40.5 to 48.3 years) as compared to study group 2 comprising of CKD cases and bearing a mean of 61.4 years (ranging from 55.3 to 64.6 years) which was consistent with the trend noted in age groups commonly affected in the CKDu scenario faced in other developing countries (Lusco et al., 2017; Wijkstrom et al., 2018). Subjects that were suffering from CKD (study group 2) were at higher age groups than CKDu cases (study group 1) owing to the general trend of CKD to manifest later in life due to the inherent potency of incurable causative factors i.e. diabetes and hypertension to induce renal damaging effects that inevitably progresses and accumulates with time on improper management. Moreover, the intrinsic reno-protective effects of humans degenerates by default over time, thereby conferring susceptibility for further aggravation of these typical risk-factors induced renal damage in older diabetic and hypertensive adult population. However the CKDu population of the taluka was stratified at a younger age composition as compared to general CKD cases. This observation highlights a possible role of an environmental factor in disease causation as it is well reported that chronic exposure to environmental nephrotoxin over a period of 25 yrs is commonly observed in environmental toxin induced nephropathy. This observation highlighted a possible role of an environmental factor in disease causation as it is well reported that environmentally induced chronic kidney disease usually manifests only on prolonged exposure to environmental nephrotoxin(s) for a period of 25 years or more. Thereby explaining the possible age composition of CKDu cases and providing supporting evidence to the common trend of CKDu to affect adults in the third to fifth decade. Moreover, a comparable frequency of occurrence of CKDu cases was noted among males (i.e. 51%) and females (49%). A similar replication of this trend was observed in CKD cases as well with a prevalence of 54% and 46% noted in males and females respectively. Thereby highlighting existence of no significant gender bias in the disease presentation scenarios (i.e. CKDu or CKD) in study groups 1 & 2 respectively which was consistent with the gender based stratification of the disease pattern noted in various affected developing countries (Badurdeen et al., 2016; Khandare et al., 2015).

Table 2.3 :Demographic, health and lifestyle characteristics of the entire study population comprising of three study groups stratified by study area

Variable (parameter)	Study group 1 (Endemic CKDu cases)			Study group 2 (Non-endemic CKD cases)			Study group 3 (True Controls)		
	M (n=58)	F (n=56)	Total (n=114)	M (n=15)	F (n=13)	Total (n=28)	M (n=62)	F (n=62)	Total (n=124)
Prevalence of unknown etiologies Yes	58* [100]	56* [100]	114* [100]	0	0	0	N.A	N.A	N.A
Prevalence of known etiologies (from histopathological analysis)									
Glomerulonephritis	0	0	0	15* [100]	13* [100]	28* [100]	N.A	N.A	N.A
Tubulonephritis	58* [100]	56* [100]	114* [100]	0	0	0	N.A	N.A	N.A
Occurrence of diabetes- Yes	0	0	0	10* [67]	9* [69]	19* [67]	0	0	0
Occurrence of hypertension- Yes	0	0	0	5* [33]	4* [31]	9* [33]	0	0	0
Occupation									
Mining	47* [81]	40* [71]	87* [76]	2 [13]	2 [15]	4 [14]	4 [6]	3 [5]	7 [6]
Farming	6 [10]	5 [9]	11 [10]	4 [30]	3 [20]	7 [25]	27 [44]	28 [45]	55 [44]
Fishing	5 [9]	11 [20]	16 [14]	9 [60]	8 [62]	17 [61]	30 [48]	28 [45]	58 [47]
Others	0	0	0	0	0	0	1 [2]	3 [5]	4 [3]
Use and exposure to chemical agrochemicals (pesticides) Yes	0	0	0	2 [13]	1 [7]	3 [10]	2 [3]	2 [3]	4 [3]
Use of organic bio-pesticides Yes	4 [7]	3 [5]	7 [6]	3 [20]	5 [38]	8 [28]	23 [37]	26 [41]	49 [40]
Presence of mines in the vicinity	Yes , Non-operational for past 10yrs (located in Chaudi village)			No			No		
Conduction of mining	No, Was conducted earlier but mining has been stopped for the last 15 yrs			No			No		
Source of drinking water									
Well (groundwater)	58* [100]	56* [100]	114* [100]	2 [13]	2 [15]	4 [14]	5 [8]	4 [6]	9 [7]
Municipality treated public supply (surface water)	0	0	0	13* [87]	11* [73]	24* [86]	57* [92]	58* [93]	115* [93]
Duration of residence(yrs)	40.3±11.2	36.5±8.1	38.4±10.9	29±13.9	35.8±9.3	32.4±8.7	37.5±7.9	34.3±6.8	35.9±7.8
Family history of CKD Yes	0	0	0	0	0	0	N.A	N.A	N.A
Use of long-term alternate/ ayurvedic medicine Yes	0	0	0	0	0	0	0	0	0
History of skeletal disorders Yes	31* [53]	30* [54]	61* [53]	2 [13]	1 [8]	3 [11]	4 [6]	5 [8]	9 [7]
History of NSAIDs consumption Yes	30* [51]	28* [50]	58* [51]	2 [13]	2 [15]	4 [14]	5 [8]	5 [8]	10 [8]
Duration of NSAIDs consumption	14.3±5.3*	10.7±6.9*	12.5±6.8*	2.8±1.4	3.4±0.9	3.1±1.3	1±1.9	1.4±1.3	1.2±1.7
Prolonged duration of NSAIDs consumption(for more than 5 yrs) Yes	24* [41]	22* [39]	46* [40]	2 [13]	1 [7]	3 [10]	3 [4]	4 [6]	7 [11]
Signs of dental fluorosis Yes	0	0	0	0	0	0	0	0	0

Abbreviations: M-males, F-females, CKD-chronic kidney disease, NSAIDs-non-steroidal anti-inflammatory drugs. Values are represented as counts with proportion of the study subjects (depicted individually for the males, females and the combined total categories) displaying positive response for each characteristic or variable as % in parenthesis, or as mean±SD wherever applicable..Differences at *p<0.05 were considered to be statistically significant.

Assessments of the lifestyle factors **(Table 2.3)** indicated that a higher proportion (around 75%) of the affected subjects of study group 1 were previously involved in mining (with 81 % of the males and 71% of the females engaged in this occupation) versus 10% of farmers and 15% of fishermen indicating that the prevalent occupation at that time in this endemic CKDu hit study area was mining. However since a ban was imposed on mining in the state of Goa of which Canacona taluka is a part of for the last 15 years (The Hindu, 2017), the affected subjects were no longer involved in mining in the present time. This observation was backed by the occurrence of a non-operational granite mine in the Chaudi village that was present in the vicinity of the study area 1, located approximately 2 kms from both of the CKDu endemic villages viz. Ponsulem and Chaudi whose study subjects constituted the study group 1 which justified the previous dominance of the mining in this area.

The occurrence of mining confers susceptibility to the residents in the vicinity of the mine to be exposed to various kinds of environmental nephrotoxins like heavy metals that could induce severe reno-toxic effects via major exposure routes like air or groundwater (Orantes et al., 2011; Senanayake and King, 2017). However the non-functionality of the mine for the past 15 years eliminates the possibility of nephrotoxin exposure via air in the current study thereby restricting it to the groundwater (Senapati et al., 2018). Groundwater can be severely contaminated with various toxins as a result of acid mine drainage from the non-operational mine whose effect can persist over several years due to continuous precipitation and dissolution of the ubiquitous sulphide mineral presents in open pits of the mine which can leach out various toxins from the aquifer (McCarthy, 2011; Sankhla et al., 2016). Therefore our further studies focussed on analysis of various environmental toxins in the groundwater which also turned out to be the primary drinking water source in the CKDu hit endemic area.

The gender bias of males among mining population just goes to indicate that certain occupations are highly dominated by males due to significant amount of physical strenuous activity involved that is preferably carried out by men. This observation does not make supporting claims of predisposition of males to developing CKDu as compared to females as the overall number of affected males & females across all occupations were similar.

Contrarily, fishing was the predominant occupation in study group 2 with a prevalence of 61% followed by farming at 25% whereas fishing was predominant among the controls, suggesting a possible lack of contribution of the occupational risk factor in the development of CKD among the study group 2 subjects as fishing negligibly predisposes the involved population to nephrotoxin exposure as established in many related studies (Wanigasuriya, 2012).

Negligibly few numbers of the pesticides have been considered as a possible etiological factor in the development of CKDu in developing countries like Sri Lanka and Central America as farming is the primary occupation in these rural areas. Farming has been invariably associated with pesticides exposure out of which few of them like DDT (banned), and glyphosate have been proved to be nephrotoxic in animal models but no significant evidence has been noted from human studies (Almaguer et al., 2014; Jayasumana et al., 2015a). However since farming is not the predominant occupation among study group 1 subjects and these potentiated nephrotoxic pesticides are not used in the taluka (as established from demographic survey, the Statistical Handbook of Goa (2016) and from the Personal communication with Zonal Canacona agricultural officer, Mr.Shivram Gaonkar), it negates the possible involvement of pesticide exposure in the development of CKDu (Ganguli, 2016; Khandare et al., 2015; Rajapakse et al., 2016). Moreover, the mode of farming practiced among the study group 1 and 2 subjects and controls is of the organic type wherein bio-pesticides (like *Trichoderma* spp, *Metarhizium* spp., pheromone traps, alkaloids, terpenoids) provided at concessional rates by the state government are utilized (Personal communication with Zonal Canacona agricultural officer, Mr.Shivram Gaonkar) which restricts the use of chemical pesticides therefore suggesting no contribution of agrochemicals in CKD development herein. The irrigation methods, type of crop cultivated (mostly paddy cultivation) and access to staple foods in each of the three study areas were also no different, further disproving the role of these few nephrotoxic pesticides in CKDu development in the current study (Kabata et al., 2016; Rajapakse et al., 2016). Drinking untreated groundwater specifically well water provides a high risk of developing chronic nephropathy especially when invaded by anthropogenic influences (like mining) owing to its remarkable contamination with various environmental nephrotoxins like heavy metals (Jayasumana et al., 2015b; Lebov et al., 2015; Sankhla et al., 2016; Wanigasuriya, 2012).

In the current study as well the entire population of study group 1 (100% prevalence) consumed untreated well water for their drinking requirements as compared to 14% and 7% of the study group 2 and 3 subjects respectively ingesting well water. The remaining proportions of these two study groups 2 and 3 relied on municipality treated surface water for consumption. The significantly higher prevalence of well-water consumption among the study group 1 subjects unequivocally suggests an evidence of environmental etiology for CKDu development in this cohort (Jayasumana et al., 2015b; Lebov et al., 2015; Sankhla et al., 2016; Wanigasuriya, 2012).

Furthermore there was the absence of familial clustering of CKD in both the study cohorts 1 and 2 suggesting the absence of a genetic predisposition of the CKD disease development in either of these regions. Ayurvedic herbal preparations have recently come to light as a potential causal in development of chronic nephropathy owing to the presence of nephrotoxic heavy metals in its constitution. In lieu with this, lack of utilisation of traditional herbal medicines (ayurvedic medicines) were noted among all the three study groups, hence this agent maybe unlikely to play a role in the disease etiology (Jayatilake et al., 2013; Ranasinghe et al., 2015).

Surprisingly, around 54% of patients in the endemic study group (study group 1) complained of a history of back pain, body pain and joint pain for an average of 10-15 years before the development of chronic renal failure, while only 11% and 7% of the subjects from study group 2 and 3 respectively reported such problems. This was found to be statistically significant. These patients of study group 1 (around 51%) also admitted to the consumption of NSAID's like paracetamol during that period to get some relief from the skeletal discomfort. Chronic consumptions of NSAIDs have been widely reported to be associated in the causation of Chronic tubulo-interstitial nephritis (CTN), the major hallmark of CKDu (De Broe and Elseviers, 2009). In the current study as well a considerable history of chronic consumption of NSAID's in relatively half the proportion of study group 1 subjects suggested that NSAID's could be one of the contributing factors in the progression of CTN to chronic renal failure indicating a possible evidence of analgesic nephropathy in this region as well(Brooks, 2009; Khandare et al., 2015; MINSAL, 2015; Wesseling et al., 2015) Moreover fluoride has been recently considered as a plausible etiological factor in CKDu development in a few affected developing countries like Sri-Lanka (Wasana et al., 2016). However in the existing study, no visible signs of dental fluorosis such as yellow and patchy discoloration of the teeth were observed in either of the study groups indicative of absence of fluoride exposure and poisoning (Correa-Rotter, 2017).This was further backed by the deficient levels of fluoride present in the drinking water of all the three study regions described in Chapter 3 of this thesis. These studies were embarked upon as the published data on the prevalence of Chronic kidney disease of unknown etiology (CKDu) in the Canacona taluka were lacking and apprehensions were being raised for the rising incidence of this disease in the taluka by the local and medical experts. Hence in order to gain a preliminary knowledge about the distribution of CKDu in this taluka, this basic demographic study was conducted to analyse the general characteristics of the CKDu stratified population against the typical CKD cases, to permit further analysis of risk factors associated with CKDu development in this region.

2.3.3.2 Analysis of various risk-factor or predictors in the development of Chronic kidney disease of unknown etiology (CKDu)

Even though a cross-sectional study may not be able to fully decipher the causation of the disease, an effort was undertaken by us to explore the plausible risk factors associated with CKDu in the Canacona taluka based on the estimated demographic and lifestyle characteristics of the study population. The pooled data of the demographic and lifestyles features for all the three study cohorts were utilised in the analysis of risk-factors/ predictors for CKDu by suitable determination of risk-ratios (Ratnayake et al., 2017). These risk ratios were estimated in three varied scenarios i.e. by comparison of CKDu cases with true controls; general CKD cases versus true controls and CKDu cases versus general CKD cases (considering general CKD as controls) to assess variations (if any) in the risk factors associated with the development of CKDu and CKD. The selected risk factors/predictors analysis for CKDu (via risk -ratio determination) in comparison with the true controls cohort is depicted in **Table 2.4**; whereas in comparison with general CKD cases (considered as controls) are represented in **Table 2.5**. The selected risk factors analysis for CKD (via risk-ratio determination) in comparison with the true controls cohort are depicted in **Table 2.6.**

The significant risk factors/predictors of chronic kidney disease of unknown etiology in a few related studies in some of the affected developing countries like Sri Lanka and Central America have been reported to be family history of kidney dysfunction (Athuraliya et al., 2011), carrying out farming as a primary occupation (Jayasumana et al., 2015 a and c), history of ayurvedic therapy (Wanigasuriya et al., 2011), history of NSAIDs (Wesseling et al., 2015) intake and consumption of groundwater(i.e. well water for drinking purposes) (Lebov et al., 2015). In current study these risk factors were analyzed separately for both the sexes and collectively as well to assess for gender bias (if any) for certain CKDu predictors.

In a univariate risk-ratio (R.R) analysis of the pooled demographic and lifestyle features for the two study cohorts (CKDu v/s true controls, **Table 2.4**) consuming well water for drinking requirements (R.R (males)- 12.4, 95% CI-5.35-28.74, p=<0.0001 and R.R (females)- 15.5, 95% CI-6.01-39.99, p=<0.0001) prevalence of skeletal disorders (R.R(males)- 8.28, 95% CI-3.12-22.02, p=<0.0001 and R.R(females)- 6.64, 95% CI-2.77-15.93, p=<0.0001), history of NSAIDs consumption (R.R(males)- 6.41, 95% CI-5.67-15.41, p=<0.0001 and R.R(females)-6.2, 95% CI-2.57-14.95, p=<0.001) and long duration of NSAIDs consumption (R.R(males)-8.55, 95% CI-2.72-26.89, p=0.0002 and R.R (females)- 6.08, 95% CI-2.24-16.59, p=0.0004) were significantly more common among CKDu patients as compared to controls with no

remarkable variations observed in either of the sexes. These observations were consistent with the risk factors that were noted for CKDu development in Central America, Sri Lanka and another neighboring Indian state i.e. Andhra Pradesh (specifically Uddanam region) (Brooks, 2009; Correa-Rotter et al., 2014; Ganguli et al., 2016; Jayasumana et al., 2015b; Khandare et al., 2015; Lebov et al., 2015; Wanasinghe et al., 2018; Wasana et al., 2016). Another interesting observation was that among the possible lifestyle factors analysed (i.e. the type of occupation carried out) previous involvement in mining conferred a significantly higher risk of CKDu development (R.R (males)-12.56, 95% CI-4.83-32.67, p= <0.0001 and R.R (females)-14.76, 95% CI- 4.83-45.07, p= <0.0001) relative to controls. Although mining was banned for the past 15 years (The Hindu, 2017) in the Canacona taluka and the CKDu affected subjects are no long involved in the same, does not accurately justify mining as an occupational risk-factor in this CKDu endemic region. The high risk ratio obtained is merely an outcome of the higher prevalence of the affected subjects previously being involved in mining in the past. However the presence of a mine (operational or non-operational in the vicinity of the CKDu hit area increased the risk of developing CKDu (R.R (males)- 12.49, 95% CI-2.78-19.75, p=0.0006 and R.R(females)- 12.47, 95% C.I-2.69-18.74, p=0.0003) as compared to the areas without the mines in its proximity. The presence of mine in the vicinity as a significant risk factor for CKDu were consistent with the results from the analysis of various risk factors in CKDu development in Central America and Uddanam region of India wherein the CKDu endemic regions were located in the close proximity of a mining area (Correa-Rotter et al., 2014; Khandare et al., 2015; Ramirez-Rubio et al., 2013).

Considering both males and females collectively, chronic consumption of untreated well water (R.R(total)- 13.78, 95% CI-7.34-25.85, p=<0.0001), occurrence of skeletal discomfort among the CKDu population (R.R (total)- 7.37, 95% CI-3.84-14.15, p=<0.0001), previous history of NSAIDs consumption(R.R (total)- 6.31, 95% CI- 3.39-11.74, p=<0.0001), prolonged intake of NSAID's for pain alleviation (R.R (total)- 7.15, 95% CI-3.37-15.18, p=<0.0001), occurrence of previous involvement in mining as the occupation (R.R (total)-13.51, 95% CI-6.54-27.96, p=<0.0001), presence of operational or non-operational mines (R.R (total)-12.48, 95% CI- 2.72-19.12, p=0.0001) in the vicinity persisted as prominent and significant risk factors in the development of chronic renal failure in the CKDu population in the Canacona taluka. (Brooks, 2009; Correa-Rotter et al., 2014; Ganguli et al., 2016; Jayasumana et al., 2015b; Khandare et al.,2015; Lebov et al., 2015; ; Ramirez-Rubio et al., 2013; Thammitiyagodage et al., 2018; Wanasinghe et al., 2018).

Table 2.4: Univariate risk ratio analysis for determination of risk factors/predictors for Chronic kidney disease of unknown etiology (CKDu) on comparison of CKDu cases with true controls.

Variable	Response	No.(%) of males)		No.(% of females)		Total No.(%)	
		Cases (study group 1) (n=58)	Control (study group 3) (n=62)	Cases (study group 1) (n=56)	Control (study group 3) (n=62)	Cases (study group 1) (n=114)	Control (study group 3) (n=124)
Occurence of diabetes	Yes	0	0	0	0	0	0
	No	58	62	56	62	114	124
Risk-ratio (95% CI)		0.06(0.002-5.47)		0.11(0.002-5.68)		0.09(0.002-5.53)	
χ^2 (p-value)		0.974		0.959		0.967	
Occurence of hypertension	Yes	0	0	0	0	0	0
	No	58	62	56	62	114	124
Risk-ratio (95% CI)		0.06(0.002-5.47)		0.11(0.002-5.68)		0.09(0.002-5.53)	
χ^2 (p-value)		0.974		0.959		0.967	
Type of occupation							
Involvement in mining	Yes	47	4	40	3	87	7
	No	11	58	16	59	27	117
Risk-ratio (95% CI)		**12.56(4.83-32.67)***		**14.76(4.83-45.07)***		**13.51(6.54-27.96)***	
χ^2 (p-value)		<0.0001		<0.0001		<0.0001	
Risk-ratio (95% CI)		61.95 (18.52-207.2)		49.16 (13.44-179.84)		53.86 (22.4-129.37)	
Involvement in farming	Yes	6	27	5	28	11	55
	No	52	35	51	34	103	69
Risk-ratio (95% CI)		0.23(0.11-0.53)		0.20(0.08-0.48)		0.22(0.12-0.39)	
χ^2 (p-value)		0.0003		0.0003		<0.0001	
Involvement in fishing	Yes	5	30	11	28	16	58
	No	53	32	45	34	98	66
Risk-ratio (95% CI)		0.18(0.07-0.43)		0.43(0.24-0.79)		0.30(0.18-0.49)	
χ^2 (p-value)		0.0001		0.0062		<0.0001	
Presence of mines in the vicinity	Yes	58	0	56	0	114	0
	No	0	62	0	62	0	124
Risk-ratio (95% CI)		12.49(2.78-19.75)		12.47(2.69-18.74)		12.48(2.72-19.12)	
χ^2 (p-value)		0.0006		0.0003		0.0001	
Usage of chemical pesticides	Yes	0	2	0	2	0	4
	No	58	60	56	60	114	120
Risk-ratio (95% CI)		0.09(0.005-1.71)		0.10(0.006-1.77)		0.052(0.003-0.87)	
χ^2 (p-value)		0.112		0.111		0.04	
Drinking water source							
Untreated well water	Yes	58	5	56	4	114	9
	No	0	57	0	58	0	115
Risk-ratio (95% CI)		**12.4(5.35-28.74)***		**15.5(6.01-39.99)***		**13.78(7.34-25.85)***	
χ^2 (p-value)		<0.0001		<0.0001		<0.0001	
Municipality treated surface water	Yes	0	57	0	58	0	115
	No	58	5	56	4	114	9
Risk-ratio (95% CI)		0.009(0.006-0.147)		0.009(0.0006-0.149)		0.0047(0.0003-0.075)	
χ^2 (p-value)		0.0009		0.0009		0.0001	
Familial history of renal disease	Yes	0	0	0	0	0	0
	No	58	62	56	62	114	124
Risk-ratio (95% CI)		0.06(0.002-5.47)		0.11(0.002-5.68)		0.09(0.002-5.53)	
χ^2 (p-value)		0.974		0.959		0.967	
Usage of ayurvedic medicine	Yes	0	0	0	0	0	0
	No	58	62	56	62	114	124
Risk-ratio (95% CI)		0.06(0.002-5.47)		0.11(0.002-5.68)		0.09(0.002-5.53)	
χ^2 (p-value)		0.974		0.959		0.967	
Previous medical history of skeletal disorders	Yes	31	4	30	5	61	9
	No	27	58	26	57	53	115
Risk-ratio (95% CI)		**8.28(3.12-22.02)***		**6.64(2.77-15.93)***		**7.37(3.84-14.15)***	
χ^2 (p-value)		<0.0001		<0.0001		<0.0001	
Past history of NSAIDs consumpton	Yes	30	5	28	5	58	10
	No	28	57	28	57	56	114
Risk-ratio (95% CI)		**6.41(5.67-15.41)***		**6.2(2.57-14.95)***		**6.31(3.39-11.74)***	
χ^2 (p-value)		<0.0001		<0.0001		<0.0001	
Prolonged Duration of NSAIDs consumption (for more than 5 yrs)	Yes	24	3	22	4	46	7
	No	34	59	34	58	68	117
Risk-ratio (95% CI)		**8.55(2.72-26.89)***		**6.08(2.24-16.59)***		**7.15(3.37-15.18)***	
χ^2 (p-value)		0.0002		0.0004		<0.0001	

Abbreviations: CKDu- chronic kidney disease of unknown etiology, CKD-chronic kidney disease, CI-confidence interval, NSAIDs-non-steroidal anti-inflammatory drugs. Statistical significance of the risk ratios were calculated by chi-square test(χ^2). Differences at *p<0.05 were considered to be statistically significant. A risk ratio >1 indicates the exposure increases the relative risk of developing the disease outcome(i.e. CKDu) and <1 indicates protection and a lower relative risk of developing the disease outcome(i.e. CKDu).Risk ratio with values that were significantly (i.e. possessing statistical significance of *p <0.05) and markedly larger than 1 were considered to be predominant risk factors in the development of CKDu and these values are highlighted in bold.Herein the risk factors for CKDu development was established to be drinking untreated well water,presence of mines in the vicinity,prevalence of skeletal disrorder,past history and prolonged intake of NSAIDs.

In simpler words, subjects that chronically consumed untreated groundwater (i.e. well water) were 13.7 times more likely to possess CKDu as compared with those who did not consume it. Subjects that had an occurrence of skeletal discomfort and chronically consumed NSAIDS for pain relief were 7.4 and 7.2 times respectively more likely to develop CKDu as opposed to those who complained of no such skeletal problems and refrained from NSAIDs consumption. Moreover subjects engaged in mining previously as the primary occupation were 13.5 times more likely to have CKDu as compared to those with no involvement in the same. The higher risk associated with mining in this CKDu hit area of the Canacona taluka is an upshot of the higher number of subjects that were previously engaged in this occupation before its non-functionality. Therefore, the odds of the mining as an occupational risk factor are not completely justifiable as these subjects are currently no longer involved in the same for the past 15 years (The Hindu, 2017), thereby decreasing the chances of occupational exposure to nephrotoxins like heavy metals via air. However the location of non-operational granite mine in the vicinity of the CKDu affected area (that comprised of a large prevalence of previous miners) increased the risk of the all of the residing subjects in developing the disease by 12 times as compared to subjects residing in mine–free areas (control region without CKD incidence) (**Table 2.4).**

This trend was completely resonated in the a univariate risk-ratio assessment of the assembled lifestyle and demographic features for the two study cohorts (CKDu v/s general CKD cases(considered as controls), **Table 2.5**). Herein also no gender bias was noted for the analysed risk factors as well, with risk factors affecting both the sexes to a comparable extent (Khandare et al., 2015). The prevalent and significant risk factors (predictors) associated with the development of CKDu as opposed to the typical development of diabetic or hypertensive CKD were the long-term consumption of untreated well water (R.R (total)-7.0, 95% C.I 2.83-17.34, p= <0.001), incidence of skeletal disorders among the CKDu population (R.R (total)-4.99, 95% C.I-1.69-14.75, p=0.003), past history of NSAIDs consumption (R.R (total)- 3.56, 95% C.I-1.41-8.98, p=0.007), chronic intake of NSAID's (R.R (total)- 3.76, 95% C.I- 1.26-11.23, p=0.017), previous existence of mining occurring among subjects of study group 1 (CKDu subjects) (R.R (total)-5.34, 95% C.I- 2.14-13.31, p=0.0003), occurrence of functional or non-functional mines in the vicinity (R.R (total)- 5.74, 95% C.I-2.69-19.01, p=0.0038) compared to the general CKD cases (study group 2) (Brooks, 2009; Hitomi et al., 2012; Jayasekara et al., 2015; Khandare et al.,2015; Lebov et al., 2015; Ramirez-Rubio et al., 2013; Ranasinghe et al., 2015; Senevirathna et al., 2012; Wasana et al., 2016).

Table 2.5:Univariate risk ratio analysis for determination of risk factors/predictors for Chronic kidney disease of unknown etiology (CKDu) on comparison of CKDu cases with typical CKD cases considered as non-endemic CKD controls.

Variable	Response	No.(%) of males)		No.(% of females)		Total No.(%)	
		Cases (study group 1) (n=58)	CKD Control (study group 2) (n=15)	Cases (study group 1) (n=56)	CKD Control (study group 2) (n=13)	Cases (study group 1) (n=114)	CKD Control (study group 2) (n=28)
Occurence of diabetes	Yes	0	10	0	9	0	19
	No	58	5	56	4	114	9
Risk-ratio (95% CI)		0.0129(0.0008-0.21)		0.0129(0.0008-0.20)		0.0065(0.0004-0.10)	
χ^2 (p-value)		0.0022		0.0022		0.0004	
Occurence of hypertension	Yes	0	5	0	4	0	9
	No	58	10	56	9	114	19
Risk-ratio (95% CI)		0.025(0.0014-0.43)		0.027(0.0016-0.478)		0.013(0.0008-0.222)	
χ^2 (p-value)		0.0107		0.014		0.003	
Type of occupation							
Involvement in mining	Yes	47	2	40	2	87	4
	No	11	13	16	11	27	24
Risk-ratio (95% CI)		**6.08(1.67-22.22)***		**4.6(1.28-16.79)***		**5.34(2.14-13.31)***	
χ^2 (p-value)		0.0064		0.002		0.0003	
Involvement in farming	Yes	6	3	5	4	11	7
	No	52	12	51	9	103	21
Risk-ratio (95% CI)		0.52(0.15-1.83)		0.30(0.09-0.93)		0.38(0.16-0.91)	
χ^2 (p-value)		0.31		0.038		0.028	
Involvement in fishing	Yes	5	9	11	8	16	17
	No	53	6	45	5	98	11
Risk-ratio (95% CI)		0.14(0.06-0.37)		0.32(0.16-0.63)		0.23(0.13-0.39)	
χ^2 (p-value)		<0.0001		0.001		<0.0001	
Presence of mines in the vicinity	Yes	58	0	56	0	114	0
	No	0	15	0	13	0	28
Risk-ratio (95% CI)		5.89(2.81-22.3)		5.65(2.01-17.6)		5.74(2.69-19.01)	
χ^2 (p-value)		0.0041		0.0036		0.0038	
Usage of chemical pesticides	Yes	0	2	0	1	0	3
	No	58	13	56	12	114	25
Risk-ratio (95% CI)		0.054(0.0027-1.07)		0.082(0.0035-1.91)		0.036(0.0019-0.68)	
χ^2 (p-value)		0.056		0.12		0.027	
Drinking water source							
Untreated well water	Yes	58	2	56	2	114	4
	No	0	13	0	11	0	24
Risk-ratio (95% CI)		**7.5(2.06-27.25)***		**6.5(1.82-23.26)***		**7.0(2.83-17.34)***	
χ^2 (p-value)		0.0022		0.004		<0.0001	
Municipality treated surface water	Yes	0	13	0	11	0	24
	No	58	2	56	2	114	4
Risk-ratio (95% CI)		0.01(0.0006-0.16)		0.011(0.0007-0.171)		0.0051(0.0003-0.082)	
χ^2 (p-value)		0.0011		0.0013		0.0002	
Familial history of renal disease	Yes	0	0	0	0	0	0
	No	58	15	56	13	114	28
Risk-ratio (95% CI)		0.027(0.005-1.31)		0.024(0.0005-1.18)		0.025(0.0005-1.24)	
χ^2 (p-value)		0.51		0.48		0.49	
Usage of ayurvedic medicine	Yes	0	0	0	0	0	0
	No	58	15	56	13	114	28
Risk-ratio (95% CI)		0.027(0.005-1.31)		0.024(0.0005-1.18)		0.025(0.0005-1.24)	
χ^2 (p-value)		0.51		0.48		0.49	
Previous medical history of skeletal disorders	Yes	31	2	30	1	61	3
	No	27	13	26	12	53	25
Risk-ratio (95% CI)		**4.01(1.08-14.89)***		**6.4(1.04-46.51)***		**4.99(1.69-14.75)***	
χ^2 (p-value)		0.038		0.045		0.003	
Past history of NSAIDs consumpton	Yes	30	2	28	2	58	4
	No	28	13	28	11	56	24
Risk-ratio (95% CI)		**3.88(1.04-14.43)***		**3.25(0.88-11.94)***		**3.56(1.41-8.98)***	
χ^2 (p-value)		0.043		0.048		0.007	
Prolonged Duration of NSAIDs consumption (for more than 5 yrs)	Yes	24	2	22	1	46	3
	No	34	13	34	12	68	25
Risk-ratio (95% CI)		**3.1(0.82-11.69)***		**4.9(0.756-34.52)***		**3.76(1.26-11.23)***	
χ^2 (p-value)		0.008		0.009		0.017	

Abbreviations: CKDu- chronic kidney disease of unknown etiology, CKD-chronic kidney disease, CI-confidence interval, NSAIDs-non-steroidal anti-inflammatory drugs. Statistical significance of the risk ratios were calculated by chi-square test(χ^2). Differences at *p<0.05 were considered to be statistically significant. A risk ratio >1 indicates the exposure increases the relative risk of developing the disease outcome(i.e. CKDu) and <1 indicates protection and a lower relative risk of developing the disease outcome(i.e. CKDu). Risk ratio with values that were significantly (i.e. possessing statistical significance of *p <0.05) and markedly larger than 1 were considered to be predominant risk factors in the development of CKDu and these values are highlighted in bold. Herein the risk factors for CKDu development were further confirmed to be drinking untreated well water,presence of mines in the vicinity,prevalence of skeletal disrorder,past history and prolonged intake of NSAIDs.

In other words, subjects that consumed untreated well water for a long-term were 7 times more likely to develop CKDu instead of typical diabetic or hypertensive nephropathy. Subjects that had a considerable prevalence of skeletal pains and consumed NSAIDS over a prolonged period were 5 and 3.7 times respectively more probable to possess CKDu with contributing analgesic etiology as opposed to the development of typical CKD (**Table 2.5).**

Furthermore subjects residing in area with mines (operational or non-operational) in their close proximity were 5.7 times more likely at risk of developing CKDu instead of manifesting the typical diabetic or hypertensive CKD even though the mine was non-operational. Mining can confer susceptibility of the subjects to be exposed to various environmental nephrotoxins via matrices like groundwater excluding air, even after mining has ceased. This is majorly possible due to contamination of the groundwater aquifer by acid mine drainage from the non-operational mine which can persists for several years even after stoppage of mining and can effectively leach out nephrotoxins (like heavy metals) from the aquifer thereby deeming it to be a suitable matrix for environmental nephrotoxin exposure (Fantong et al., 2009; McCarthy, 2011; Khandare et al., 2015; Sankhla et al., 2016).This explains the increased risk of developing CKDu in the endemic region of the Canacona taluka which is located in close proximity of a non-operational granite mine (in Chaudi) and the absence of CKDu development in the control regions and areas with diabetic/hypertensive CKD residents owing to the lack of mines in these regions. Thereby further suggesting that the causation of the CKDu disease in the endemic region of the Canacona taluka might be related to an environmental causal agent and not associated with occupational exposure to nephrotoxins via air owing to the non-functionality of the mine for a long-time(15 yrs) (Khandare et al., 2015, Obrador et al., 2017; Wimalawansa, 2016) (**Table 2.5).**

Canacona taluka being a rural area extensively relies on untreated groundwater for their drinking requirement being more popular among the residents of study area 1. Due to their poor financial status and economic constraints faced by these residents, treatment of groundwater becomes unaffordable due to which it serves as a major route for exposure to various environmental nephrotoxins like heavy metals when disturbed by anthropogenic activities like mining (Fantong et al., 2009; Sankhla et al., 2016). In the current study as well the higher significant risk-ratios indicated that exposure to well water conferred a higher risk of developing CKDu, thereby providing supporting evidence that CKDu is of an environmental etiology (Khandare et al., 2015; Ranasinghe et al., 2015; Wasana et al., 2016; Wanasinghe et al., 2018). The higher risk of exposure to anthropogenically contaminated groundwater with environmental toxins was additionally backed by the presence of non-

operational granite mine in the vicinity of the CKDu affected study area and the predominance of previous involvement in mining as the common occupational risk factor in the CKDu population (Khandare et al., 2015; Senevirathna et al., 2012). Mining is a major anthropogenic activity that has been well reported to disturb various exposure matrices like groundwater aquifer or air, with high tendency to contaminate these matrices with various environmental nephrotoxins (McCarthy, 2011; Sankhla et al., 2016). However in the current study although mining was established to the occupational risk factor, it is not completely defensible due to non-operation of the mine present in the vicinity of the CKDu hit area for past 15 yrs., due to which the affected subjects are not presently involved in mining. Hence the occupational propensity to be exposed to nephrotoxins via air associated with mining can be safely ruled out limiting it to exposure via groundwater. The groundwater can be gravely contaminated with nephrotoxins like heavy metals for several years even post discontinuation of mining as a result of acid mine drainage ,a persistent environmental hazard of mining (Orakwue et al., 2016). This groundwater contamination with nephrotoxins could have occurred in this CKDu affected region of the Canacona taluka as well. Furthermore since the affected subjects of this CKDu-endemic area in this taluka comprised of mixture of occupations i.e. previous miners, farmers and agricultures, suggests that source of nephrotoxin exposure is possibly environmental (like groundwater) and common among the CKDu affected subjects thereby highlighting that the causation of this disease is not occupational but rather related to an environmental factor (Abiola, 2017; Ratnayake et al., 2016; Rajapakse et al., 2016).

In the demographic study, half of the CKDu population did report complaints of skeletal disorders due to which they resorted to NSAIDS consumption for pain relief which justifies the common occurrence of these factors among the CKDu study population suggesting that the CKDu etiology in this study region could also be potentially associated with analgesic nephropathy as reported in an endemic CKDu case study in a sub-district of Andhra Pradesh, India (Khandare et al., 2015) and Nicaragua of Central America (Brooks, 2009).

Other potential risk factors (predictors) that were analysed for CKDu in comparison with true controls **(Table 2.4)** and general CKD cases **(Table 2.5)** were familial history, farming as a primary occupation, history of ayurvedic therapy, diabetes, and hypertension. These selected predictors were found to possess low risk-ratios and no statistical significance in both of the sets of comparative analysis (**Table 2.4 and 2.5**), hence were not believed to be risk factors associated with the induction and development of CKDu. A familial history of kidney dysfunction usually indicates an involvement of a genetic factor in the causation of the

disease. However since familial clustering was uncommon among the CKDu subjects of study group 1 and persons of families residing in this study area developed this condition, it is greatly unlikely that this disease is genetically predisposed but rather triggered by an environmental causal agent. These results were consistent with the observations obtained from the study of CKDu in Sri Lanka and Uddanam region of Andhra Pradesh (Ganguli, 2016; Jayatilake et al., 2013).

Amongst the lifestyle factors analysed in previous related studies of CKDu in developing countries, specifically Central America, the farming community was noted to be the most vulnerable population which possessed the highest risk of developing CKDu as compared to non-farmers. This vulnerability is attributed to exposure to plausible disease modifiers like prolonged dehydration, heat stress and environmental renal toxicants like pesticides, and soil borne heavy-metals like lead, cadmium and arsenic (which can also be simultaneously present in the pesticides as well) (Jayasumana et al., 2016; Orantes-Navarro et al., 2017).However in the current study, farming was overpowered by the prevalence of the previous involvement in mining as the major occupation among the CKDu subjects of study group 1,which potentially rules out the general bias of CKDu to majorly affect farmers only, thereby negating an established trend of CKDu development in this region. These observations were consistent with the presence of a lack of occupational bias in the CKDu scenario in the Uddanam region of India, Sri Lanka & some parts of Central America (Butler-Dawson et al., 2018; Kabata et al., 2016; Khandare et al., 2015; Wanigasuriya et al., 2007). The absence of the predominance of farming among the subjects of study group1 plausibly eliminates the association of CKDu with the etiology of Balkan endemic nephropathy. Balkan endemic nephropathy (BEN) is a renal dysfunction predominant among the residents situated alongside the Danube River tributaries in Bosnia, Serbia, Romania, and Bulgaria (Stefanovic, 1998). BEN is majorly caused by mycotoxins which gradually advancing tubulo-interstitial disease that ultimately progresses to end-stage renal failure. This disease mainly affects the rural farming community, depicts familial clustering and possesses raised incidence of renal pelvis and ureter tumors (Pavlovic, 2014), all of which were significantly absent among the subjects of study group 1, suggesting no correlation of the etiology of the CKDu cases with BEN.

Although mining was determined to be the predominant risk factor due to the larger involvement of the affected subjects in mining in the past (previously), it cannot be concluded that the CKDu developed is an occupational induced nephropathy i.e. triggered from mining. It has been well established that the presence of mining increases the risk of

development of chronic kidney damage via possible exposure to environmental nephrotoxins via air and water (Fantong et al., 2009; McCarthy, 2011; Sankhla et al., 2016). However since granite mining in the Canacona taluka has been banned and non-operational for the past 15 years and the affected subjects have no longer been engaged in the same, eliminates the possibility of contracting nephropathy/ kidney disease due to the occupational exposure to various nephrotoxins via the major source i.e. air (Khandare et al., 2015; Nanayakkara et al., 2014). Moreover the occurrence of a mixed occupational constitution in the affected cohort i.e. comprising of previous miners, farmers and fishermen, provides supporting evidence to the CKDu disease in this taluka to be of the non-occupational kind. Thereby suggesting that the CKDu affected subjects comprising of mixed occupations are possibly exposed to nephrotoxins from common environmental source like groundwater (drinking water), indicating the etiology of this disease to be environmentally induced (Abiolam2017; Ganguli, 2016; Levine et al., 2016; Obrador et al., 2017; Wimalawansa, 2016).

A previous history of consumption of ayurvedic or herbal preparations has been reportedly associated with development of chronic renal failure. Most of these constitutions are not formally approved by regulatory bodies like the FDA (Food and Drug Administration) and hence contain elements that induce kidney failure (Wanigasuriya et al. 2011). In Belgium, common consumption of Chinese herb Stephania tetrandra as a part of the slimming routine significantly resulted in development of chronic tubulointerstitial nephritis, the histopathological presentation of CKDu. On considerable withdrawal of this herb, a drastic decrease in CTN cases were noted indicating a possible role of ayurvedic preparations in CKDu development (Stiborova et al., 2016). In the current study absence of history of ayurvedic medicine excludes its causal risk associated with induction of CKDu among study group 1; which was in agreement with risk factor analysis of CKDu in Sri Lanka & Uddanam region (India) (Khandare et al., 2015; Rajapakse et al., 2016; Ranasinghe et al., 2015).

Moreover diabetes and hypertension were not significant predictors for the development of CKDu in study group 1 (Gifford et al., 2017; Lusco et al., 2017). However, in a univariate risk-ratio analysis of the collective demographic and lifestyle features for the two study cohorts (general CKD cases v/s true controls, **Table 2.6),** diabetes (R.R (males)- 8.26, 95% CI-0.511-133.75, p=0.0019 and R.R (females)- 8.55, 95% CI-0.53-138.41, p=0.0017) and hypertension (R.R (males)- 4.33, 95% CI- 0.25-74.3, p=0.009 and R.R (females)- 4.05, 95% CI-0.23-70.9, p=0.0011) were observed to be more significant and prevalent (common) among the subjects of study group 2 (i.e. CKD cases) as compared to true controls (i.e. study group 3) with no significant gender bias noted. Considering the risk-ratios of the males and

females collectively, diabetes (R.R (total)-8.48, 95% CI-0.521-136.1, p=0.0018) and hypertension (R.R (total)- 4.0, 95% CI-0.24-72.6, p=0.011) persisted as significant risk factors associated with the development of chronic kidney disease among the subjects of study group 2 as opposed to controls providing unequivocal evidence on the role of diabetes and hypertension to serve as traditional risk factors that characterize Chronic kidney disease.(Hill et al., 2016; Hu and Coresh, 2017). In other words, the prevalence of pre-existing medical conditions like diabetes and hypertension among the subjects are 8.4 and 4 times respectively more likely to develop Chronic kidney disease as compared to those who do not possess such medical histories. The etiology of Chronic kidney disease in study area 2 associated with traditional causals (i.e. diabetes and hypertension) was further reinforced by the reduced prevalence of the general predicted risk factors for CKDu like farming, dominance of mining, consumption of untreated groundwater, familial clustering, history of ayurvedic therapy & NSAIDs consumption. Thereby suggesting that etiology of Chronic kidney disease among study group 2 subjects was linked to known risk-factors viz. diabetes &hypertension with no significant genetic predisposition or contribution from environmental causals associated with food or drinking water (Levey et al., 2015; Hill et al., 2016).

Therefore, risk factor analysis via determination of risk-ratios using the lifestyle and demographic characteristics of the study population permitted the suitable prediction of various risk factors (Lebov et al., 2015; Ratnayake et al., 2017; Senevirathna et al., 2012) that were significantly and positively associated with the development of CKDu among the subjects of study cohort 1.These risk factors included the prolonged consumption of untreated groundwater (well-water) for their drinking requirements, presence of a non-operational mine in the vicinity and predominance of previous involvement in mining as the major occupation in the study area 1 (CKDu hit endemic region) that conferred susceptibility to prolonged exposure to various environmental nephrotoxins and associated induction of CKDu development. This study also suggested the occurrence of skeletal discomfort and concomitant long-term duration of NSAIDs for pain relief proved to be auxiliary etiological factors in triggering renal failure linked with the progression of Chronic kidney of unknown etiology. These established risk factors for CKDu development in the Canacona taluka were consistent with a few related analysis of the CKDu prevalence and etiology in endemic areas of the affected developing countries like the sub-district of Andhra Pradesh, India and North Central Province of Sri Lanka as described above. Thereby providing supporting evidence to the contribution of these risk factors for appropriate characterization of CKDu in the Canacona taluka.

Table 2.6:Univariate risk ratio analysis for determination of risk factors/predictors for Chronic kidney disease(CKD) on comparison of CKD cases with those without chronic renal disease(i.e. true controls)

Variable	Response	No.(% of males)		No.(% of females)		Total No.(%)	
		CKD cases (study group 2) (n=15)	True controls (study group 3) (n=62)	CKD cases (study group 2) (n=13)	True controls (study group 3) (n=62)	CKD cases (study group 2) (n=28)	True controls (study group 3) (n=124)
Occurence of diabetes	Yes	10	0	9	0	19	0
	No	5	62	4	62	9	124
Risk-ratio (95% CI)		**8.26(0.511-133.75)***		**8.55(0.53-138.41)***		**8.48(0.521-136.1)***	
χ^2 (p-value)		0.0019		0.0017		0.0018	
Occurence of hypertension	Yes	5	0	4	0	9	0
	No	10	62	9	62	19	124
Risk-ratio (95% CI)		**4.33(0.25-74.3)***		**4.05(0.23-70.9)***		**4.0(0.24-72.6)***	
χ^2 (p-value)		0.009		0.011		0.011	
Type of occupation							
Involvement in mining	Yes	2	4	2	3	4	7
	No	13	58	11	59	24	117
Risk-ratio (95% CI)		2.07(0.42-10.25)		2.91(0.59-17.17)		2.53(0.79-8.06)	
χ^2 (p-value)		0.37		0.17		0.12	
Involvement in farming	Yes	3	27	4	28	7	55
	No	12	35	9	34	21	69
Risk-ratio (95% CI)		0.46(0.16-1.31)		0.68(0.29-1.61)		0.56(0.29-1.10)	
χ^2 (p-value)		0.15		0.38		0.09	
Involvement in fishing	Yes	9	30	8	28	17	58
	No	6	32	5	34	11	66
Risk-ratio (95% CI)		1.04(0.76-2.02)		1.06(0.82-2.27)		1.09(0.91-1.84)	
χ^2 (p-value)		0.39		0.23		0.15	
Presence of mines in the vicinity	Yes	0	0	0	0	0	0
	No	15	13	62	62	28	124
Risk-ratio (95% CI)		0.039(0.012-3.37)		0.030(0.006-2.96)		0.033(0.008-3.32)	
χ^2 (p-value)		0.59		0.41		0.46	
Usage of chemical pesticides	Yes	2	2	1	2	3	4
	No	13	60	12	60	25	120
Risk-ratio (95% CI)		0.65(0.15-6.71)		0.86(0.12-7.01)		0.71(0.14-5.5)	
χ^2 (p-value)		0.52		0.96		0.65	
Drinking water source							
Untreated well water	Yes	2	5	2	4	4	9
	No	13	57	11	58	24	115
Risk-ratio (95% CI)		1.65(0.35-7.71)		1.91(0.48-9.67)		1.75(0.65-5.93)	
χ^2 (p-value)		0.64		0.28		0.22	
Municipality treated surface water	Yes	13	57	11	58	24	115
	No	2	5	2	4	4	9
Risk-ratio (95% CI)		0.094(0.076-0.11)		0.090(0.071-0.12)		0.092(0.079-0.11)	
χ^2 (p-value)		0.546		0.41		0.97	
Familial history of renal disease	Yes	0	0	0	0	0	0
	No	15	62	13	62	28	124
Risk-ratio (95% CI)		0.03(0.008-1.91)		0.045(0.009-2.01)		0.043(0.008-2.1)	
χ^2 (p-value)		0.48		0.45		0.46	
Usage of ayurvedic medicine	Yes	0	0	0	0	0	0
	No	15	62	13	62	28	124
Risk-ratio (95% CI)		0.03(0.008-1.91)		0.045(0.009-2.01)		0.043(0.008-2.1)	
χ^2 (p-value)		0.48		0.45		0.46	
Previous medical history of skeletal disorders	Yes	2	4	1	5	3	9
	No	13	58	12	57	25	115
Risk-ratio (95% CI)		0.21(0.04-1.02)		0.10(0.01-0.74)		0.14(0.04-0.51)	
χ^2 (p-value)		0.37		0.96		0.53	
Past history of NSAIDs consumpton	Yes	2	5	2	5	4	10
	No	13	57	11	57	24	114
Risk-ratio (95% CI)		0.16(0.03-0.77)		0.19(0.04-0.87)		0.17(0.05-0.52)	
χ^2 (p-value)		0.52		0.87		0.31	
Prolonged Duration of NSAIDs consumption (for more than 5 yrs)	Yes	2	3	1	4	3	7
	No	13	59	12	58	25	117
Risk-ratio (95% CI)		0.27(0.05-1.50)		0.12(0.015-0.98)		0.18(0.05-0.69)	
χ^2 (p-value)		0.24		0.87		0.33	

Abbreviations: CKD-chronic kidney disease, CI-confidence interval, NSAIDs-non-steroidal anti-inflammatory drugs. Statistical significance of the risk ratios were calculated by chi-square test(χ^2). Differences at *p<0.05 were considered to be statistically significant. A risk ratio >1 indicates the exposure increases the relative risk of developing the disease outcome(i.e. CKDu) and <1 indicates protection and a lower relative risk of developing the disease outcome(i.e. CKDu). Risk ratio with values that were significantly (i.e. possessing statistical significance of *p <0.05) and markedly larger than 1 were considered to be predominant risk factors in the development of CKD and these values are highlighted in bold. Herein the risk factors for general CKD development in study group 2 was established to be diabetes and hypertension.

The current study therefore strongly advocated an environmental etiology of the CKDu disease in the taluka that needed further investigation. Hence a descriptive environmental monitoring for various nephrotoxins in major exposure matrices i.e. food and water was conducted which has been elaborately described in Chapter 3 of this thesis.

2.3.4 Biochemical analysis of various clinical markers of kidney function for establishment of the disease pattern of Chronic Kidney disease of unknown etiology (CKDu)

2.3.4.1 General assessment of various renal function biomarkers of the study population

Biochemical analysis of various biomarkers of renal function is a prerequisite for accurate establishment of disease pathology (i.e. glomerular or tubular injury) (Gowda et al., 2010; Ratnayake et al., 2017). Since study group 1 subjects (i.e. CKDu endemic population) and study group 2 (i.e. typical CKD patients) have already progressed to end stage of renal disease i.e. chronic renal failure, assessments of biomarkers in urine of both these study cohorts 1 & 2 is unfeasible owing to absence of urine production for quantitative analysis. Biochemical measurements in the blood were possible but in order to establish the disease pattern (i.e. glomerular or tubular pathology) the study population would have to be subjected to repeated and frequent biochemical measurements to preserve consistency in the results which would inevitably involve tremendous amount of pricking and inconvenience, hence was avoided in the current study. Thus in order to maintain uniformity in the representation of the assessed biochemical parameters measured in urine and blood in the current study, previous medical records of the estimated renal function biomarkers over a 6 month period (with a 4 week interval) prior to kidney failure manifestation were comprehensively analysed and the average of each measured parameter was reported. The medical records were evaluated solely after attainment of written informed consent from the hospital management and patients. However, the healthy controls (i.e. study group 3 subjects) were subjected to renal function biochemical marker analysis during the course of the current study (for a 6 month period with a 4 week interval) in spot urine and blood samples collected from each of these subjects as these subjects were healthy enough for conduction of clinical assessments (Wijkstrom et al., 2018). These analyses were performed in study group 3 to assess for signs or impending risks of developing kidney damage and proper validation of their recruitment as true controls (i.e. without any kidney damage).The mean levels of various biochemical parameters analysed for the subjects of the three study cohorts (i.e. CKDu endemic cases, CKD non-endemic cases and true control groups) are presented in **Table 2.7.**

Table 2.7: The biochemical characteristics of the entire study population

Parameter	Study group 1 (Endemic CKDu cases)			Study group 2 (Non-endemic CKD cases)			Study group 3 (Healthy controls)			Reference range limits
	Total(N=114)	Male(n=58)	Female(n=56)	Total(N=28)	Male(n=15)	Female(n=13)	Total(N=124)	Male(n=62)	Female(n=62)	
Serum creatinine	19.42±3.2*©	19.83±3.8 *©	19.01± 2.6 *©	7.18±0.6*	7.23±0.9*	7.12±0.5*	0.54±0.22	0.56±0.25	0.51+0.14	0.3-1.4 mg/dL
Urinary creatinine	78.0±4.5*©	79.13±6.1 *©	76.87±3.3 *©	193.12±5.9*	193.66±8.3*	192.58±4.6*	255.5±5.5	256.4±5.9	254.63±5.3	220-300 mg/dL
Serum urea	177.02±5.3*©	182.33±5.6*©	180.5±4.9 *©	106.85±6.9*	108.28±7.8 *	105.41±6.2*	11.09±3.9	11.13±4.8	11.05±3.5	7-20 mg/dL
Urinary urea	0.54±0.05 *©	0.55±0.11*©	0.53±0.04 *©	0.98±0.09 *	0.99±1.1*	0.97±0.06*	1.68±0.2	1.71±0.4	1.65±0.1	1.3-2.1 g/dL
Serum uric acid	35.97±2.3*©	36.41±1.5*©	35.52±2.5 *©	19.08±2.6*	19.12±1.6*	19.04±3.2*	2.35±0.4	2.24±0.3	2.45±0.5	uptill 3.5 mg/dL
Urinary uric acid	3.35±0.15 *©	3.41±0.23*©	3.29±0.14 *©	9.96±0.9*	10.09±1.1*	9.83±0.7*	27.95±4.3	29.55±6.3	26.36±3.3	12.5-37.5 mg/dL
Serum protein	5.56±0.3*©	5.69±0.6*©	5.43±0.2 *©	**1.56±0.2***	**1.66±0.3***	**1.46±0.1***	7.58±1.3	7.67±1.7	7.49±1.2	6-8 g/dL
Urinary protein	16.81±3.3*©	17.16±3.9*©	16.44±2.6*©	**204.76±6.9***	**205.5±7.9***	**203.71±6.5***	6.53±1.1	6.65±1.3	6.41±0.9	uptill 10 mg/dL
Serum albumin	4.03±0.25	4.13±0.13	3.92±0.23	**0.075±0.26***	**0.085±0.02***	**0.065±0.03***	3.75±0.4	3.9±0.6	3.6±0.5	3.5-4.5 g/dL
Urinary albumin	2.62±0.1*	2.71±1.1*	2.53±1.0*©	**134.58±5.6 ***	**136.53±6.7***	**133.63±5.4***	0.89±0.1	1.01±0.12	0.77±0.09	0.5-2.3 mg/dL
Serum b2M	**0.26±0.05 *©**	**0.28±0.07 *©**	**0.24±0.03***	2.57±0.9	2.75±1.0	2.39±0.8	2.48±0.5	2.36±0.9	2.60±0.4	1.8-3 mg/L
Urinary b2M	**0.723±0.10*©**	**0.741±0.13 *©**	**0.705± 0.09*©**	0.023±0.009	0.024± 0.0013	0.022±0.008	0.0163± 0.00023	0.0165± 0.00028	0.0160± 0.00031	0.15-0.3 mg/L
Serum NAG	**0.00089± 0.00006*©**	**0.00083± 0.00005*©**	**0.00097± 0.00007*©**	0.0095± 0.00004	0.01± 0.00008	0.009± 0.00003	0.0077± 0.00005	0.0079± 0.0009	0.0078± 0.00004	uptill 0.01 U/L
Urinary NAG	**15.5±3.3*©**	**17.34±3.9*©**	**13.78±2.9*©**	0.55±0.2	0.57±0.4	0.53±0.1	0.36±0.15	0.38±0.2	0.34±0.1	0.6-1.2 U/L
Serum bicarbonates	**8.19±1.1*©**	**8.25±1.2 *©**	**8.14±0.9 *©**	24.85±2.3	25.2±2.7	24.5±2.1	28.84±2.2	27.9±2.3	29.78±2.1	20-30 mEq/L
Serum phosphates	**1.03±0.09 *©**	**1.11±0.1 *©**	**0.96±0.08 *©**	4.01±0.2	4.08±0.4	3.94±0.1	4.48±0.18	4.55±0.22	4.41±0.16	3.5-4.5 mg/dL

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; b2M-β2-microglobulin, NAG-N-acetyl-β-D-glucosaminidase. Data are derived from two independent experiments with each parameter of an individual experiment measured in triplicates. Values are represented as mean±SD.The units for each measured parameter are represented in their corresponding reference limits. Differences at *$p<0.05$ were considered to be statistically significant as compared to the true controls(study group 3). Differences at © $p<0.05$ were considered to be statistically significant as compared to the non-endemic CKD cases(study group 2). Remarkably large differences in the values of a particular parameter in either study group 1 or 2 as compared to the other groups respectively are highlighted in bold. CKDu in study group 1 was found to be minimally proteinuric with lowered and close to normal levels of the high molecular weight (HMW) proteins viz. albumin (which abundantly constitutes the plasma proteins) being noted in the urine of these subjects. The lack of dominance of albumin in the urinary excreted proteins indicated an absence of glomerular injury in these subjects because albumin is prevented from filtration through the glomerulus under normal functioning of the same due to its HMW. However significantly higher levels of proteinuria(i.e. increased excretion of proteins in the urine and remarkably elevated albumin levels in the urine) were noted in study group 2 subjects(values emphasized in bold) highlighting the occurrence of glomerular injury in these subjects. On the contrary, significantly elevated levels of low molecular weight (LMW) proteins like b2M and NAG were noted in the urine of study group1(CKDu affected)subjects (values emphasized in bold) signifying occurrence of tubular injury in these subjects as LMW proteins are ideally reabsorbed by the proximal tubules under normal functioning. Moroever significantly decreased levels of serum electrolytes viz. bicarbonates and phosphates were noted in the study group 1 subjects as these electrolytes were not reabsorbed and hence eliminated in the urine due to the tubular injury inflicted.

Among the biochemical parameters analysed **(Table 2.7)**, significantly higher levels of basic markers of renal function viz. serum urea, creatinine and uric acid were noted in study group 1 subjects (i.e. CKDu endemic cases) as compared to typical CKD cases (i.e. study group 2). These elevated levels are primary indicators of disturbance in the kidney function as these substances being significant by-products of basic metabolism are deemed to be eliminated from the body at a constant excretion rate. However, owing to induced renal injury, the excretion of these substances is compromised due to which levels are remarkably elevated in blood (García-Trabanino et al., 2015). Although there were elevations in levels (> reference limit) of these parameters in study groups 1 & 2, rise was more significant in study group 1.

Serum urea, creatinine and uric acid are usually eliminated from the body as a result of tubular secretion in the urine. Hence the comparative increase in these metabolic by-products in serum of the study group 1 subjects is an outcome of the insufficiency in the inherent tubular elimination of the same from the serum into the urine via tubular secretion. This decreased clearance from the body (in the urine) and enhanced accumulation of urea, uric acid and creatinine in the serum are characteristic of tubular pathology as noted in chronic tubular interstitial nephritis (the major histopathological diagnosis of CKDu in developing countries), thereby indicating the possible existence of a tubular injury among study group 1 subjects (Wesseling, et al., 2016; Wijkström et al., 2018). This was backed by the existence of a good correlation between serum creatinine with urea and uric acid respectively possessing correlation coefficient values of 0.971 ($p=0.002$) and 0.99 ($p=0.004$) for study group 1 as opposed to a reduced correlation coefficients of 0.116 ($p=0.065$) and 0.166 ($p=0.049$) for study group 2, indicating that increased levels of serum creatinine, urea and uric acid among the CKDu endemic cases (study group 1) are attributed to a tubular injury **(Table 2.9A) (**Wijkström et al., 2018**).** The existence of a lower association between serum creatinine, urea and uric acid in study group 2 indicates the nature of renal injury to be non-tubular (Ratnayake et al., 2017). The elevated levels of urea, creatinine and uric acid in the serum were consistent with decreased levels of the same in the urine supported by significant ($p<0.05$) negative correlations **(Table 2.9)** as noted among the study group 1 subjects indicating signs of kidney failure as the rate of excretion is decreased due to improper filtration (García-Trabanino et al., 2015; Selvarajah et al., 2016). These findings were validated by the observance of similar values reported for these biomarkers in the study of tubular interstitial nephropathic cases in Sri Lanka and Central America (García-Trabanino et al., 2015; Selvarajah et al., 2016; Wesseling, et al., 2016; Wijkström et al., 2018), thereby providing supporting evidence to the existence of a tubular dysfunction among the CKDu

endemic cases in the Canacona taluka. However these indicators are not sufficient for confirmation of tubular injury, but necessitate analysis of other biomarkers for analysis of changes in renal function (glomerular/ tubular) to make a reasonable conclusion.

Clinically the presence of substantial quantities of protein in urine is one of the initial signs of most kidney diseases. Ideally the total quantities of proteins in the plasma are maintained at 6-8 g/dL (reference levels), however on renal injury (i.e. glomerular or tubular), these levels drop down owing to excessive elimination of the same in the urine. Proteinuria mainly refers to the enhanced excretion of urinary proteins like albumin or varied plasma proteins (i.e. tubular proteins). The quantitation of leakage of urinary protein (proteinuria) is central to the diagnosis, detection, treatment and prognosis of renal disease.. Thus proteinuria serves as a vital marker in the risk-assessment of renal damage in both the general populace and patients affected by chronic kidney disease (CKD) (Inker et al., 2014; Vassalotti et al., 2016). Thus precise identification & quantitation of proteinuria are critical. Most guidelines recommend the estimation of protein or albumin in first-void early morning spot urine samples. Even though 24h assessment of urine proteinuria is a golden standard for quantification, spot urine for protein or albumin estimation serves as a practical alternative due toinconvenience associated with 24h sample collection (Jha et al., 2013).

Prior investigations of the scientific implications of urinary protein profiles have classified the leaked (eliminated) plasma proteins as high or low molecular weight (MW). Albumin, is a high MW (60 kDa), that constitutes the major portion of the plasma proteins and is ideally never filtered by the glomerulus owing to its large size, ideally resulting in plasma concentration of 3-5 g/dL (reference levels). However the occurrence of these high MW proteins in the urine (albuminuria) reveals a glomerular toxicity (pathology) as a result of induced remarkable intensification in the effective pore-size of the glomerulus. Moreover, albuminuria is also further evident during the inefficiency in tubular reabsorption providing a preliminary indication of tubular dysfunction (Levin et al., 2013; NIH, 2015; Levey et al., 2015; Hu and Coresh, 2017)**.** The National Kidney Foundation have classified albuminuria as albumin excretion in the urine exceeding the reference limit of 2.3 mg/dL or 23 mg/L and proteinuria as protein elimination in the urine surpassing the reference levels of 5-10 mg/dL or 50-100 mg/L. Therefore albumin and protein excretion surpassing the above mentioned reference levels are crucial indicators of renal dysfunction (Fisher et al., 2013; Unnikrishnan et al., 2017). Earlier, urine albumin was quantified to detect emerging nephropathy in patients exposed to traditional risk factors like diabetes and hypertension wherein an albumin excretion rate (AER) of $\geq$ 30 mg/L signified a variation in the structural integrity of the

glomerular capillary wall. However, current guidelines endorse estimating albuminuria in all CKD patients owing to prognostic significance of albuminuria for outcomes of renal disease & mortality (Levey et al., 2015; Lentine et al., 2017; Webster et al., 2017).

The minor component of plasma proteins includes low molecular weight (LMW) proteins like β2-microglobulin (b2M) (12kDA) and are usually present in the plasma at levels of 3mg/L (reference level). These proteins possess a small size due to which they are freely filtered and excreted at the glomerulus into the ultra-filtrate but are effectively reabsorbed and catabolized by the proximal tubule usually resulting in urinary concentration of less than 0.3 mg/L (reference limit). Therefore amplified intensities of these proteins in the urine are deliberated to reveal impairment in the tubular function due to which they are not reabsorbed but excreted (Simons, 2018; Wang et al., 2016; Gifford et al., 2017). Quantification of some of these proteins and calculation of their ratios has been widely used in efforts to improved definition of the nature of proteinuria, its etiology and clinical repercussions.

In the current study as well, significantly higher levels of urinary proteins (mean=204.76 mg/dL with min-max=199.62-210.6 mg/dL) and albumin (mean=134.58 mg/dL with min-max=133.15-141.36 mg/dL) were noted among the subjects of study group 2 that were suffering from diabetic or hypertensive CKD as compared to study group 1 subjects comprising of CKDu endemic cases (mean (urinary protein)= 16.81 mg/dL with min to max= 15.56-19.07 mg/dL) and mean (urinary albumin)= 2.62 mg/dL with min-max= 2.39-2.89 mg/dL) (**Table 2.7**).These levels resulted in correspondingly opposite values of serum protein and albumin levels wherein low values were noted in the study group 2 cohort (mean(serum proteins)=1.56 g/dL, min to max-1.31-1.79 g/dL and mean(serum albumin)=0.075 g/dL, min-max-0.055-0.11 g/dL) as compared to study group1 subjects owing to enhanced excretion of these substances in the urine. The quantities of albumin and protein in the serum remained significantly higher in the CKDu subjects (study group 1) [mean (serum protein)= 5.56 g/dL, min-max=5.31-5.79 g/dL and mean (albumin)=4.03 g/dL, min-max=3.81-4.19 g/dL] as the urinary elimination of these proteins were considerably reduced as compared to hypertensive or diabetic CKD cases. Contrarily the levels of low molecular weight tubular proteins i.e. β2-microglobulin (b2M) in the urine were significantly elevated in the CKDu endemic cases (study cohort 1) (mean (urinary b2M)= 0.723 mg/L, min to max= 0.669-0.806 mg/L as compared to study group 2 (mean (urinary b2M)= 0.023 mg/L with min-max= 0.020-0.028 mg/L). These levels were consistent with the decreased levels of b2M in the serum in the study group 1 cohort (mean (b2M)= 0.26 mg/L, min-max= 0.21-0.32 mg/L) owing to

enhanced excretion of the same during tubular injury as compared to study group 2 subjects (mean (b2M)= 2.57 mg/L min to max= 1.09-2.99 mg/L).

Therefore assessment of proteinuria aids in discriminating between tubulointerstitial and glomerular diseases and monitoring of the renal disease progression and assessment of the response therapy (Lentine et al., 2017; Webster et al., 2017). Previous studies like the analysis by Wang and his group (Wang et al., 2016) and Jelaković and his colleagues (Jelaković et al., 2015) have depicted huge differences in the urinary protein composition excreted in renal tubular and glomerular disorders and found that LMW proteins like b2M and HMV proteins like albumin predominated these disorders respectively, which enabled them to successfully distinguish between tubular proteinuria and glomerular proteinuria. Cabral & his team (Cabral et al., 2015) had also demonstrated that the average molecular weight of the urinary proteins in tubular proteinuria is remarkably smaller than glomerular proteinuria. These previous findings paralleled results of current study **(Table 2.7)** wherein LMV proteins (i.e.b2M) & HMV proteins (i.e. albumin) dominated urinary protein constitution of study group 1 (i.e. CKDu cases) and study group 2 subjects respectively, suggesting occurrence of tubular & glomerular dysfunction in these respective groups.

Therefore the common observation from all these studies was that, low-level (minimal) proteinuria/albuminuria is considered indicative of tubulointerstitial renal disease, whereas higher-levels (>1 g/24 h of protein and >300 mg/24h of albumin) are believed to represent causal glomerular pathology (Venkatachalam et al., 2015). Proteinuria of greater more than 1.5 gm/24h are considered as diagnostic indicator of nephrotic syndrome specifically compromise in glomerular permeability due to glomerular injury. Diabetes and hypertension, the leading causals of the nephrotic syndrome, are well known for the induction of glomerulonephropathy during chronic kidney disease which is majorly diagnosed when the protein and albumin levels in the urine surpass 1g/24 h and 500mg/24h respectively (Fisher et al., 2013; Lentine et al., 2017; Unnikrishnan et al., 2017). This explains the significantly increased average levels of urinary albumin and protein among the subjects of study group 2 as this population are primarily exposed to major risk factors of chronic kidney disease development i.e. diabetes and hypertension (Fisher et al., 2013; Hill et al., 2016; Hu and Coresh, 2017). The absence of these risk-factors in the study population of group 1 (i.e. CKDu endemic cases) justifies the minimal levels of urinary albumin and protein levels noted like other non-glomerular diseases indicative of the existence of tubular pathology instead of a glomerular injury among the CKDu endemic cases (Rajapakse et al., 2016; Lunyera et al., 2016). These observations were further supported by the significant excessive excretion of

tubular low MW proteins (i.e. b2M) in the urine of the study group 1 subjects as compared to study group 2 cohort, signifying impairment in the tubular functioning depictive of tubulointerstitial nephropathy (Gifford et al., 2017; Lozier et al., 2016; Venkatachalam et al., 2015; Wijetunge et al., 2018). These findings were consistent with the observations from nephrologists involved in CKDu screening across various developing countries like Sri Lanka and Central America wherein they have noted CKDu to be a minimally-proteinuric pathology displaying a sub-nephrotic range similar to other tubulointerstitial disease (Gifford et al., 2017; Nanayakkara et al., 2012a and b; Herrera et al., 2014)

Further support to this conclusion was provided by calculation of correlation coefficients for the elimination of total proteins and albumin, the excretion of total protein and b2M and for the elimination of albumin and b2M for all the three study groups **(Table 2.9B).** Urinary proteins and albumin had a correlation coefficients of 0.018 ($p=0.058$) and 0.913 ($p=0.004$) and proteins and b2M had a coefficients of 0.856 ($p=0.001$) and 0.0004 ($p=0.112$) in study groups 1 and 2 respectively. This association analysis indicated that albumin constitutes the major portion of the excreted urinary protein profile of study group 2 subjects with a very small portion occupied by b2M.This is justified owing to the normal dominance of albumin proteins in the plasma hence in the case of glomerular injury, the release of these proteins into the urine is inevitable due to failure in prevention of glomerular filtration owing to its large size (Fisher et al., 2013; Levey et al., 2015, Unnikrishnan et al., 2017). On the contrary, the significantly ($p<0.01$) stronger correlation between urinary b2M and protein and weaker correlation between urinary albumin and the protein in study group 1 subjects indicated that the urinary protein excretion of these subjects is dominated by LMV proteins (i.e.b2M) with near optimal levels of albumin, indicative of tubular injury (Gifford et al., 2017; Venkatachalam et al., 2015; Wijetunge et al., 2018). Moreover the negative correlation between albumin and b2M in both the study groups 1 ($r= -0.188$, $p=0.011$) and 2 ($r= -0.158$, $p=0.012$) **(Table 2.9B)** indicates that the source of origin of these urinary markers is different owing to the varied nature of the renal injury with the former being due to glomerular disturbances and the latter being due to tubular disorders (Gifford et al., 2017, Siriwardhana et al., 2014). Therefore this correlation analysis provided supportive evidence to the existence of a pathological proteinuria (i.e. glomerular proteinuria predominated by albumin) and minimal proteinuria (i.e. tubular proteinuria predominated by b2M) among the study group 1 and 2 subjects respectively, reflective of related glomerular and tubular disorders in the CKDu cases and the general CKD cases. These observations of tubular dysfunction among the study group 1 subjects was further backed by the presence of strong relationships between

urinary b2M and serum creatinine (r=0.913, p=0.003), serum urea (r=0.995, p=0.009) and serum uric acid (r=0.991, p=0.006) and weak associations between urinary albumin and serum creatinine (r=0.112, p=0.32), urea (r=0.101, p=0.128) and uric acid (r=0.124, p=0.19) **(Table 2.9B)** indicating that all these markers viz.b2M,creatinine, urea and uric acid are commonly handled by the proximal tubules with the exception of albumin (i.e. handled by the glomerulus). Hence during tubular injuries, the handling of these parameters is severely disturbed which causes marked elevations (above the reference limit) of these biomarkers in their respective biological matrices like urine and serum. Therefore justifying the closely proportional rise in the levels of urinary b2M, serum urea, serum creatinine and serum uric acid (depicted by strong correlations) with no significant elevations observed for urinary albumin (indicated by weak correlation between albumin and serum urea, serum creatinine and serum uric acid), thereby signifying the presence of a renal tubular pathology among the CKDu endemic cases of study group 1 (Herrera et al., 2014; Wesseling et al., 2016).

Thus overall, these correlation findings obtained in the current study were in accordance with the trends of associations between various renal function biomarkers observed during the study of tubular and glomerular disorders (Athuraliya et al., 2011; Gifford et al., 2017; Levey et al., 2015; Nanayakkara et al., 2012 a and b; Siriwardhana et al., 2014; Wanigasuriya et al., 2017; Wijetunge et al., 2018), justifying the existence of tubular and glomerular nephropathy among the study group 1 and 2 subjects respectively.

Since the renal pathology among the CKDu endemic cases (i.e. study group 1) in the taluka was established to be a tubulointerstitial nephropathy, it was unavoidably mandatory to assess various markers of tubular function among the study population for confirmation of the tubular pathology. One such characteristic marker of tubular function is the estimation of tubular specific enzyme viz. N-acetyl-β-D-glucosaminidase (NAG) in the urine and plasma. NAG is a high molecular weight (150kDa) enzyme that precludes the filtration from the glomerular membrane attributed to its enormous size. It is primarily localized to the lysosomal compartment of epithelial cells with significant role in glycoprotein breakdown and is situated along the walls (brush border) of the proximal tubule. NAG is ideally excreted in very low amounts (of <1.2 U/L) in the urine which is consequential of the normal exocytosis and pinocytosis process in the tubular epithelial cells (Simons, 2018). Pathological injury of the proximal tubules disturbs the homeostatic lysosomal/plasma membrane interaction in the cells that consequently results in an enhanced loss of NAG enzyme into the urine (enzymuria).Hence an increase in urinary NAG excretion is directly associated with the pathologically induced lesion triggered structural breakdown and functional perturbation of

the proximal tubules ,thereby serving as a significant indicator of tubular dysfunction (damage) and diagnostic marker of tubular injury (Moriguchi et al., 2003; Moriguchi et al., 2009; Wang et al., 2016). According to previous studies like that of (Bazzi et al., 2015), the urinary enzyme profiles of two different study cohorts distinguished by the type of renal pathology viz. glomerulonephopathy (nephrotic proteinuria) and tubulonephropathy (non-nephrotic proteinuria) subjects were evaluated and it was found that the urinary NAG levels were consistently and significantly higher ($p<0.05$) in majority of the subjects affected by the tubular pathology as compared to glomerular dysfunction over a 1 year evaluation period. Moreover , the NAG levels in the glomerulonephropathy cases sustained at an approximately normal range owing to the absence of distinctive tubular damage in such patients. Furthermore NAG enzyme biomarker has been widely used in previous studies for monitoring of the effects of various environmental toxin like lead, cadmium, arsenic and NSAIDs on the tubular functional integrity and prediction of tubular nephrotoxicity in CKDu Results from these previous studies have indicated significant elevations ($p<0.05$) in the levels of NAG in toxin exposed subjects as compared to controls highlighting the causal role of nephrotoxins in inducing tubular injury instead of glomerular damage that ultimately manifests as chronic tubulointerstitial nephritis, the hallmark of CKDu pathology (Gifford et al., 2017; Laws et al., 2015; Nanayakkara et al., 2012a and b; Ramírez-Rubio et al., 2013; Sabath and Robles-Osorio, 2012). In a separate study that involved the comparison of the NAG excretion between a cohort of workers subjected to cadmium (a tubular toxin) and dry-cleaning benzene solvent (a glomerular toxin), significant rise in NAG were noted in urine of cadmium exposed subjects with optimal levels observed in benzene exposed subjects further justifying the relevance of NAG as tubular dysfunction diagnostic indicator (Franchini et al., 2005; Meyer et al., 2004).

Our results (Table 2.7) were in accordance with the findings of the previous biochemical assessment of the CKDu cases in Sri Lanka and Central America and similarly demonstrated significantly($p<0.005$) elevated quantities of the NAG enzyme in the urine of study cohort 1 comprising of CKDu cases (mean(urinary NAG)=15.5/L with min-max= 12.36-18.41 U/L) as compared to near optimal levels in the study group 2 subjects (mean(urinary NAG)= 0.55 U/L with min-max= 0.51-0.59 U/L).Thus indicating the presence of tubular nephropathy among the CKDu endemic cases which additionally suggests the possible involvement of nephrotoxins in the causation of the disease (Gifford et al., 2017). This further explains the presence of glomerular injuries among the diabetic and hypertensive subjects of study group 2 owing to the occurrence of normal levels of the tubular specific enzyme (NAG) in the urine

(Levey et al., 2015). Further evidence for this conclusions were provided by a significantly strong and linear correlation (r= 0.897, p= 0.012) between urinary NAG and urinary b2M levels and weak correlation (r= -0.0052, p= 0.103) between urinary albumin and NAG values among the subjects of study group 1 (Table 2.9B), indicating the specificity of these markers (i.e. b2M and NAG) for tubular function, owing to which the damage in the tubules causes a simultaneous rise in both of these proteins. The weak correlation between urinary albumin and NAG in this study group suggest the differential functional specificity of these markers with former pertaining to glomerular and latter for tubular function, hence higher elevations in urinary NAG were not concurrent with minimal urinary albumin rise (Herrera et al., 2014; Nanayakkara et al., 2012a and b; Wanigasuriya et al., 2017). However in the study group 2 very weak correlations were noted for urinary b2M and NAG (r=0.0011, p=0.069) **(Table 2.9B)**, owing to the minimal rise in both of these markers due to occurrence of a non-tubular pathology. This was further supported by poor correlations between urinary albumin & NAG (r=-0.0048, p=0.148) **(Table 2.9**), owing to the glomerular pathological manifestation in this study group which prevents concomitant increase in tubular specific NAG marker with glomerular related albumin levels (Fisher et al., 2013; Unnikrishnan et al., 2017).

Overall, these findings provided supporting evidence to presence of tubular pathology among CKDu cases (study group 1) & glomerular injury among diabetic & hypertensive study group 2. These observations were consistent with trends of renal tubular & glomerular dysfunction markers noted in comparative assessment of CKDu tubular pathology with CKD glomerular manifestation for purpose of distinguishing CKDu from diabetic & hypertension induced CKD in Sri Lanka & Central America (Gifford et al., 2017; Herrera et al., 2014; Laws et al., 2015; Nanayakkara et al., 2012a and b; Wanigasuriya et al., 2017).

Other characteristic markers of tubular function involve assessment of native tubular handling of electrolytes like bicarbonate & phosphate. These electrolytes are filtered at the glomerulus but are reabsorbed by proximal tubules to maintain the electrolyte balance in the body crucial for metabolic functions like maintaining cellular pH, synthesis of carbohydrates & proteins for cellular repair etc. Under ideal conditions of good health, owing to negligible excretion of these electrolytes in the urine, levels of the bicarbonate & phosphate in the serum should be maintained at normal reference levels of 23-30 mEq/L & 3-4.5 mg/dL respectively. Hence, analysis of these electrolytes in serum are critical for evaluation of variations in tubular ability to maintain homeostatic equilibrium of these electrolytes as excessive excretions of the same in urine manifests in < normal levels in the serum significantly indicating faulty tubular reabsorption, reflective of tubular damage. The test for these

electrolytes is essential for management & diagnosis of renal proximal tubular nephropathy & acid-base & water imbalance conditions (Philip and Jacob, 2018; Sharma et al., 2015; Sobhonslidsuk et al., 2017). Results of observational studies pertaining to comparison of electrolyte profiles of glomerular & tubular cases depicted 10-15 folds of significant ($p<0.05$) reduction in levels of these electrolytes viz. bicarbonate & phosphates in subjects suffering from tubular disorders as compared to the diabetes/ hypertension induced glomerular nephropathy cases (Rodriguez-Nóvoa et al., 2010).Thus providing supporting evidence to diagnostic significance of these electrolyte markers in assessment of tubular dysfunction.

Our results **(Table 2.7)** parallely demonstrated significantly decreased levels (< normal reference limit) of serum bicarbonate (mean=8.19 mEq/L with min-max=8.10-8.39 mEq/L) & phosphates (mean=1.03 mg/dL with min-max=0.93-1.16 mg/dL) in study cohort 1 (i.e. CKDu endemic cases) as compared to near optimal levels noted in diabetic & hypertensive nephropathy cases of study group 2 [mean (bicarbonate)= 24.85 mEq/L with min-max=24.0-27.1 mEq/L & mean (phosphate)=4.01 mg/dL with min-max=3.90-4.25 mg/dL]. These findings were consistent with observations of previous biochemical studies of CKDu wherein reduced electrolyte profiles of serum bicarbonate & phosphate were typical of failure in tubular reabsorption owing to tubular injury & optimal levels of these electrolytes were noted in glomerular functional disruption (Kupferman et al., 2016; Wijkström et al., 2018), thus providing supporting evidence to prevalence of tubular pathology & glomerular nephropathy among CKDu endemic and diabetic/hypertensive-CKD subjects respectively.

Further support to these findings was provided by presence of strong and linear correlations of serum b2M and bicarbonates ($r=0.988, p=0.0054$); serum b2M and phosphates ($r=0.896, p=0.0039$); serum NAG and bicarbonates($r=0.896$, $p=0.0036$) and serum NAG and phosphates($r=0.901$, $p=0.0059$) among the subjects of study group 1 **(Table 2.9A),** with simultaneous and significantly proportional elevations noted in each set of these markers signifying the common origin of all of these urinary markers i.e. from tubular dysfunction (Wijkström et al., 2013). Moreover weak or negligible correlations were observed between serum albumin and bicarbonate ($r=-0.105$, $p=0.0017$) and serum albumin and phosphate ($r=-0.103$, $p=0.005$) **(Table 2.9A)**, justifying the absence of commonality in the source of these markers with serum bicarbonates and phosphates levels decreasing due to tubular disorders and serum albumin quantities negligibly reducing (almost retaining normality) owing to the lack of glomerular disturbances (Kupferman et al., 2016; Suwazono et al., 2015; Wijkström et al., 2018). On the other hand, the strength of association between serum b2M and bicarbonates ($r=0.00015$, $p=0.051$); serum b2M and phosphates ($r=0.00021$, $p=0.068$); serum

NAG and bicarbonates (r=0.00012, p=0.60) and serum NAG and phosphates (r=0.00009, p=0.18) were significantly reduced in the study group 2 as compared to study cohort 1 (**Table 2.9A).** This was attributed to the absence of significant excretions of these markers into the urine causing the retention of normalcy of the same in the serum due to the presence of non-tubular (i.e. glomerular) disturbances among study group 2 subjects. This was further backed by observance of negligible and inverse correlations between serum albumin & bicarbonates (r=-0.091, p=0.148) and serum albumin and phosphates (r=-0.088, p=0.171) **(Table 2.9A)**, indicating the differential origin of these markers wherein albumin decrease and bicarbonate or phosphate reduction in the serum arises from glomerular and tubular pathological manifestations respectively (Levey et al., 2015; Fisher et al., 2013). All in all, these findings and correlation assessments were in accordance with the study of CKDu and diabetic or hypertensive CKD cases by (Kupferman et al., 2016; Ratnayake et al; 2017; Suwazono et al., 2015), in similarly affected regions of Sri Lanka and Central America. Therefore justifying the existence and contribution of tubular and glomerular pathologies in the development of chronic renal failure among the subjects of study group 1 and 2 respectively (Gifford et al., 2017; Herrera et al., 2014; Wanigasuriya et al., 2017; Wijkstrom et al., 2018).

2.3.4.2 Estimation of various selectivity indices (like urinary ACR, PCR, BCR, NCR, APR & Alb-B2M ratio) of nephropathy specific proteinuria (i.e. glomerular or tubular) and analysis of its diagnostic utility in detection/identification of renal pathology

Previous studies related to screening CKD globally have utilized the above mentioned urinary biomarkers of renal function (i.e. protein, albumin, b2M, NAG) as a ratio of creatinine for accurate identification & detection of the type of pathology (i.e. glomerular or tubular) manifested in chronic kidney disease. Creatinine is a major by-product of metabolism involving creatinine phosphate for energy generation (ATP) that is generated & eliminated at a constant rate from the body. Thus correction of these parameters for creatinine concentration by expressing as ratio of creatinine assists in reduction of spread of measured data points by diminishing variations in levels of these parameters owing to diuresis triggered concentration fluctuations (Ratnayake et al., 2017). Hence, estimated urinary parameters of renal function (i.e. protein, albumin, b2M, NAG) are expressed as a ratio of creatinine to give urinary protein to creatinine ratio (uPCR), urinary albumin to creatinine ratio (uACR), urinary β2-microglobulin to creatinine ratio (uBCR) & urinary N-acetyl-β-D-glucosaminidase (NAG) to creatinine ratio (uNCR) respectively, that are depicted in **Table 2.8.**

Table 2.8: Comparison of the estimated selectivity indices typical of glomerular or tubular nephropathy for the entire study population comprising of three study groups stratified by the type of nephropathy distribution

Selectivity index	Study group 1 (Endemic CKDu cases)			Study group 2 (Non-endemic CKD cases)			Study group 3 (Healthy controls)			Reference cutoff
	Total (N=114)	Male(n=58)	Female(n=56)	Total(N=28)	Male(n=15)	Female(n=13)	Total(N=124)	Male(n=62)	Female(n=62)	
uPCR	215.51± 4.3*	216.85 ±4.8*	213.86 ±3.6*	1060.38 ±5.6*	1064.76 ±6.8*	1059.4± 5.5*	25.88± 1.3	25.97± 2.2	25.17± 0.9	up till 200 mg/g-Creatinine
uACR	33.58± 3.6*	34.26± 3.9*	32.91± 3.5*	705.08± 6.1*	715.32± 6.9*	703.16± 5.5*	3.48± 1.1	3.93± 1.3	3.02± 0.9	30-300 mg/g-creatinine
uBCR	9.26±1.5 *©	9.36±1.8*©	9.17±1.2 *©	0.12± 0.05	0.124 ±0.09	0.114 ±0.04	0.063± 0.008	0.064± 0.0010	0.0628 ±0.007	0.1-0.3 mg/g creatinine
uNCR	2.25±0.9*©	2.47±1.1 *©	2.03±0.8 *©	0.032 ±0.0005	0.033± 0.0009	0.031± 0.0004	0.015± 0.0003	0.0168±0.0006	0.149± 0.0002	0.12-0.43 U/ mmol creatinine
uAPR	0.155± 0.07	0.1579± 0.09	0.154± 0.06	0.67± 0.15*	0.672± 0.18*	0.663± 0.12*	0.13± 0.06	0.15± 0.07	0.12± 0.04	<0.4=tubular nephropathy; >0.4=glomerular nephropathy
uALb/b2M	3.63± 0.16	3.66± 0.20	3.59± 0.14	5916.5± 8.2*	5961.1±6.6*	5872.16± 9.9*	55.23± 6.0	61.41± 6.4	47.93± 5.8	Not applicable

The major urinary biochemical parameters(protein, albumin, β2-microglobulin and N-acetyl-β-D-glucosaminidase)were normalized for creatinine and expressed as a ratio of creatinine to account for diuresis associated dilutions in the levels of these parameters. Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. The selectivity indices specific for glomerular diseases are uPCR,uACR,uAPR and uAlb/b2M and for tubular disorders are uBCR and uNCR. Data are derived from two independent experiments with each parameter of an individual experiment measured in triplicates. Values are represented as mean±SD. The units for each measured parameter are represented in their corresponding reference limits. Differences at *$p<0.05$ were considered to be statistically significant as compared to the true controls(study group 3). Differences at ©$p<0.05$ were considered to be statistically significant as compared to the non-endemic CKD cases(study group 2).Significantly elevated levels of urinary expression of tubular nephropathy specific indices viz.uBCR and uNCR and near optimal or lowered levels of glomerular injury specific indices viz.uACR,uPCR,uAPR and uAlb/b2M ratio were noted in the study group 1 confirming the manifestation of tubular injury in CKDu development in these subjects.Contrarily reversal of this trend characterised by significantly increased expression of glomerular injury specific indices were noted in study group 2 subjects confirming the dominance of glomerular damage in these subjects.

Most of these measured parameters under good health conditions are present at very minimal levels in the urine. Therefore adjustments of these biomarkers for creatinine permits the low level detection of these measured parameters which are critical for the sensitive and specific detection of the manifestation of tubular or glomerular nephropathy during chronic renal failure (Ratnayake et al., 2017; Vassalotti et al., 2016).

Under metabolic homeostatic conditions the loss of protein in the urine is generally less than 80 mg/24h. However, on renal injury, this urinary protein loss progressively enhances with elevated levels of proteinuria transpiring as a result of disturbances in the tubular function, glomerular filtration barrier, or both (Gowda et al., 2010; Inker et al., 2014; Vassalotti et al., 2016; Thomas et al., 2015). In tubular nephropathy, like chronic tubulointerstitial nephritis, urinary protein loss protein pattern is selective for low molecular weight proteins like β_2-microglobulin which usually comprises a very small proportion (1.5-5%) of the plasma proteins (Hettinga, et al., 2015; Laidoudi et al., 2016; Simons, 2018). Therefore these previous studies suggest that measurements of urinary albumin and urinary β_2-microglobulin normalized for creatinine are critical for accurate detection of the disruptions or variations in the renal tubular of glomerular handling of these native plasma proteins. The findings suggest a differential tubular reabsorption of the two proteins (Lentine et al., 2017; Vassalotti et al., 2016).

Majority of the epidemiologists and nephrologists dedicated to CKDu screening have reported CKDu to be a minimally or non-proteinuric disease displaying sub-nephrotic amounts of protein in the urine out of which only a very small portion (<15%) is the high molecular weight plasma-rich protein viz. albumin, which is distinctive of tubular pathologies (Gifford et al., 2017; Orantes et al., 2014). Hence, diagnostic tools like urine dipstick proteinuria that are commonly used for detection of high range albuminuria common to hypertensive and diabetes induced CKD manifestation are not suitable for appropriate detection of CKDu. Therefore these estimated urinary parameters (i.e. protein and albumin) are expressed as a ratio of creatinine to give urinary protein to creatinine ratio (uPCR) and urinary albumin to creatinine ratio (uACR). uPCR and uACR are sensitive and widely used biomarkers for identification of low or minimal levels of protein and albumin during diagnosis of CKDu cases (Jayasumana et al., 2016; Ratnayake et al., 2017). It has been used by Jafar et al. in 2007 as a successful screening tool for CKD cases in Indo-Asian populaces. Further, it has been shown to be capable of effective discovery of early stage CKD in patients with hypertension or diabetes (Inker et al., 2014; Jafar et al., 2007). According to the National Kidney Foundation the generally accepted cut-off for proteinuria is a uPCR surpassing the

reference limit of 200 mg/g and normal being < 200 mg/g. This foundation further classifies albuminuria based on the extent of albumin excretion in the urine into two broad categories, which are microalbuminuria with a uACR of 30-300mg/g (reference limit) and macroalbuminuria with a uACR > 300mg/g (Levey et al., 2015; Vassalotti et al., 2016).

Our results **(Table 2.8)** depicted a significantly($p<0.05$) low mean-level uPCR of 215.51 mg/g (min-max= 212.56-222.75 mg/g creatinine) and low average level uACR of 33.58 mg/g (min-max= 32.62-36.6 mg/g creatinine) among the CKDu endemic subjects (study group 1) with normal levels noted in the true controls [uPCR (mean)= 25.88 mg/g with min-max= 24.49-26.61 mg/g) and uACR (mean)= 3.48 mg/g with min-max= 2.73-4.44 mg/g). The levels of uPCR and uACR in the study group 1 subjects (CKDu endemic cases) were slightly above the reference cut-off values and correlated poorly($r=0.018$, $p=0.058$) **(Table 2.9B)** indicating that the reduced amounts of excreted urinary proteins comprised of a very small proportion of the native plasma dominating protein viz. albumin which highlighted the disease in this cohort to be lowly proteinuric and manifesting in the minimal microalbuminuria range, characteristic of tubular dysfunction (Gifford et al., 2017). These findings were consistent with the uACR and uPCR values obtained during the screening of CKDu cases in Sri-Lanka and Central America (Raines et al., 2014; Ratnayake et al., 2017) providing supporting evidence to our observations of the CKDu cases in Canacona taluka and all over the world to be minimally proteinuric. Moreover, large numbers of CKDu cases are usually manifested in the range of microalbuminuria that cannot be detected by basic urine dipstick proteinuria as dipstick measures higher albumin values exceeding 300mg/g-Creatinine. This trend of microalbuminuria was also noted among the CKDu endemic cases (study group 1) of the taluka in the current study as well, which was validated by a similar observation noted in screening in related endemic regions of Sri-Lanka and Central America (Orantes-Navarro et al., 2016; Selvarajah et al., 2016; Vela Parada et al.,2014). Thereby providing supporting evidence to the CKDu cases in this taluka and other developing countries to be largely present in the range of microalbuminuria (30–300 mg/g-Creatinine).

Table 2.9:Correlation assessments between the biochemical characteristics and nephropathy specific selectivity indices of the entire study population for establishment of the pattern of renal disease presentation (tubular or glomerular) in the study groups 1 and 2.

A

		Correlation coefficient(r)								
Parameter	Study group	Serum creatinine	Serum urea	Serum uric acid	Serum protein	Serum albumin	Serum b2M	Serum NAG	Serum bicarbonates	Serum phosphates
Serum creatinine	1	**1**	**0.971**	**0.99**	**-0.411**	-0.105	**-0.893**	**-0.827**	**-0.963**	**-0.905**
	2	**1**	0.116	0.166	-0.123	-0.295	-0.0056	-0.0042	0.0003	0.0006
	3	**1**	0.056	0.031	-0.069	-0.0023	**0.014**	**0.0102**	**0.0065**	**0.0098**
Urinary creatinine	1	**-0.995**	**-0.795**	**-0.754**	**0.245**	0.102	**0.901**	**-0.897**	**0.914**	**0.923**
	2	-0.107	-0.146	-0.151	0.128	0.123	0.0014	0.013	0.0112	0.003
	3	-0.145	0.0015	-0.0101	0.0023	0.0015	0.0014	0.0011	0.0101	0.005
Serum urea	1	**0.971**	**1**	**0.903**	**-0.384**	-0.115	**-0.905**	**-0.914**	**-0.876**	**-0.906**
	2	0.116	**1**	0.136	-0.113	-0.181	-0.0085	-0.0102	0.0113	0.008
	3	0.056	**1**	0.0041	0.0072	0.0003	0.0024	0.0019	0.123	0.01
Urinary urea	1	**-0.856**	**-0.896**	**-0.815**	**0.296**	0.112	**0.91**	**-0.904**	**0.936**	**0.924**
	2	-0.154	-0.236	-0.155	0.135	0.163	0.0016	0.0112	0.00121	0.006
	3	-0.069	-0.071	-0.0119	0.0033	0.0018	0.0021	0.0016	0.0123	0.009
Serum uric acid	1	**0.99**	**0.903**	**1**	**-0.387**	-0.118	**-0.921**	**-0.914**	**-0.888**	**-0.925**
	2	0.116	0.136	**1**	-0.113	-0.181	-0.0085	-0.0102	0.0113	0.008
	3	0.031	0.0041	**1**	0.0083	0.0004	0.0031	0.0023	0.0114	0.0015
Urinary uric acid	1	**-0.753**	**-0.876**	**-0.947**	**0.289**	0.115	**0.98**	**-0.916**	**0.946**	**0.942**
	2	-0.108	-0.212	-0.212	0.137	0.173	0.0019	0.0121	0.00131	0.009
	3	-0.0035	-0.0045	-0.087	0.0043	0.0025	0.002	0.0018	0.0223	0.01
Serum protein	1	**-0.411**	**-0.384**	**-0.387**	**1**	0.102	**0.712**	**0.779**	**0.186**	**0.192**
	2	-0.123	-0.113	-0.113	**1**	**0.908**	0.015	0.009	0.012	0.014
	3	-0.069	0.0072	0.0083	1	0.0083	0.0021	0.0008	0.0024	0.00015
uPCR	1	**0.265**	**0.236**	**0.213**	**-0.458**	-0.015	**-0.745**	**-0.148**	**0.0015**	**0.0009**
	2	0.168	0.103	0.103	**-0.917**	**-0.889**	-0.0002	-0.00013	0.0015	0.0021
	3	0.0012	0.0056	0.0062	0.0093	0.00028	0.0003	0.0019	0.0246	0.014
Serum albumin	1	-0.105	-0.115	-0.118	0.102	**1**	-0.161	-0.112	-0.105	-0.103
	2	*-0.295*	-0.181	-0.181	**0.908**	**1**	-0.151	-0.101	-0.091	-0.088
	3	-0.0023	0.0003	0.0004	0.0083	**1**	0.0015	0.0015	0.00146	0.000156
uACR	1	0.112	0.101	0.124	-0.118	-0.109	-0.163	-0.00015	-0.0018	-0.0013
	2	0.142	0.116	0.116	**-0.943**	**-0.998**	-0.149	-0.00012	-0.0014	-0.001
	3	-0.0012	0.0025	0.0028	0.0087	0.0078	-0.0004	-0.00018	-0.00246	-0.00014
Serum b2M	1	**-0.893**	**0.995**	**0.991**	**0.801**	-0.178	**1**	**0.974**	**0.988**	**0.896**
	2	-0.0056	-0.0085	-0.0085	0.015	-0.151	**1**	0.00015	0.00015	0.00021
	3	0.014	0.0024	0.0031	0.0021	0.0015	**1**	0.0014	0.0001	0.00012
uBCR	1	**0.913**	**0.995**	**0.991**	**0.801**	-0.178	**-0.997**	**-0.865**	**-0.901**	**-0.865**
	2	0.0079	0.0159	0.0159	-0.011	-0.174	-0.0013	-0.0015	-0.0009	-0.00018
	3	0.023	0.00114	0.0019	0.00114	0.00012	-0.0003	-0.00011	0.000246	0.00014
Serum NAG	1	**-0.827**	**-0.914**	**-0.914**	**0.779**	-0.112	**0.974**	**1**	**0.896**	**0.901**
	2	-0.0042	-0.0102	-0.0102	0.009	-0.101	0.00015	**1**	0.00012	0.00009
	3	0.0102	0.0019	0.0023	0.0008	0.0015	0.0014	**1**	0.00005	0.000065
uNCR	1	**0.936**	**0.927**	**0.963**	**-0.756**	-0.127	**-0.981**	**-0.919**	**-0.963**	**-0.954**
	2	0.0069	0.0126	0.0126	-0.014	-0.109	-0.00056	-0.000015	-0.00075	-0.00069
	3	0.0059	-0.0015	-0.0018	0.00011	0.0003	-0.00012	-0.00009	0.00036	0.00015
Serum bicarbonate	1	**-0.963**	**-0.876**	**-0.888**	**0.186**	-0.105	**0.988**	**0.896**	**1**	**0.945**
	2	0.0003	0.0113	0.0113	0.012	-0.091	0.00015	0.00012	**1**	0.00013
	3	0.0065	0.123	0.0114	0.0024	0.00146	0.0001	0.00005	**1**	0.00009
Serum phosphates	1	**-0.905**	**-0.906**	**-0.925**	**0.192**	-0.103	**0.896**	**0.901**	**0.945**	**1**
	2	0.0006	0.008	0.008	0.014	-0.088	0.00021	0.00009	0.00013	**1**
	3	0.0098	0.01	0.0015	0.00015	0.00016	0.00012	0.000065	0.00009	**1**
uAPR	1	-0.091	-0.085	-0.075	0.0185	0.00145	-0.0013	-0.00019	-0.00011	-0.00013
	2	0.164	0.112	0.112	**-0.893**	**-0.912**	-0.003	-0.00065	-0.0009	-0.0001
	3	0.0056	0.0018	0.0026	0.0015	0.00015	-0.0015	-0.00025	-0.000074	-0.000054
uAlb/b2M	1	-0.106	-0.099	-0.088	0.106	0.108	-0.0026	-0.00015	-0.00035	-0.00025
	2	0.159	0.135	0.135	**-0.901**	**-0.927**	-0.0016	-0.00047	-0.00027	-0.0017
	3	0.0064	0.0034	0.0041	0.0003	0.00123	-0.001	-0.00031	-0.000054	-0.000047

Please refer the table legend below

B

Parameter	Study group	Correlation coefficient(r)								
		Urinary creatinine	Urinary urea	Urinary uric acid	uPCR	uACR	uBCR	uNCR	uAPR	uAlb/ b2M ratio
Serum creatinine	1	**-0.995**	**-0.856**	**-0.753**	0.265	0.112	**0.913**	**0.936**	-0.091	-0.106
	2	-0.107	-0.154	-0.108	0.168	0.142	0.0079	0.0069	0.164	0.159
	3	-0.145	-0.0069	-0.0035	-0.0014	-0.0012	0.023	0.0059	0.0056	0.0064
Urinary creatinine	1	**1**	**0.812**	**0.805**	-0.186	-0.113	**-0.954**	**-0.906**	0.093	0.011
	2	**1**	0.132	0.112	-0.213	-0.135	-0.0021	-0.0056	-0.116	-0.107
	3	**1**	0.0031	0.005	0.0035	0.0023	0.0016	0.0015	0.0028	0.0031
Serum urea	1	**-0.896**	**-0.896**	**-0.876**	0.236	0.101	**0.995**	**0.927**	-0.085	-0.099
	2	-0.146	-0.236	-0.212	0.103	0.116	0.0159	0.0126	0.112	0.135
	3	0.0015	-0.071	-0.0045	0.0056	0.0025	0.00114	-0.0015	0.0018	0.0034
Urinary urea	1	**-0.754**	**1**	**0.857**	-0.178	-0.116	**-0.965**	**-0.91**	0.089	0.01
	2	0.132	**1**	0.146	-0.313	-0.147	-0.0033	-0.0065	-0.121	-0.109
	3	0.0031	**1**	0.009	0.0023	0.0027	0.0015	0.0011	0.0038	0.0025
Serum uric acid	1	**-0.754**	**-0.815**	**-0.947**	0.213	0.124	**0.991**	**0.963**	-0.075	-0.088
	2	-0.146	-0.236	-0.212	0.103	0.116	0.0159	0.0126	0.112	0.135
	3	-0.0101	-0.0119	-0.087	0.0062	0.0028	0.0019	-0.0018	0.0026	0.0041
Urinary uric acid	1	**0.805**	**0.857**	**1**	-0.177	-0.189	**-0.975**	**-0.924**	0.009	0.013
	2	0.112	0.146	**1**	-0.346	-0.151	-0.0034	-0.0068	-0.141	-0.119
	3	0.005	0.009	**1**	0.0035	0.0017	0.0019	0.0014	0.0028	0.0035
Serum protein	1	0.245	0.296	0.289	**-0.458**	-0.118	**-0.801**	**-0.756**	0.0185	0.106
	2	0.128	0.135	0.137	**-0.917**	**-0.943**	-0.011	-0.014	**-0.893**	**-0.901**
	3	0.0023	0.0033	0.0043	0.0093	0.0087	0.00114	0.00011	0.0015	0.0003
uPCR	1	-0.186	-0.178	-0.177	**1**	0.018	**0.856**	**0.201**	0.0013	0.00117
	2	-0.213	-0.313	-0.346	**1**	**0.913**	0.00035	0.00031	**0.914**	**0.927**
	3	0.0035	0.0023	0.0035	**1**	0.0014	0.0021	0.0017	0.0031	0.0047
Serum albumin	1	0.102	0.112	0.115	-0.015	-0.109	-0.178	-0.127	0.00145	0.108
	2	0.123	0.163	0.173	**-0.889**	**-0.998**	-0.174	-0.109	**-0.912**	**-0.927**
	3	0.0015	0.0018	0.0025	0.00028	0.0078	0.00012	0.0003	0.00015	0.00123
uACR	1	-0.113	-0.116	-0.189	0.018	1	-0.188	-0.0052	0.0029	0.00113
	2	-0.135	-0.147	-0.151	**0.913**	**1**	-0.158	-0.0048	**0.945**	**0.957**
	3	0.0023	0.0027	0.0017	0.0014	1	-0.00021	-0.00015	0.0042	0.0049
Serum b2M	1	**-0.954**	**-0.965**	**-0.975**	**-0.856**	-0.188	**-0.997**	**-0.981**	-0.0013	-0.0026
	2	0.0014	0.0016	0.0019	-0.0002	-0.149	-0.0013	-0.00056	-0.003	-0.0016
	3	0.0014	0.0021	0.002	0.0003	-0.0004	-0.0003	-0.00012	-0.0015	-0.001
uBCR	1	**-0.954**	**-0.965**	**-0.975**	**0.856**	-0.188	**1**	**0.897**	-0.025	-0.075
	2	-0.0021	-0.0033	-0.0034	0.00035	-0.158	**1**	0.0011	-0.018	-0.066
	3	0.0016	0.0015	0.0019	0.0021	-0.0002	**1**	0.00009	-0.00034	-0.00021
Serum NAG	1	**-0.897**	**-0.904**	**-0.916**	**-0.148**	-0.0002	**-0.865**	**-0.919**	-0.00019	-0.00015
	2	0.013	0.0112	0.0121	-0.00013	-0.0001	-0.0015	-0.000015	-0.00065	-0.00047
	3	0.0011	0.0016	0.0018	0.0019	-0.0002	-0.00011	-0.00009	-0.00025	-0.00031
uNCR	1	**-0.906**	**-0.91**	**-0.924**	**0.201**	-0.0052	**0.897**	**1**	-0.00013	-0.00016
	2	-0.0056	-0.0065	-0.0068	0.00031	-0.0048	0.0011	**1**	-0.0015	-0.0024
	3	0.0015	0.0011	0.0014	0.0017	-0.0002	0.00009	**1**	-0.00031	-0.00025
Serum bicarbonate	1	**0.914**	**0.936**	**0.946**	**0.0015**	-0.0018	**-0.901**	**-0.963**	-0.00011	-0.00035
	2	0.0112	0.00121	0.00131	0.0015	-0.0014	-0.0009	-0.00075	-0.0009	-0.00027
	3	0.0101	0.0123	0.0223	0.0246	-0.0025	0.00025	0.00036	-0.000074	-0.000054
Serum phosphates	1	**0.923**	**0.924**	**0.942**	**0.0009**	-0.0013	**-0.865**	**-0.954**	-0.00013	-0.00025
	2	0.003	0.006	0.009	0.0021	-0.001	-0.00018	-0.00069	-0.0001	-0.0017
	3	0.005	0.009	0.01	0.014	-0.0001	0.00014	0.00015	-0.000054	-0.000047
uAPR	1	0.093	0.089	0.009	0.0013	0.0029	-0.025	-0.00013	**1**	-0.0156
	2	-0.116	-0.121	-0.141	**0.914**	**0.945**	-0.018	-0.0015	**1**	**0.926**
	3	0.0028	0.0038	0.0028	0.0031	0.0042	-0.00034	-0.00031	**1**	0.000047
uAlb/b2M	1	0.011	0.01	0.013	0.00117	0.00113	-0.075	-0.00016	-0.0156	**1**
	2	-0.107	-0.109	-0.119	**0.927**	**0.957**	-0.066	-0.0024	**0.926**	**1**
	3	0.0031	0.0025	0.0035	0.0047	0.0049	-0.00021	-0.00025	0.000047	**1**

Table 2.9:

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; b2M-β2-microglobulin, NAG-N-acetyl-β-D-glucosaminidase, uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. 'A' represents the correlation coefficients(r-values) calculated between the serum based biomarkers of tubular and glomerular function and the complete set of biochemical characteristics (serum based biomarkers and urinary based tubular or glomerular nephropathy specific selectivity indices) for the three study cohorts.' B' represents the correlation coefficients(r-values) calculated between the urinary based tubular or glomerular nephropathy specific selectivity indices and the complete set of biochemical characteristics (serum based biomarkers and urinary based tubular or glomerular nephropathy specific selectivity indices) for the three study cohorts.

Values are represented as Pearson's correlation coefficient (r) with statistically significant associations between any given two biochemical parameters identified via calculation of their respective p-values. Differences at $p<0.05$ were considered to be statistically significant correlations which are highlighted in bold. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. For units of each biochemical parameter, kindly refer Table 2.7 and 2.8.

Furthermore the trend of PCR and ACR noted among the CKDu endemic cases (study group 1) was completely contradictory to those observed among the diabetic and hypertensive CKD cases of study group 2.The average uPCR (1.06 g/g creatinine with min-max= 1.055-1.067 g/g creatinine) and uACR values (705.08 mg/g creatinine with min-max= 702.19-718.43 mg/g creatinine) in study group 2 (Table 2.8) significantly exceeded the quantities noted in study group 1 subjects and were largely beyond the reference cutoff values. Moreover a stronger and linear correlation ($r=0.913$, $p=0.004$) **(Table 2.9B)** was noted between the uACR and uPCR values indicating that the significant ($p<0.05$) and proportional rise in the excreted urinary proteins was strongly associated with the elevation in albumin levels. This is reflective of the dominance of the urinary protein constitution by albumin that is typically manifested during disruptions in glomerular filtration. Hence the disease in this study cohort 2 was observed to be nephrotically proteinuric and in the macroalbuminuria range of proteins (with uACR exceeding the cutoff of 300 mg/g), that is characteristic of glomerular dysfunction. Proteinuria with uPCR levels surpassing 1g/g Creatinine and Albuminuria with uACR levels in the macroalbuminuria range (exceeding 300 mg/g) are distinctive of glomerular disorders wherein alterations in the selective glomerular permeability by risk factors like diabetes and hypertension can cause severe leakage of these proteins in the urine (that are ideally precluded from glomerular ultrafiltration due to their large size) and hence ultimately manifests in the nephrotic syndrome (Thomas et al., 2015; Yang et al., 2017). Therefore the manifestation of glomerular nephropathy among the study group 2 subjects is explained by the widespread occurrence of the causal agents i.e. diabetes and hypertension among this cohort (as previously described in our demographic study). These findings corroborated with the comparative study of the CKDu and CKD cases by Ratnayake et al. (2017) thereby providing supporting evidence to the manifestation of glomerular nephropathy among the study group 2 cohort and high risk-group of patients affected by prolonged diabetes and hypertension in general. Moreover the absence of glomerular dysfunction inducing risk factors viz. hypertension and diabetes justifies the absence of the development of the glomerular pathology among the study group 1 subjects (CKDu endemic cases) which explains the occurrence of a tubular disease pattern comprising of minimal proteinuria and microalbuminuria (Gifford et al., 2017). Hence uACR and uPCR were suitably used to differentiate the albumin and protein excretion patterns among the CKDu and CKD cases in the current study for proper identification of the disease pathology i.e. tubular and glomerular respectively.

Although urine centred tests are reliable in CKDu screening, protein or albumin tests can yield negative outputs in the progressed minimal or non-proteinuric forms of tubular nephropathies which challenges and falters the screening process, by possibly granting healthy verdicts for to affected tubular nephropathic patients. Therefore uACR and uPCR alone will not suffice in early and reliable diagnosis of tubular injuries. This fact necessitates the requirement of more sensitive and selective markers of tubular function for proper confirmation of the manifestation of tubular pathology among the CKDu cases for improvement of patient safety and morbidity reduction (Simons, 2018).

Owing to this, two distinctive markers viz. low molecular weight (LMV) protein, β2-microglobulin (b2M) and high molecular weight (HMV) enzyme, N-acetyl-β-D-glucosaminidase (NAG) pertaining to the tubular functions of reabsorption and lysosomal based protein degradation respectively were considered for analysis in the present study. It has recently emerged that urinary estimation of these HMV and thus un-filterable enzyme-NAG and LMV protein-b2M are useful in the assessment of proximal tubular disorders (Hettinga et al., 2015; Venkatachalam et al., 2015). These target markers viz. b2M and NAG are present at very low abundance of 1.5-5% and 0-0.25% respectively in the plasma as opposed to the high abundance of albumin (40%).Therefore under good health conditions urinary leakage of b2M and NAG will not be as high as albumin excretion, thus a sensitive and specific method for detection of these markers is mandatory for disease identification. Hence these urinary markers are generally expressed as ratio of creatinine to obtain b2M to creatinine ratio (uBCR) and NAG to creatinine ratio (uNCR) to allow the low-level detections of the same and were adopted in the current study as well. According to the National kidney foundation, the accepted cut-off for uBCR is 0.3 mg/g-Creatinine and uNAG is 0.57U/mmol creatinine (Laidoudi et al., 2016; Simons, 2018).

In the current study **(Table 2.8),** significantly($p<0.05$) higher values of uBCR (mean= 9.26 mg/g creatinine with min-max= 9.13-9.41 mg/g creatinine) and uNCR (mean= 2.25 U/mmol creatinine with min-max=1.99-2.88 U/mmol creatinine) were observed among the CKDu endemic subjects (study group 1) as compared to study group 2 subjects [mean (uBCR)=0.12 mg/g with min-max= 0.119-0.121 mg/g creatinine) and mean (uNCR)=0.032U/mmol creatinine with min-max= 0.030-0.034 U/mmol creatinine) and true-controls (mean (uBCR) = 0.063 mg/g creatinine with min-max= 0.0622-0.0641 mg/g creatinine) and mean (uNCR)=0.015U/mmol creatinine with min-max=0.0147-0.0174 U/ mmol creatinine). Under normal health conditions b2M is freely filtered at the glomerulus followed by successive and almost complete reabsorption at the proximal tubules. Therefore the increased urinary

elimination of LMV proteins-b2M after creatinine adjustments serve as a specific indicator for tubular disorders i.e. disruption of tubular function (Laidoudi et al., 2016; Simons, 2018). This explains the significantly increased uBCR values evidenced in the CKDu cases of the study group 1 and optimal values of BCR noted in the study group 2 subjects (Table 2.8) confirming the existence of a tubular disorders and glomerular dysfunction in these cohorts respectively.

Another approach at identifying tubular disorder is by the estimation of urinary excretion of tubular specific enzymes like NAG. NAG is distinctly localised to the lysosomal compartment of the proximal tubule cells and being of higher molecular weight precludes glomerular filtration. Hence the NAG content in the urine after normalisation with creatinine cannot originate from disorders in glomerular structural integrity and functional filtration thereby restricting its origin solely to structural damage of proximal tubules which makes this target marker selective for detection of tubular injuries (Moriguchi et al., 2009; Sabath and Robles-Osorio, 2012; Simons, 2018; Wang et al., 2016). This fact justifies the occurrence of significantly elevated levels of urinary NCR (well beyond the reference cut-off) among the study group 1 subjects and normal levels in the study group 2 population further providing supporting evidence to the manifestation of tubular and glomerular pathologies among the respective CKDu affected subjects and general CKD cases.

The existence of elevated values of uBCR and uNCR among the study group 1 CKDu endemic cases **(Table 2.8)** were consistent with the similarly observed values in the clinical analysis of the CKDu cases in endemic regions of Central America and Sri Lanka (Athuraliya et al., 2011; Herrera et al., 2014; Laws et al., 2015; Siriwardhana et al., 2014; Wanigasuriya et al. 2017). Thereby providing supporting evidence to existence of a tubular proteinuria pattern comprising of elevated LMW proteins (b2M) and HMV enzymes (NAG) and reduced HMV protein (albumin) among the CKDu endemic cases in the taluka signifying the histopathological diagnosis of CKDu in the region to be Chronic tubulointerstitial nephritis, just like the CKDu scenario faced in other developing countries (i.e. Sri Lanka and Central America) as well (Gifford et al., 2017; Wijkstrom et al., 2018). Further support to these conclusions was provided by the existence of a significantly strong relationship between uBCR and uPCR ($r=0.856$, $p=0.0012$) and weak correlation between uACR and uPCR ($r=0.018$, $p= 0.058$) **(Table 2.9B)** among the study group 1 subjects indicating the predominance of the excreted urinary protein profile by LMV proteins (i.e. b2M) and not HMV protein viz. albumin, distinctive of the tubular proteinuria pattern commonly manifested during tubular pathologies (Wijkstrom et al., 2018). An obvious inverse

correlation was also noted between uBCR and uACR (r= -0.188, p= 0.011) **(Table 2.9)** indicative of the differential origin of these markers with the former arising from tubular dysfunction and latter arising from glomerular damage. This prevalent tubular damage among the CKDu cases was further backed by the presence of a strong correlation (r=0.89,p<0.01) that existed between uBCR and uNCR (r=0.897, p= 0.012) along with a simultaneous very weak inverse association (r= -0.0052, p= 0.103) noted between uACR and uNCR **(Table 2.9B)** depicting that the concurrent elevation in the markers viz. BCR and NCR is due to their origin from a common cause (i.e. tubular dysfunction) (Nanayakkara et al., 2012 a and b). This explains the reduced correlation between uACR and uNCR among these subjects due to the different causal agents (i.e. glomerular and tubular dysfunctions respectively) associated with their emergence (Ramírez-Rubio et al., 2015). These findings were well in agreement with the results obtained during the correlation assessment of various biomarkers associated with tubulointerstitial pathology manifested among the CKDu cases in Sri Lanka and Central America (Herrera et al., 2014; Nanayakkara et al., 2012a and b; Ramírez-Rubio et al., 2015; Wijkstrom et al., 2018).Thereby providing supporting evidence to the existence of a tubular pathological manifestation among the CKDu cases in the Canacona taluka.

Contrarily, the presence of optimal values of uBCR and uNCR among the study group 2 subjects comprising of general CKD cases **(Table 2.8)** and a weak correlation between these markers (r=0.0011, p=0.069) **(Table 2.9B)** highlighted the existence of a glomerular pathological manifestation in this cohort evident from the absence of excessive urinary excretions of tubular specific proteins (i.e.b2M and NAG). This conclusion of the occurrence of glomerular pathology was strongly backed by the existence of weak relationships between uBCR and uPCR (r=0.00035, p=0.112), strong association between uACR and uPCR (r=0.91, p=0.004) and negative correlation between uACR and uBCR (r= -0.158, p=0.012) **(Table 2.9B)**, indicative of the presence of glomerular proteinuric pattern among these subjects that was predominated by HMV protein (albumin) with negligible amounts of LMV protein (b2M). Moreover the occurrence of a weak inverse correlation between uNCR and uACR (r= -0.0048, p=0.148) **(Table 2.9B)** further indicated that these markers do not originate from a common source (i.e. glomerular dysfunction in this case), further supporting glomerular nephropathic development among these subjects. Overall these findings from the pathological analysis of the study cohort 2 were validated by the observance of similar results during the study of diabetic and hypertensive induced glomerular nephropathy cases by the following study groups (Jha et al., 2014; Thomas et al., 2015; Unnikrishnan et al., 2017; Yang et al., 2017). Thereby suggesting that the glomerular nephropathy among the study group 2 subjects

was also triggered by the typical risk-factors viz. diabetes and hypertension which was supported by the large prevalence of these risk factors in this study cohort (as estimated in our demographic study), thereby establishing its causative role. Therefore uBCR and uNCR were utilised as selective indices for estimation of the disturbances in tubular function and structural damage respectively for the accurate and appropriate diagnostic confirmation of tubular pathological manifestation in CKDu prevalent in the Canacona taluka.

It has been well established that pattern of proteinuria manifested during glomerular and tubular nephropathies are significantly different. In glomerular proteinuria, there is pathological dominance of HMV proteins-albumin and near optimal levels of LMV protein-β2-microglobulin with the trend being reversed in tubular proteinuria with largely significant above optimal levels of LMV proteins- β2-microglobulin and minimal levels of HMV proteins-albumin tending towards the normoalbuminuria (normal quantities) or microalbuminuria range (Inker et al., 2014; Vassalotti et al., 2016). Therefore for appropriate prediction of the protein pattern it is indispensable to estimate the proportion of albumin constituting the excreted protein to estimate the inclination for glomerular or tubular presentation. This was possible by calculating the ratio of uACR to uPCR to give a urinary albumin to protein ratio (uAPR) and by dividing the uACR by uBCR to give uAlb/b2M that respectively provides the albumin content of the protein and an estimate of the approximate predominance of albumin and b2M in the protein constitution. According to the National Kidney foundation, the standard reference values of uAPR > 0.4 predicts glomerular nephropathy and < 0.4 suggests tubulointerstitial nephropathy (Jelakovic et al., 2013; Lentine et al., 2017; Shroff et al., 2016; Sise et al., 2015; Smith et al., 2011; Vassalotti et al., 2016). In the current study **(Table 2.8),** a significantly lower uAPR value (below the cutoff - 0.4) of 0.155 (min-max= 0.153-0.164) and uAlb/b2M ratio of 3.63 (min-max=3.57-3.89) was noted in the CKDu endemic cases (study group 1) in the Canacona taluka respectively indicating that the excreted proteinuria comprises of reduced protein levels (minimally proteinuric) with albumin amounting to a small proportion (15.5 %) of the total protein content and b2M remarkably prevailing the protein constitution.

Further support to this conclusion was provided by the independent determination of the strength of association of these two ratios (i.e. uAPR and uAlb/b2M ratio) with each of the estimated biochemical indices via calculation of their correlation coefficients **(Table 2.9B)**. uAPR was found to poorly correlate with uACR ($r=0.05$, $p<0.05$) and uAlb/b2M ratio ($r=0.0029$, $p=0.087$) just like uAlb/b2M ratio with uACR ($r=0.0011$, $p=0.103$) with presence of simultaneous negative associations between uAPR and uBCR ($r= -0.025$, $p=0.12$) and

uAlb/b2M ratio and uBCR (r= -0.075, p=0.36) **(Table 2.9B)** among these study subjects. Thereby confirming the presentation of a relatively reduced albumin composition and significantly elevated dominance of b2M in the total urinary protein makeup of this cohort which is reflective of the tubular nephropathic pattern of loss of urinary proteins (i.e. tubular proteinuria). Our biochemical analysis and correlation findings of significantly ($p<0.05$) lower uAlb/b2M ratio and a uAPR <0.4 among the study group 1 subjects of this taluka **(Table 2.8 and 2.9B)** were in accordance with the clinical and retrospectively studied biopsied cohort of CKDu cases by Ohisa et al (2008) wherein a clinical uAPR of <0.40 confirmed the existence of tubulointerstitial injuries with demonstration of high specificity and sensitivity. Moreover our findings were in agreement with the observations of low uAPR values (less than the threshold of 0.4) noted in the biochemical screening of CKDu cases in the Balkan region and Central America (Jelaković et al., 2013; Smith et al., 2011; Valdés et al., 2015). Thereby providing confirmatory evidence to the tubular origin of the pathological manifestation of chronic renal failure among the CKDu cases of the Canacona taluka.

Contrarily, a significantly ($p<0.05$) higher mean uAPR of 0.67 (min-max=0.66-0.673) and mean uAlb/b2M ratio of 5875.66 (min-max=5865.71-5967.4) were noted among the diabetic and hypertensive CKD cases of study group 2 **(Table 2.8),** indicative of the occurrence of the remarkably large levels of total protein (pathologically proteinuric) with albumin significantly governing the urinary protein constitution as compared to b2M and accounting for 67% of the total urinary protein makeup. These findings were reinforced by uAPR strongly and significantly correlating with uACR (r=0.945, p=0.005) and uAlb/b2M ratio (r=0.926, p=0.0021) comparable to uAlb/b2M ratio with uACR (r=0.957, p=0.0103) in addition to uBCR negatively associating with uAPR (r=-0.018, p=0.321) and uAlb/b2M (r= -0.066, p=0.156) **(Table 2.9B)**. Thus depicting the significant dominance of the eliminated urinary protein profile by HMV protein-albumin which completely overshadowed the negligible occurrence of LMV protein-b2M, that is commonly manifested in the glomerular proteinuric pattern. These findings **(Table 2.8 and 2.9B)** were corroborated with the observance of similar values in the study of clinical disease presentation of diabetes induced glomerular proteinuric patients. Herein, uAPR was successfully utilised for valuable determination of the probable source of leakage of proteins specifically when conduction of kidney biopsies are not feasible due to high risks involved (Hong et al., 2016; Thomas et al., 2015). Thereby providing confirmatory evidence to existence of glomerular nephropathic disease presentation among the CKD cases of study group 2 patients affected by uncontrolled diabetes and hypertension.

Moreover uAPR and the uAlb/b2M ratio were used as significant tools for establishment of a diagnosis and confirmation of the tubular and glomerular pathological manifestation of CKDu and CKD cases in the study population of the Canacona taluka.

2.3.5 Demographic based prediction of the pattern of presentation of the clinical renal function markers for the assessment of CKDu susceptible risk groups in the study population

2.3.5.1 Age, sex and occupation based distribution and prevalence of various biochemical markers of renal function in the study population

A crucial step in the epidemiological screening and management of a disease is the assessment of various demographically stratified risk groups that are highly susceptible or more prone towards development of the disease. In order to achieve this purpose, the preliminary step involves the analysis of the demographic distribution of the biochemical characteristics (i.e. renal function markers) of the study population majorly stratified by age, sex and occupation (Jayatilake et al., 2013). Hence this was also undertaken in the current study

Previous epidemiological studies of CKDu in other developing countries like Central America have suggested the CKDu disease mainly affects higher age groups, males and individuals involved in farming (Almaguer et al., 2014; Orantes-Navarro et al., 2014).

Hence the current study desired to validate these findings by conducting a preliminary analysis of the differences (if any) in the presentation and prevalence of these biomarkers in different ranges across various age groups, both sexes and the type of occupation prevalent in the study population. We also investigated the extent to which the typical CKDu risk factors may affect the prevalence of various renal function markers in the abnormally high ranges and tried to verify that male sex and agricultural occupation are not conventional risk factors in our study.

Firstly, the prevalence and range distribution of the estimated serum based clinical biomarkers of general renal and tubular function along with urinary based tubular or glomerular nephropathy specific selectivity indices were analysed across two major demographic variable/risk-factor (i.e. age, sex) in the study population, which are presented in [**Figure 2.1; Tables 2.10] and [Figure 2.2; Table 2.11]** respectively.

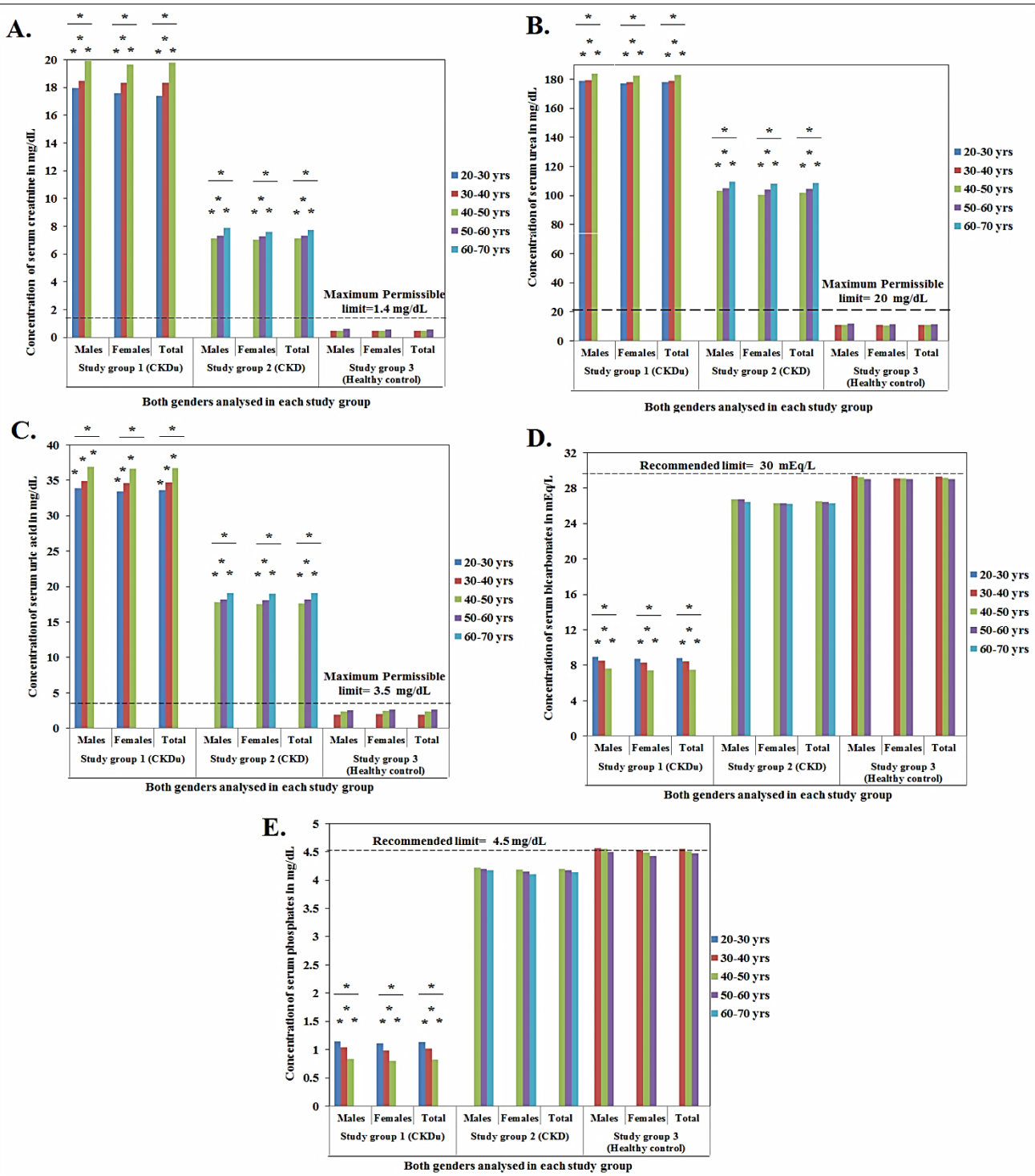


Figure 2.1. Age and sex based stratification of the levels of serum based general renal and tubular function biochemical markers of the entire study population for identification of the risk-groups predisposed to CKDu development.

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; b2M-β2-microglobulin, NAG-N-acetyl-β-D-glucosaminidase.Study group(cohort) 1, 2 & 3 consists of CKDu affected,diabetes/hypertension induced CKD affected & healthy controls subjects respectively. The number of males (M) / females (F)/total number of subjects(total) in study group 1,2&3 are 58(M)/56(F)/114(total); 15(M)/13(F)/28(total) and 62(M)/62(F)/124(total) respectively.The age based distribution(i.e.number of subjects under each age category) among males,females and total number of subjects (combination of both sexes) in all three study groups are detailed in the affixed table(**Table 2.10)** of this figure.(A)Concentration of serum creatinine in various age groups among the male sex,female sex and both sexes combined in all three study groups(B) Concentration of serum urea in age and gender stratified sub-groups of the three study cohorts population (C) Concentration of serum uric acid in age and gender stratified sub-groups of the three study cohorts' population (D) Concentration of serum bicarbonates in age and gender stratified sub-groups of the three study cohorts' population (E) Concentration of serum phosphates in the age and gender stratified sub-groups of the three study cohorts' population. Values are represented as mean concentrations of a given biochemical parameter in age based stratified sub-groups of the population i.e. subjects in the age group of 20-30yrs,30-40yrs,40-50yrs,50-60yrs and 60-70yrs under each of the broad population segments stratified by gender i.e.males,females & total number of subjects (i.e.both sexes combined) in all three study groups. All concentration values are in mg/dL except for serum bicarbonates which is depicted in mEq/L. Differences at *p<0.05 were considered to be significant as compared to healthy controls(group 3) & CKD affected group(group 2) and also relative to lower age categories (within a given study group) . The WHO established permissible limits for serum creatinine=1.4mg/dL;serum urea=20 mg/dL; serum uric acid=3.5 mg/dL and recommended limit for serum bicarbonates=30mEq/L & serum phosphates=4.5 mg/dL. The absence of a bar representation of the levels of a given biochemical marker for a particular age category among males,females & total number of subjects in each of the study groups indicated no subjects in that particular age category. No gender bias in renal function markers' expression were observed evident from comparable levels noted in both sexes in all groups.Moreover, levels of markers like serum creatinine,urea,uric acid increased and serum bicarbonates & phosphates reduced with rising age with significant(*p<0.05) prevalence of their levels at higher and lower ranges respectively being observed in higher age groups (i.e.40-50yrs) of study group 1.This was ascribed to the bioaccumulative potencies of the CKDu causing toxins which caused accumulation and aggravation of tubular damage effects with prolonged exposure (i.e.age),that was evident from corresponding escalation in expression of general serum renal function markers(that severely rises on renal injury) and reduction in tubular function markers(viz.serum bicarbonates & phosphates) with increasing age.The details on the range of minimum to maximum values of the concentration of each biochemical marker & prevalence distribution(%) of the concentration in various ranges for different age groups of each sex and both sexes combined(i.e. total number of subjects) in all study groups are listed in **Table 2.10**. For more details of the levels of each biochemical parameter, kindly refer Table 2.7.

Table 2.10: Age and sex dependent stratification of the prevalence of various serum based general renal and tubular function biochemical markers of the entire study population for identification of the population risk-groups prone to CKDu development.

2.10 A. Age and sex based distribution of serum based general renal function biochemical markers viz. creatinine, urea and uric acid

Age in years	Sex	Study group	Number of subjects in each category	Parameter								
				Serum creatinine			Serum urea			Serum uric acid		
				Mean in mg/dL (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/dL)	Mean in mg/dL (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/dL)	Mean in mg/dL (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/dL)
20-30 yrs	M	1	3	17.96 (17.89-18.03)	I=5.17%	A=0.45-0.5	178.77 (178.36-179.15)	G=5.17%	A=10-10.5	33.84 (33.40-33.98)	F=5.17%	A=1.0-2
		2	-	-	-	B=0.5-0.55	-	-	B=10.5-11	-	-	B=2.0-3.0
		3	-	-	-	C=0.55-0.6	-		C-11-11.5	-	-	C=17-18
	F	1	3	17.58 (17.54-17.96)	I=5.17%	D=0.6-0.65	177.03 (175.64-177.89)	G=5.17%	D=11.5-12	33.42 (33.13-33.48)	F=5.17%	D=18-19
		2	-	-	-	E=7-7.5	-	-	E=100-105	-	-	E=19-20
		3	-	-	-	F=7.5-8	-	-	F=105-110	-	-	F=33-34
	Total	1	6	17.38 (17.54-18.03)	I=5.17%	G=16.5-17	177.90 (175.64-179.15)	G=5.17%	G=175-180	33.63 (33.13-33.98)	F=5.17%	G=34-35
		2	-	-	-	H=17-17.5	-	-	H=180-185	-	-	H=35-36
		3	-	-	-	I=17.5-18	-	-		-	-	I=36-37
30-40 yrs	M	1	22	18.46 (18.12-18.79)	J=13.7%; K=32.7%	J=18-18.5	179.56 (178.44-180.23)	G=36.21%; H= 1.72%		34.89 (34.42-35.33)	G=12.06%; H=25.86%	
		2	-	-	-	K=18.5-19	-	-		-	-	
		3	26	0.49 (0.47-0.53)	A=19.35%; B=22.58%		10.84 (10.48-10.91)	A=11.71%; B=24.19%		1.88 (1.83-2.03)	A=19.35%; B=22.58%	
	F	1	22	18.33 (18.01-18.68)	J=8.9%; K=30.4%	L=19.5-20	178.05 (176.69-180.12)	G=35.71%; H=3.57%		34.57 (34.29-35.17)	G=7.14%; H=32.14%	
		2	-	-	-	M=19.5-20	-	-		-	-	
		3	26	0.46 (0.45-0.52)	A=20.9%; B=20.9%		10.72 (10.34-10.87)	A=19.6% B=24.19%		1.96 (1.89-2.15)	A=20.9%; B=20.9%	
	Total	1	44	18.34 (18.01-18.79)	J=11.3%; K=38.59%		178.80 (176.69-180.23)	G=35.96%; H=2.63%		34.73 (34.29-35.33)	G=9.73%; H=29%	
		2	-	-	-		-	-		-	-	
		3	52	0.48 (0.47-0.53)	A-=20.13%; B=21.74%		10.78 (10.34-10.91)	A=15.65%; B=24.19%		1.92 (1.83-2.15)	A-=20.13%; B=21.74%	
40-50 yrs	M	1	33	19.91 (19.53-20.01)*	L=56.89%		183.94 (180.89-184.81)*	H=56.9%		36.91 (36.52-36.96)*	I=56.89%	
		2	1	7.13	E=6.6%		103.23	E=6.6%		17.78	C=6.6%	
		3	26	0.495 (0.49-0.57)	A=3.2%, B=38.7%		10.89 (10.65-10.91)	B=41.93%		2.35 (1.91-2.41)	A=3.2%, B=38.7%	
	F	1	31	19.63 (19.55-19.66)*	L=55.36%		182.35 (180.13-182.70)*	H=55.36%		36.62 (36.5-36.69)*	I=55.36%	
		2	1	7.03	E=6.6%		100.61	E=6.6%		17.53	C=6.6%	
		3	26	0.48 (0.47-0.54)	A=4.8; B=37.1%		10.63 (10.51-10.85)	B=41.93%		2.43 (1.94-2.49)	A=4.8; B=37.1%	
	Total	1	61	19.77 (19.53-20.01)*	L=56.14%		183.15 (180.13-184.81)*	H=56.14%		36.77 (36.5-36.96)*	I=56.89%	
		2	2	7.13 (7.03-7.13)	E=6.6%		101.92 (100.6-103.2)	E=6.6%		17.66 (17.53-17.78)	C=6.6%	
		3	52	0.488 (0.47-0.57)	A=4%; B=37.9%		10.76 (10.51-10.91)	B=41.93%		2.39 (1.91-2.49)	A=4%; B=37.9%	
50-60 yrs	M	1	-	-	-		-	-		-	-	
		2	6	7.34 (7.23-7.48)	E=40%		104.89 (103.59-104.91)	E=40%		18.21 (17.86-18.46)	C=6.67% D=33.33%	
		3	10	0.60 (0.55-0.61)	C=3.22%; D=12.90%		11.59 (11.51-11.63)	D=16.13%		2.56 (2.51-2.58)	C=16.13%	
	F	1	-	-	-		-	-		-	-	
		2	5	7.27 (7.11-7.39)	E=38.46%		103.96 (102.16-104.85)	E=38.46%		18.10 (17.69-18.39)	C=7.69%; D=30.77%	
		3	10	0.57 (0.53-0.59)	B=4.83%; C=1.29%		11.25 (11.05-11.28)	C=16.13%		2.68 (2.56-2.72)	C=16.13%	
	Total	1	-	-	-		-	-		-	-	
		2	11	7.31 (7.11-7.48)	E=39.29%		104.43 (102.16-104.91)	E=39.29%		18.16 (17.86-18.46)	C=7.18%; D=32.05%	
		3	20	0.59 (0.53-0.61)	B=4.83%; C=2.23%; D=12.9%		11.35 (11.05-11.63)	C=16.13% D=16.13%		2.62 (2.51-2.72)	C=16.12%	
60-70 yrs	M	1	-	-	-		-	-		-	-	
		2	8	7.89 (7.61-7.93)	F=53.33%		109.54 (107.79-109.69)	F=53.3%		19.13 (18.28-19.55)	D=6.67% E=46.67%	
		3	-	-	-		-	-		-	-	
	F	1	-	-	-		-	-		-	-	
		2	7	7.59 (7.51-7.69)	F=53.18%		107.96 (106.71-108.90)	F=53.18%		19.01 (18.15-19.41)	D=7.69% E=46.15%	
		3	-	-	-		-	-		-	-	
	Total	1	-	-	-		-	-		-	-	
		2	15	7.74 (7.51-7.93)	F=53.25%		108.75 (106.71-109.69)	F=53.25%		19.09 (18.15-19.55)	D=7.18%; E=46.41%	
		3	-	-	-		-	-		-	-	

Please refer the table legend in the next page

2.10 B. Age and sex based distribution of serum based tubular function associated biochemical markers viz. bicarbonates and phosphates

Age in years	Sex	Study group	Number of subjects in each category	Parameter					
				Serum bicarbonates			Serum phosphate		
				Mean in mEq/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mEq/L)	Mean in mg/dL (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/dL)
20-30 yrs	M	1	3	8.37 (8.33-8.39)	C=5.17%	A=8.1-8.2	1.14 (1.12-1.16)	C=5.17%	A=0.9-1.0
		2	-			B=8.2-8.3			B=1.0-1.1
		3	-			C=8.3-8.4			C=1.1-1.2
	F	1	3	8.32 (8.31-8.33)	C=5.17%	D=24-25	1.11 (1.10-1.12)	C=5.17%	D=3.9-4.0
		2	-			E=25-26			E=4.0-4.1
		3	-			F=26-27			F=4.1-4.2
	Total	1	6	8.34 (8.31-8.39)	C=5.17%	G=27-28	1.12 (1.1-1.16)	C=5.17%	G=4.2-4.3
		2	-			H=28-29			H=4.3-4.4
		3	-			I=29-30			I=4.4-4.5
30-40 yrs	M	1	22	8.27 (8.23-8.29)	B=37.93%		1.06 (1.03-1.09)	B=37.93%	J=4.5-4.6
		2	-						
		3	26	29.41 (29.23-29.77)	I=30.64%; H=26.92%		4.59 (4.53-4.61)	J=56.9%	
	F	1	22	8.23 (8.21-8.26)	B=37.93%		1.02 (1.00-1.05)	B=37.93%	
		2	-						
		3	26	29.84 (29.44-30.01)	I=30.64%; H=26.92%		4.53 (4.51-4.56)	J=56.9%	
	Total	1	44	8.25 (8.21-8.29)	B=37.93%		1.04 (1.00-1.09)	B=37.93%	
		2	-						
		3	52	29.62 (29.23-30.01)	I=30.64%; H=26.92%		4.56 (4.51-4.61)	J=56.9%	
40-50 yrs	M	1	33	8.17 (8.16-8.19)*	A=56.89%		0.98 (0.97-0.99)*	A=56.89%	
		2	1	26.7	F=6.67%		4.22	G=6.67%	
		3	26	28.46 (28.27-29.18)	H=32.25%; I=9.67%		4.48 (4.44-4.57)	I=32.25%; J=9.67%	
	F	1	31	8.12 (8.10-8.14)*	A=55.36%		0.94 (0.93-0.95)*	A=55.36%	
		2	1	24	F=6.67%		4.2	G=6.67%	
		3	26	26.1	H=32.25%; I=9.67%		4.42 (4.41-4.51)	I=32.25%; J=9.67%	
	Total	1	61	8.14 (8.10-8.19)	A=56.13%		0.96 (0.93-0.99)*	A=56.13%	
		2	2	26.42	F=6.67%		4.21	G=6.67%	
		3	52	28.58 (28.27-29.24)	H=32.25%; I=9.67%		4.45 (4.41-4.57)	I=32.25%; J=9.67%	
50-60 yrs	M	1	-						
		2	6	25.71 (24.90-25.93)	D=13.33%; E=26.66%		4.08 (4.03-4.14)	F=13.33%; E=26.66%	
		3	10	27.33 (27.06-27.12)	G=16.29%		4.39 (4.36-4.45)	H=16.29%	
	F	1	-						
		2	5	25.23 (24.61-25.48)	D=13.18%; E=23.07%		4.04 (4.0-4.11)	F=13.18%; E=23.07%	
		3	10	27.77 (27.64-28.35)	G=16.29%		4.35 (4.33-4.41	H=16.29%	
	Total	1	-						
		2	11	25.47 (24.61-25.93)	D=13.25%; E=24.86%		4.06 (4.0-4.14)	F=13.33%; E=26.66% J54	
		3	20	27.55 (27.06-28.35)	G=16.29%		4.37 (4.33-4.45)	H=16.29%	
60-70 yrs	M	1	-						
		2	8	24.8 (24.51-24.91)	D=54.33%		3.97 (3.91-3.99)	E=54.33%	
		3	-						
	F	1	-						
		2	7	24.5 (24.1-24.81)	D=50.77%		3.91 (3.90-3.93)	E=50.77%	
		3	-						
	Total	1	-						
		2	15	26.42 (24.1-24.91)	D=52.86%		3.94 (3.90-3.99)	E=52.86%	
		3	-						

(A) represents prevalence distribution of various serum based general renal function biochemical markers viz. creatinine, urea and uric acid for different age categories and sexes in all the three study cohorts.(B) represents prevalence distribution of various serum based tubular function associated biochemical markers viz. bicarbonates and phosphates for different age categories and sexes in all the three study cohorts.Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; b2M-β2-microglobulin, NAG-N-acetyl-β-D-glucosaminidase; M-male; F-female. Values are represented as mean with the range of minimum to maximum levels of each biochemical parameter depicted as well. The prevalence of the biochemical markers(i.e. general renal function[A] and tubular biomarkers[B]) in various ranges of their levels for each age and sex category of the three study groups are represented by prevalence % and annotated by uppercase letters(i.e. A to M) for convenience. The annotations for each analysed biochemical marker vary and have been independently described. The prevalence % denotes the proportion of subjects from the total number in their respective age & sex categories bearing biochemical markers at a given range of their levels. For example the prevalence % annotation of 'A' in the case of serum creatinine denotes the percentage of the total population in each age and sex category possessing levels in the range of 0.45-0.5 mg/dL. Differences at *$p<0.05$ were considered to be statistically significant as compared to the healthy controls(group 3) & the CKD affected group(group 2) and also relative to the lower age categories (in a given study group).No gender bias in renal function markers'expression were noted evident from similar levels observed in both sexes in all study groups.Moreover levels of markers like serum creatinine,urea,uric acid increased and serum bicarbonates & phosphates decreased with increasing age with significant(*$p<0.05$) prevalence of their levels at higher and lower ranges respectively being noted in higher age groups (i.e.40-50yrs) of study group 1.This was attributed to the bioaccumulative potencies of toxins that were deemed responsible for CKDu causation which caused accumulation and aggravation of tubular damage effects with increased exposure (i.e.age),that was evident from corresponding increase in expression of general serum renal function markers and decrease in tubular function markers(viz.serum bicarbonates & phosphates) with rising age.For detailed units of biochemical parameter,refer Table2.7

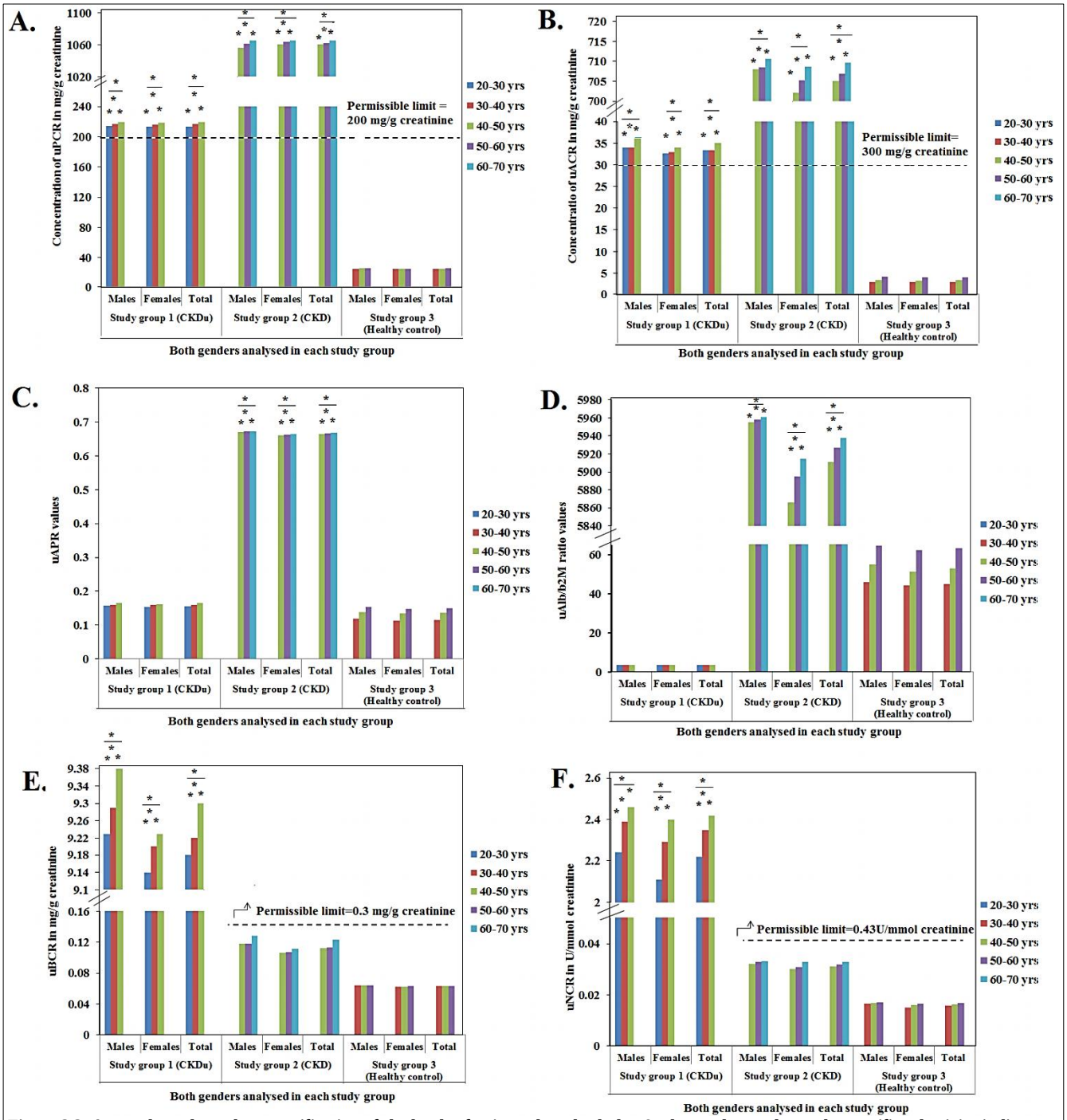


Figure 2.2 .Age and sex dependent stratification of the levels of urinary based tubular & glomerular nephropathy specific selectivity indices of the entire study population for identification of the risk-groups susceptible to CKDu development.

Abbreviations:CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. Study group(cohort)1, 2 & 3 consists of CKDu affected,diabetes/hypertension induced CKD affected & healthy controls subjects respectively. The number of males(M)/ females(F)/total number of subjects(total) in study group 1,2& 3 are 58(M)/56(F)/114(total);15(M)/13(F)/28(total)& 62(M)/62(F)/124(total) respectively.The age based distribution(i.e.number of subjects under each age category) among males,females&total number of subjects (combination of both sexes) in all three study groups are detailed in the affixed table(**Table 2.11)** of this figure. (A)Concentration of uPCR in various age groups among the males,females & both sexes combined in all 3study groups(B)Concentration of uACR in age and gender stratified sub-groups of three study cohorts'population (C) uAPR levels in age & gender stratified sub-groups of the three study cohorts' population (D)uAlb/b2M ratio values in age & gender stratified sub-groups of the three study cohorts' population (E)Concentration of uBCR in age & gender stratified sub-groups of the three study cohorts' population (F)Concentration of uNCR in age and gender stratified sub-groups of the three study cohorts' population. Values are represented as mean concentrations of a given nephropathy specific selectivity index(renal injury specific biochemical markers) estimated independently in various age groups(i.e. 20-30;30-40;40-50;50-60& 60-70yrs) of male, female & total number of subjects(i.e. both sexes combined) in all 3 study groups. All concentration values are in mg/g creatinine except uNCR which is in U/mmol creatinine. uAPR & uAlb/b2M being ratios are devoid of units.Differences in a particular study cohort at *p<0.05 were considered to be significant as compared to other 2 cohorts and also relative to lower age categories (in a given study group).The WHO permissible limits for uPCR=200mg/g creatinine uACR=300mg/g creatinine; uBCR=0.3 mg/g creatinine & uNCR=0.43U/mmol creatinine. uAPR<0.4 =tubular nephropathies & > 0.4=glomerular disorders. No bar representation of levels of a given nephropathy specific marker for a given age category among males,females & total subjects in each study group indicated no subjects in that age category.No gender bias in tubular dysfunction biomarkersexpression viz. uBCR, uNCR in group 1 & glomerular injury markers viz. uPCR, uACR**,** uAPR, uAlb/b2M expression in group 2 were noted, evident from similar levels of respective markers noted in both sexes in all groups.Also,levels of tubular damage markers in group1 & glomerular injury markers in group 2 escalated with rising age with significant (*p<0.05) prevalence of their levels at higher ranges noted in older age groups (i.e.40-50yrs in group 1& 60-70yrs in group 2). This higher tubular injury markers levels noted at higher ages(i.e.40-50 yrs) in group 1 was ascribed to bioaccumulative potencies of CKDu causing toxins that caused accumulation & aggravation of tubular damage effects with chronic exposure (i.e.age), evident from higher tubular injury markers'levels with rising age.Higher glomerular damage markers' levels noted at higher ages (i.e.60-70 yrs) in group 2 was ascribed to natural degeneration in glomerular function with age (i.e.> 50yrs) which enhances susceptibility of glomerulus to damage by risk factors (diabetes,hypertension).Details on range of min to max values of biomarker concentration & its prevalence distribution (%)in various ranges for age groups of each sex & both combined(i.e. total number) in all 3 groups are listed in **Table 2.11**. For detailed units of biochemical parameter, kindly refer Table 2.8.

Table 2.11: Age and sex dependent stratification of the prevalence of various urinary based tubular and glomerular nephropathy specific selectivity indices of the entire study population for identification of the population risk-groups susceptible to CKDu development.

2.11 A. Age and sex based distribution of urinary based glomerular function biochemical markers viz. uPCR, uACR, uAPR and uAlb/b2M

Age in years	Sex	Study group	Number of subjects in each category	Parameter											
				uPCR			uACR			uAPR			uAlb/b2M ratio		
				mean in mg/g (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in mg/g (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean (min-max)	prevalence	Annnotation for the ranges of the biomarker levels	mean (min-max)	prevalence	Annnotation for the ranges of the biomarker levels
20-30 yrs	M	1	3	214.49 (214.44-215.58)	E=5.17%	A=24-25	33.93 (33.91-33.97)	E=5.17%	A=2-3.0	0.1582 (0.01580-0.1590)	F=5.17%	A=0.10-0.12	3.631 (3.630-3.634)	B=5.17%	A=3.57-3.59
		2	-	-	-	B=25-26	-	-	B=3-4.0			B=0.12-0.14			B=3.63-3.65
		3	-	-	-	C=26-27	-	-	C=4-5.0			C=0.14-0.16			C=3.65-3.67
	F	1	3	213.01 (212.56-215.13)	D=1.72%; E=3.45%	D=212-214	32.65 (32.62-32.70)	D=5.17%	D=32-33	0.1532 (0.1530-0.1534)	D=5.17%	D=0.153-0.154	3.575 (3.570-3.580)	A=5.17%	D=3.87-3.89
		2	-	-	-	E-214-216	-	-	E=33-34			E=0.154-0.155			E=40-50
		3	-	-	-	F=216-218	-	-	F=36-37			F=0.158-0.159			F=50-60
	Total	1	6	213.75 (212.56-215.58)	D=1.72%; E=4.31%	G=218-220	33.33 (32.62-33.97)	D and E= 5.17%	G=702-705	0.1557 (0.01530-0.159)	D and F= 5.17%	G=0.159-0.160	3.603 (3.57-3.634)	A and B= 5.17%	G=60-70
		2	-	-	-	H=220-222	-	-	H=705-708			H=0.163-0.164			H=5860-5910
		3	-	-	-	I=1055-1060	-	-	I=708-711			I=0.660-0.662			I=5910-5960
30-40 yrs	M	1	22	216.76 (216.57-217.86)	F=37.93%	J=1060-1065	33.98 (33.93-34.00)	E=37.93%	J=714-717	0.1584 (01582-0.1589)	F=37.93%	J=0.662-0.664	3.638 (3.632-3.645)	B=37.93%	J=5960-6010
		2	-	-	-	K=1065-1070	-	-				K=0.664-0.666			
		3	26	25.37 (25.25-25.74)	B=41.93%		2.80 (2.78-2.89)	A=41.93%		0.118 (0.11-0.133)	A=25.80%; B=16.2%	L=0.670-0.672	45.83 (44.12-51.25)	E=32.25%; F=9.67%	
	F	1	22	216.21 (216.13-217.67)	F=37.28%		32.89 (32.67-32.98)	D=37.28%		0.1538 (0.1532-0.1539)	D=37.28%		3.631 (3.630-3.638)	B=37.28%	
		2	-	-	-		-	-							
		3	26	24.57 (24.49-24.91)	A=41.93%		2.76 (2.73-2.82)	A=41.93%		0.113 (0.109-0.131)	A=25.80%; B=16.2%		44.12 (43.89-50.27)	E=32.25%; F=9.67%	
	Total	1	44	216.48 (216.13-217.86)	F=37.61%		33.44 (32.67-34)	D and E= 37.61%		0.1561 (0.1532-0.1589)	D=37.28%; F=37.93%		3.634 (3.630-3.645)	B=37.60%	
		2	-	-	-		-	-							
		3	52	24.97 (24.49-25.74)	A and B=41.93%		2.78 (2.73-2.89)	A=41.93%		0.115 (0.109-0.133)	A=25.80%; B=16.2%		44.97 (43.89-51.25)	E=32.25%; F=9.67%	
40-50 yrs	M	1	33	219.09 (218.36-222.72)	G=55.17%; H=1.7%		36.13 (36.01-36.6)	F=56.9%		0.1593 (0.159-0.164)	G=55.17% ; H=1.72%		3.670 (3.651-3.890)	C=53.74; D=3.44%	
		2	1	1055.91	I=6.6%		708.11	I=6.6%		0.67	L=6.67%		5955.5	I=6.67%	
		3	26	25.46 (25.32-25.89)	B=41.93%		3.25 (3.23-3.89)	B=41.93%		0.138 (0.127-0.153)	B=24.19%; C=17.77%		54.89 (52.78-69.32)	F=35.48%; G=6.45%	
	F	1	31	218.59 (218.01-218.63)	G=55.15%		33.91 (33.13-33.97)	E=55.36%		0.1549 (0.1541-0.155)	E=55.36%		3.658 (3.650-3.660)	C=53.35%	
		2	1	1060.07	I=6.6%		702.19	G=6.6%		0.66	K=6.67%		5865.71	H=6.67%	
		3	26	25.08 (25.01-25.13)	B=41.93%		3.11 (3.05-3.68)	B=41.93%		0.135 (0.123-0.145)	B=25.80%; C=16.12%		51.13 (50.14-67.58)	F=35.48%; G=6.45%	
	Total	1	61	219.14 (218.01-222.72)	G=55.16%; H=1.7%		35.02 (33.13-36.6)	E and F= 56.9%		0.157 (0.1541-0.164)	E=55.36%; G=55.17% ; H=1.72%		3.664 (3.65-3.89)	C=53.54; D=3.44%	
		2	2	1059.99	I=6.6%		705.15	G and I= 6.6%		0.665	K and L=6.67%		5910.6	H and I= 6.67%	
		3	52	25.27 (25.01-25.89)	B=41.93%		3.18 (3.05-3.89)	B=41.93%		0.136 (0.123-0.153)	B=24.99%; C=16.94%		53.01 (50.14-69.32)	F=35.48%; G=6.45%	
50-60 yrs	M	1	-		-		-	-							
		2	6	1061.36 (1061.07-1064.38)*	J=40%		708.53 (708.23-709.09)	I=40%		0.6718 (0.6710-0.6720)	L=40%		5958.15 (5957.3-5960)	I=40%	
		3	10	25.89 (25.47-26.61)	B=12.9%; C=3.2%		4.18 (4.04-4.44)	C=16.13%		0.153 (0.151-0.160)	C=16.29%		64.46 (63.5-68.26)	G=16.29%	
	F	1	-	-	-		-	-							
		2	5	1061.18 (1060.01-1064.15)*	J=38.46%		705.31 (705.11-707.36)	H=38.46%		0.6629 (0.6620-0.6639)	J=.38.46%		5895.2 (5868.2-5909.3)	H=38.46%	
		3	10	25.28 (25.09-25.63)	B=16.12%		3.89 (3.56-3.99)	B=16.13%		0.148 (0.142-0.150)	C=16.29%		62.36 (61.12-63.13)	G=16.29%	
	Total	1	-	-	-		-	-							
		2	11	1061.27 (1061.01-1064.38)*	J=39.29%		706.92 (705.11-709.09)	H=38.56%; I=40%		0.6673 (06620-0.6720)	J=38.46%; L=40.0%		5926.67 (5868.2-5960.0)	H=38.46%; I=40%	
		3	20	25.58 (25.09-26.61)	B=14.51%; C=3.2%		4.04 (3.59-4.44)	B and C= 16.13%		0.15 (0.142-0.160)	C=16.29%		63.41 (61.12-68.26)	G=16.29%	
60-70 yrs	M	1	-	-	-		-	-							
		2	8	1065.54 (1065.12-1067.41)*	K=53.3%		710.67 (710.16-718.43)*	I=46.67%; J=6.67%		0.6729 (0.6722-0.673)*	L=53.33%		5960.9 (5958.13-5967.4)*	I=46.67%; J=6.67%	
		3	-	-	-		-	-							
	F	1	-	-	-		-	-							
		2	7	1065.29 (1065.08-1067.04)*	K=53.18%		708.69 (705.23-709.74)*	H=7.69%; I=46.15%		0.6648 (0.6640-0.6650)*	K=53.13%		5914.53 (5903.6-5917.13)*	H=7.69%; I=46.15%	
		3	-	-	-		-	-							
	Total	1	-	-	-		-	-							
		2	15	1065.42 (1065.08-1067.41)*	K=53.25%		709.68 (705.23-718.43)*	H=7.69%; I=46.41%; J=6.67%		0.6688 (0.6640-0.673)*	K=53.13% ; L=53.13%		5937.71 (5903.6-5967.4)*	H=7.69%; I=46.41%; J=6.67%	
		3	-	-	-		-	-							

Please refer the table legend in the next page

2.11 B. Age and sex based distribution of urinary based tubular function biochemical markers viz. uBCR and uNCR

Age in years	Sex	Study group	Number of subjects in each category	Parameter					
				uBCR			uNCR		
				mean in mg/g (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in U/mmol (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in U/ mmol creatinine
20-30 yrs	M	1	3	9.23 (9.22-9.26)	J=5.17%	A=0.0620-0.0625	2.28 (2.27-2.30)	J=5.17%	A=0.014-0.015
		2	-	-		B=0.0625-0.0630			B=0.015-0.016
		3	-	-		C=0.0630-0.0635			C=0.016-0.017
	F	1	3	9.14 (9.13-9.16)	I=5.17%	D=0.0635-0.0640	1.91 (1.9-1.93)	I=5.17%	D=0.017-0.018
		2	-	-		E=0.0640-0.0645			E=0.030-0.031
		3	-	-		F=0.10-0.11			F=0.031-0.032
	Total	1	6	9.18 (9.13-9.26)	I and J=5.17%	G=0.11-0.12	2.09 (1.90-2.3)	I and J=5.17%	G=0.032-0.033
		2	-	-		H=0.13-0.141			H=0.033-0.034
		3	-	-		I=9.12-9.22			I=1.-2
30-40 yrs	M	1	22	9.29 (9.25-9.31)	J=37.93%	J=9.22-9.32	2.39 (2.29-2.46)	J=37.93	J=2-3.0
		2	-			K=9.32-9.42			
		3	26	0.0637 (0.0636-0.06405)	D=22.5%; E=19.35%		0.0165 (0.0164-0.01701)	C=33.87%; D=8.6%	
	F	1	22	9.20 (9.18-9.21)	I=37.28%		2.05 (1.95-2.07)	I=1.78%; J=37.5%	
		2	-						
		3	26	0.0624 (0.0622-0.0626)	A=22.5%; B=19.35%		0.015 (0.0147-0.0153)	A=9.6%; B=32.25%	
	Total	1	44	9.22 (9.18-9.31)	I=37.28%; J=37.93%;		2.22 (1.95-2.46)	I=1.78%; J=37.71%	
		2	-	-					
		3	52	0.0630 (0.0622-0.06405)*	A=22.5%; B=19.35%; D=22.5%; E=19.35%;		0.0157 (0.0147-0.0170)*	A=9.6%; B=32.25% ; C=33.87%; D=8.6%	
40-50 yrs	M	1	33	9.38 (9.33-9.41)	K=56.9%		2.86 (2.80-2.88)	J=56.89%	
		2	1	0.118	G=6.6%		0.032	G=6.6%	
		3	26	0.0638 (0.0637-0.06408)*	D=22.5%; E=19.35%		0.0167 (0.0166-0.0172)*	C=38.70%; D=3.22%	
	F	1	31	9.23 (9.21-9.26)	J=55.36%		2.17 (2.14-2.19)	J=55.36%	
		2	1	0.106	F=6.6%		0.03	E=6.6%	
		3	26	0.0627 (0.0625-0.0631)*	B=22.5%; C=19.35%		0.0160 (0.0153-0.0162)*	B=38.70%; C=3.22%	
	Total	1	61	9.30 (9.21-9.41)	J=55.36%; K=56.9%		2.51 (2.14-2.88)	J=56.13%	
		2	2	0.112	F and G=6.6%		0.031	E and G=6.6%	
		3	52	0.0632 (0.0625-0.06408)	B=22.5%; C=19.35%; D=22.5%; E=19.35%;		0.0163 (0.0153-0.0172)	B=38.70%; C=38.70%; D=3.22%	
50-60 yrs	M	1	-	-					
		2	6	0.1185 (0.1182-0.1199)	G=40%		0.0329 (0.0321-0.0338)	G=26.67%; H=13.33%	
		3	10	0.06405 (0.0640-0.0641)	E=16.13%		0.0171 (0.0170-0.0174)	D=40%	
	F	1	-	-	-				
		2	5	0.1071 (0.1063-0.1097)	F=38.46%		0.0307 (0.0301-0.0319)	E=23.07%; F=15.38%	
		3	10	0.0631 (0.0630-0.632)	C=16.13%		0.0166 (0.0164-0.0168)	C=40%	
	Total	1	-	-	-				
		2	11	0.1128 (0.1063-0.1199)	C and E=16.13%		0.0318 (0.0301-0.0338)	E=23.07%; F=15.38%; G=26.67%; H=13.33%	
		3	20	0.0636 (0.0630-0.0641)	F=40%; G=38.6%		0.0168 (0.0164-0.0174)	C and D=40%	
60-70 yrs	M	1	-						
		2	8	0.1345 (0.1191-0.1410)	G=20%; H=33.33%		0.0331 (0.0326-0.034)	G=33.33%; H=20%	
		3	-	-					
	F	1	-	-					
		2	7	0.1115 (0.1093-0.1180)	F=15.38%; G=38.46%		0.0328 (0.0318-0.033)	G=38.46%; F=15.38%	
		3	-	-					
	Total	1	-	-					
		2	15	0.1230 (0.1093-0.1410)	F=15.38%; G=29.23%; H=33.33%		0.0329 (0.0318-0.034)	F=15.38%; G=35.85%	
		3	-	-					

Table 2.11:-(A) represents prevalence distribution of various urinary based glomerular function biochemical markers viz. uPCR,uACR, uAPR and uAlb/b2M for different age categories and sexes in all the three study cohorts.(B) represents prevalence distribution of various urinary based tubular function biochemical markers viz. uBCR and uNCR for different age categories and sexes in all the three study cohorts.Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio; M-male; F-female. Values are represented as mean with the range of minimum to maximum levels of each selectivity index depicted as well. The units for uPCR, uACR, uBCR are mg/g creatinine and uNCR is U/mmol creatinine. uAPR and uAlb/b2M being ratios are devoid of units. The prevalence of the urinary glomerular nephropathy(A) and tubular nephropathy(B) based selectivity indices in various ranges of their levels for each age and

sex category of the three study groups are represented by prevalence % and annotated by uppercase letters(i.e. A to M) for convenience. The annotations for each estimated selectivity index varies and have been independently described. The prevalence % denotes the proportion of subjects from the total number in their respective sex categories of a particular age group bearing the selectivity index at a given range of their levels. The total number of males (M)/females (F) in study group one, two and three are 58(M)/56(F); 15(M)/13(F) and 62(M)/62(F) respectively. For example the prevalence % annotation of 'A' in the case of PCR denotes the percentage of the total population in a particular age and sex category possessing levels in the range of 24-25 mg/g creatinine. Differences in a particular study cohort at *p<0.05 were considered to be statistically significant as compared to the other two cohorts (wherever applicable) and also relative to the lower age categories (in a given study group). No gender bias in tubular dysfunction specific selectivity indices'or biomarkers' expression viz. uBCR,uNCR in study group 1 and glomerular injury markers' viz. uPCR, uACR**,** uAPR and uAlb/b2M expression in study group 2 were noted,evident from similar levels of the respective markers being observed in both sexes in all study groups.Moreover levels of tubular injury markers in study group 1 and glomerular nephropathy specific markers in study group 2 elevated with increasing age with significant (*p<0.05) prevalence of their levels at higher ranges being noted in higher age groups (i.e.40-50yrs in study group 1 and 60-70yrs in study group 2). This elevated levels of tubular injury markers being noted at the higher age group(i.e.40-50 yrs) in study group 1 was attributed to the bioaccumulative potencies of toxins that were deemed responsible for CKDu causation which caused accumulation and aggravation of tubular damage effects with increased exposure (i.e.age),that was evident from the increase in expression of tubular injury markers with rising age. Moreover the escalated levels of glomerular injury markers being noted at the higher age group(i.e.40-50 yrs) in study group 2 was attributed to the inherent or natural degeneration and compromise in the glomerular function with age(i.e.beyond 50yrs) which increases the susceptibility of glomerulus to damage by major risk factors(diabetes,hypertension), visible from increase in glomerular damage markers' levels with age.For detailed units of biochemical parameter, kindly refer Table 2.8.

As shown in **Figures 2.1 and 2.2** & **Tables 2.10 and 2.11,** the values of biomarkers of renal function and nephropathy selectivity indices did not vary significantly with sex but showed considerable variation with age in all the three study groups. The mean values of the biomarkers of tubular dysfunction [i.e. serum creatinine, serum urea, serum uric acid (Figure 2.1; Table 2.10A) and tubular nephropathy specific selectivity indices i.e. uBCR, uNCR (Figure 2.2; Table 2.11B)] in study group 1 (CKDu cases) were found to be abnormally and significantly well beyond the reference limits and were present at similar ranges of their levels in both males and females of various age groups.

Other markers of tubular injuries like serum bicarbonates and serum phosphates (Figure 2.1; Table 2.10B) were found to be significantly reduced below the reference limit in this study group with the range of their values being prevalent to the same extent in both the sexes. Moreover this study group 1 demonstrated near normal levels (within permissible limits) of the glomerular impairment markers (i.e. uAPR, uPCR, uAPR, uAlb/b2M) (Figure 2.2; Table 2.11A) with similar range of values in both males and females, confirming the prevalence of tubular injury in this study group (Rajapakse et al., 2016).

However these target markers of tubular dysfunction depicted near optimal levels (within the reference limits) in the study group 2 (comprising of CKD cases) with range of their values being comparable between both genders (Figure 2.1; Table 2.10B and Figure2.2 Table 2.11B), indicative of the presence of non-tubular injuries in this study group (Smith et al., 2011). Hence this study group depicted a significant elevation in the values of glomerular injury markers (i.e. uAPR, uPCR, uAPR, uAlb/b2M) with uniform prevalence in the abnormally higher ranges in both the sexes (Figure 2.2; Table 2.11A).

Therefore the prevalence of uniform consistency in the levels of various biomarkers of glomerular and tubular dysfunction in both the sexes of the study group 2 and 1 indicate the

absence of a gender bias in the disease development in both these groups (Figures 2.1 and 2.2 & Tables 2.10 and 2.11). In other words, as per our findings sex is not a risk factor for the development of either tubular nephropathy (CKDu) or glomerular dysfunction (CKD) in the Canacona taluka. These findings were unusual and contradictory to the previous studies of CKDu in Central America which established the male gender to be the major risk group prone to the development of CKDu (Orantes et al., 2017; Vela Parada et al., 2014).However this lack of gender bias in CKDu distribution in the Canacona taluka was in agreement with the deficiency of gender based vulnerability in the CKDu pattern in the Uddanam region of Andhra Pradesh (India) (Ganguli, 2016); and some parts of Sri Lanka (Badurdeen et al., 2016; Rajapakse et al., 2016; Wanigasuriya et al., 2011) and Central America (Ascencio et al., 2016 Orantes-Navarro et al., 2016; Laux et al., 2012).

Since no significant gender bias for CKDu disease induction was observed, various renal function biomarkers were further stratified by age. In study group 1 and 2, it was observed that proportion of the populations with elevated levels of the biomarkers (tubular and glomerular respectively) significantly exceeding the reference limits increased with increasing age groups to a uniform extent in both sexes (Figures 2.1 and 2.2 & Tables 2.10 and 2.11). Moreover the higher age groups in these study cohorts i.e. > 40yrs in study group 1 and > 60 yrs. in study group 2 had larger prevalence of the biomarkers in considerably higher ranges of their levels. However the affected age groups in study group 1 and 2 were restricted to 50 yrs. and 70 yrs. respectively. As indicated in Figures 2.1 and 2.2 & Tables 2.10 and 2.11, the highest abnormal range levels (significantly beyond the reference limits) of both the serum based tubular injury biomarkers (Figure 2.1; Table 2.10) and associated urinary based tubular nephropathy specific selectivity indices(Figure 2.2; Table 2.11B) were largely prevalent (i.e. highest proportion/percentage of the study subjects possessing significantly disturbed levels of each measured biochemical characteristic, that is increased levels of uBCR and uNCR along with simultaneous reduction in the concentrations of serum bicarbonates and phosphates) at approximately 55% and 50% in the age category of 40-50 yrs and 30-40 yrs respectively for both genders in study group 1. Whereas in study group 2, significantly elevated levels of glomerular dysfunction markers were highly prevalent at roughly 58% and 42% in the age groups of 60-70 yrs. and 50-60 yrs. respectively for both sexes (Figure 2.2; Table 2.11 A). This observed difference in trend of the tubular and glomerular disorders being exhibited at significantly different stages of life in the Canacona taluka was supported by similar previous studies of the CKDu disease in the endemic regions of Central America and Sri Lanka that reported the inclination of CKDu disease towards

affecting the earlier age groups (specifically in the 3rd-5th decade) since the causality is mainly linked to exposure to environmental nephrotoxins (Lebov et al., 2015; Orantes et al., 2014; Orantes-Navarro et al., 2016; Raines et al., 2014; Wanigasuriya, 2012).

It has already been well reported that a nephrotoxin exposure beyond 25 yrs is significant enough to induce considerable amount of tubular dysfunction that manifests in renal failure owing to the propensity of the triggered nephrotoxic effects to aggravate and accumulate with time. Therefore explaining the earlier induction (at the 3rd decade) of CKDu in this taluka in comparison to the typical diabetes and hypertensive induced CKD cases of study group 2 that manifests at an advanced age (Abiola, 2017; Sabath and Robles-Osorio, 2012).

It also highlighted that the etiology of CKDu in the Canacona taluka is related to an environmental factor possibly environmental nephrotoxins like heavy metals etc as it was unlinked to traditional causals like diabetes and hypertension as established from our demographic analysis (Weaver et al., 2015; Gifford et al., 2017).

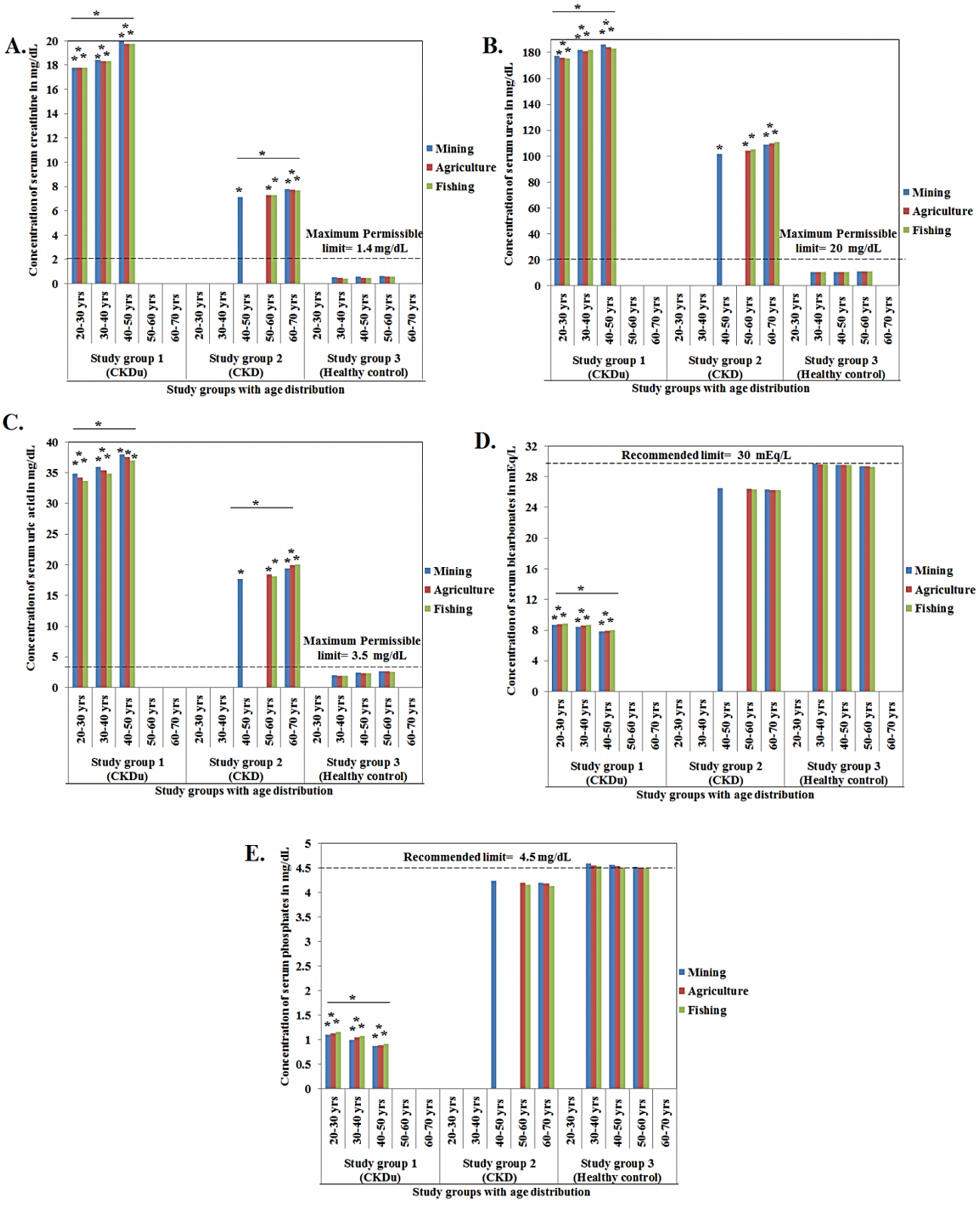
This trend was contrary to the glomerular pathological manifestation of CKD cases in the study group 2 wherein the disease developed later in life. This is attributed to inherent ability of the incurable causative factors(i.e. diabetes and hypertension) to exert severe nephrotoxic effects that only aggravates and accumulates with time as a result of natural degradation of the structural and functional integrity of the glomerulus and degeneration of the intrinsic reno-protective effects with increasing age (Defina et al., 2016). Thus explaining the induction of glomerular damage at a later time (> 45yrs) in the study group 2 subjects which is restricted to an age bar of 70 yrs.

Following age and sex based stratification of the biochemical markers, the prevalence and range distribution in the levels of various serum based general renal and tubular function markers and urinary based nephropathy specific selectivity indices in men and women of different age groups differentiated by occupation (i.e. mining **(Table 2.12 A),** fishing **(Table 2.12B)** and farming **(Table 2.12C)** in all three study cohorts were analysed. The results of this occupational based stratification of serum based general renal and tubular function markers and urinary based nepropathy specific selectivity indices (i.e. tubular and glomerular dysfunction specific markers) in different age groups in all the three study groups were summarized in **Figure 2.3.**

This analysis was done to check for predisposition of any occupation type to be prevalent at the highest range of values of each biomarker and nephropathy specific selectivity indices for suitable assessment of occupational risk groups prone to CKDu development (Jayatilake et al., 2013).

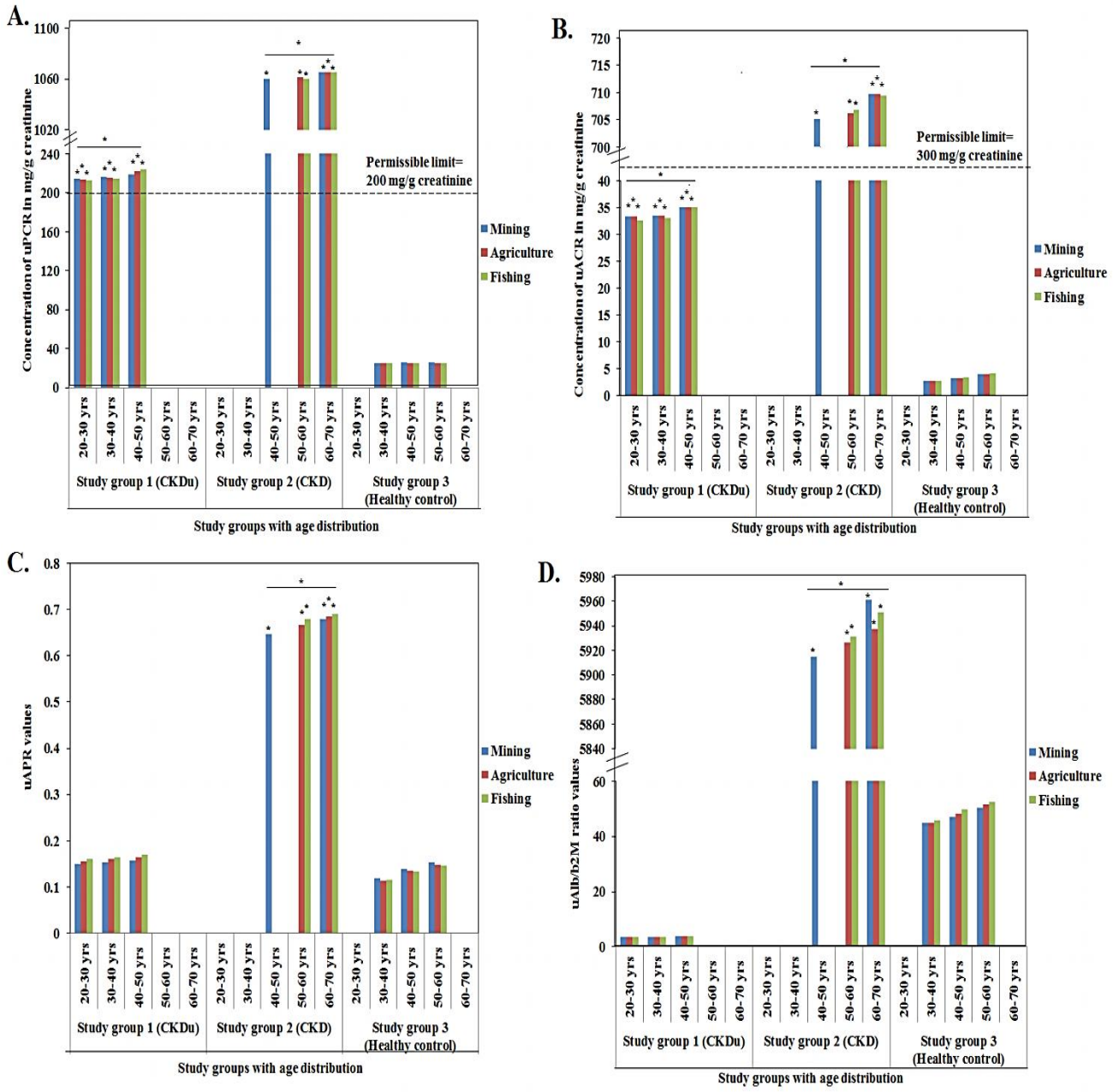
Figure 2.3. Occupation based stratification of the levels of serum based general renal and tubular function based biochemical markers and urinary based glomerular or tubular nephropathy specific selectivity indices of the entire study population for identification of the occupational risk-groups prone to CKDu development.

[I.] ***Occupation based stratification of the levels of serum based general renal and tubular function based biochemical markers***



Note:- Figure continued on the next page

[II.] ***Occupation based stratification of the levels of <u>urinary based glomerular or tubular nephropathy specific selectivity indices</u>***



Note:- Figure continued and figure legend on the next page

[II.] ***Occupation based stratification of the levels of <u>urinary based glomerular or tubular nephropathy specific selectivity indices</u>***

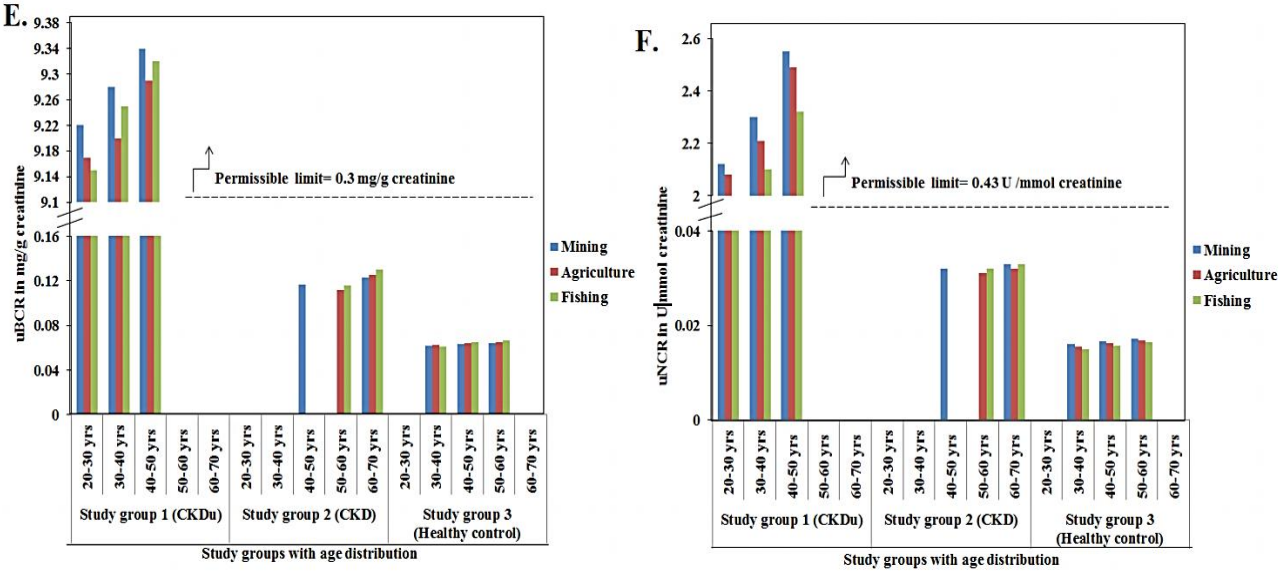


Figure 2.3:

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; b2M-β2-microglobulin; NAG-N-acetyl-β-D-glucosaminidase; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. Study group(cohort) 1, 2 & 3 consists of CKDu affected,diabetes/hypertension induced CKD affected & healthy controls subjects respectively. The total number of subjects in study groups 1,2 and 3 are 114,28 and 124 respectively.In this,the number of subjects involved in mining previously(M), agriculture(A) and fishing(F) occupations in study group one, two and three are 87(M)/11(A)/16(F); 4(M)/7(A)/17(F)and 7(M)/55(A)/62(F) respectively. The age based distribution(i.e. the number of subjects in each age category) among the mining(previously involved),agricultural and fishing communities in all three study groups are detailed in the affixed table (**Table 2.12)** of this figure.

[I.] ***Occupation based stratification of the levels of <u>serum based general renal and tubular function based biochemical markers</u>***

(A)Concentration of serum creatinine in various occupational groups under each age category in all three study groups (B)Concentration of serum urea in population sub-groups stratified by occupation and age in all three study cohorts (C)Concentration of serum uric acid in occupational and age stratified sub-groups of the three study cohorts' population (D) Concentration of serum bicarbonates in occupational and age stratified sub-groups of the three study cohorts' population (E) Concentration of serum phosphates in occupational and age stratified sub-groups of the three study cohorts' population.

[II.] ***Occupation based stratification of the levels of <u>urinary based glomerular or tubular nephropathy specific selectivity indices</u>***

(A)Concentration of uPCR in various occupational groups under each age category in all three study groups (B) Concentration of uACR in population sub-groups stratified by occupation and age in all three study cohorts (C) uAPR levels in occupational and age stratified sub-groups of the three study cohorts' population (D) uAlb/b2M ratio values in occupational and age stratified sub-groups of the three study cohorts' population (E) Concentration of uBCR in occupational and age stratified sub-groups of the three study cohorts' population (F) Concentration of uNCR in occupational and age stratified sub-groups of the three study cohorts' population

Values are represented as mean of the concentrations of serum based renal function biochemical marker(I) or urinary based nephropathy specific selectivity indices[II] estimated independently in subjects involved in different occupations (i.e.mining(previously),agriculture and fishing) belonging to various age groups (i.e. 20-30yrs,30-40yrs,40-50yrs,50-60yrs and 60-70yrs) in each of the three study groups. All concentration values of serum based general renal and tubular function markers[I] are in mg/dL except for serum bicarbonates which is depicted in mEq/L. All concentration values of urinary based nephropathy specific selectivity indices(or renal dysfunction specific biomarkers)[II] are in mg/g creatinine except for uNCR which is depicted in U/mmol creatinine. uAPR and uAlb/b2M being ratios are devoid of units.Differences in a particular study cohort at *$p<0.05$ were considered to be significant as compared to the other two cohorts (wherever applicable) and also relative to the lower age categories (in a given study group).No occupational bias in serum based tubular function markers'(viz.bicarbonates & phosphates) and urinary based tubular nephropathy specific markers'(viz.uBCR,uNCR) expression in study group 1 and glomerular injury markers' viz. uPCR, uACR**,** uAPR and uAlb/b2M expression in study group 2 were observed, which was visible from comparable levels of the respective markers being observed in all occupational groups (viz.mining,fishing and farming) in all study cohorts. Moreover, levels of tubular injury markers in study group 1 and glomerular nephropathy specific markers in study group 2 escalated with rising age with significant (*$p<0.05$) prevalence of their levels at higher ranges being noted in higher age groups (i.e.40-50yrs in study group 1 and 60-70yrs in study group 2). This heightened levels of tubular injury markers being noted at the higher age groups(i.e.40-50 yrs) in study group 1 was accredited to the bioaccumulative potencies of toxins that were reckoned responsible for CKDu causation which caused accumulation and aggravation of tubular damage effects with prolonged exposure (i.e.age),that was evident from the elevation in expression of tubular injury markers with increasing age. Moreover the increased levels of glomerular injury markers being observed at the higher age group(i.e.40-50 yrs) in study group 2 was ascribed to the inherent or natural degeneration cum compromise in the glomerular function with age(i.e.beyond 50yrs) which enhances the susceptibility of glomerulus to damage by major risk factors(like diabetes, hypertension), evident from increase in glomerular injury markers' concentrations with age. The details on the range of minimum to maximum values of the concentration of each biochemical marker cum nephropathy specific selectivity index & prevalence distribution(%) of these concentrations in various ranges for different occupational groups under individual age categories in all three study groups are listed in **Table 2.12**. For details on the levels of each biochemical parameter and selectivity index, refer Tables 2.7 and 2.8.

Table 2.12: Occupation based stratification of the prevalence of various serum based general renal and tubular function based biochemical markers and urinary based glomerular or tubular nephropathy specific selectivity indices of the entire study population for identification of the occupational risk-groups susceptible to CKDu development.

2.12 A. <u>Mining occupation</u> based prevalence distribution of:-

(i a) Serum based general renal function biochemical markers

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter								
MINING												
				Serum creatinine			Serum urea			Serum uric acid		
				mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)
Age 20-30 yrs	M	1	1	17.98	I=2.12%	A=0.45-0.5	178.87	G=2.12%	A=10-10.5	33.87	F=2.12%	A=1.0-2
		2		-		B=0.5-0.55			B=10.5-11			B=2.0-3.0
		3		-		C=0.55-0.6			C-11-11.5			C=17-18
	F	1	1	17.63	I=2.05%	D=0.6-0.65	177.36	G=2.05%	D=11.5-12	33.45	F=2.05%	D=18-19
		2		-		E=7-7.5			E=100-105			E=19-20
		3		-		F=7.5-8			F=105-110			F=33-34
	Total	1	2	17.8	I=2.08%	G=16.5-17	178.12	G=2.08%	G=175-180	33.66	F=2.08%	G=34-35
		2		-		H=17-17.5			H=180-185			H=35-36
		3		-		I=17.5-18						I=36-37
Age 30-40 yrs	M	1	19	18.49 (18.16-18.83)	J=8.51%; K=31.9%	J=18-18.5	179.59 (178.47-180.25)	G=34.04%; H=6.38%		34.93 (34.46-35.37)	G=34.04%; H=6.38%	
		2				K=18.5-19						
		3	2	0.52 (0.49-0.53)	A=25%; B=25%		10.88 (10.49-10.95)	A=25%; B=25%		1.91 (1.86-2.07)	A=25%; B=25%	
	F	1	16	18.37 (18.06-18.68)	J=10%; K=30%	L=19.5-20	178.09 (176.72-180.42)	G=35%; H=5%		34.61 (34.33-35.20)	G=35%; H=5%	
		2				M=19.5-20						
		3	1	0.49	A=23%		10.76	A=23%		1.99	A=23%	
	Total	1	35	18.41 (18.06-18.83)	J=9.25%; K=30.95%		178.84 (176.72-180.26)	G=34.52%; H=5.69%		34.77 (34.33-35.37)	G=34.52%; H=5.69%	
		2	-									
		3	3	0.50 (0.49-0.53)	A=24%; B=25%		10.81 (10.49-10.95)	A=24%; B=25%		1.96 (1.86-2.07)	A=24%; B=25%	
Age 40-50 yrs	M	1	27	19.95 (19.57-20.01)*	L=57.44%		184.01 (180.92-184.85)*	H=57.44%		36.95 (36.56-36.99)*	I=57.44%	
		2	1	7.16	E=50%		103.53	E=50%		17.81	C=50%	
		3	1	0.51	B=25%		10.91	B=25%		2.38	B=25%	
	F	1	23	19.67 (19.59-19.66)*	L=54.75%		183.01 (180.16-182.74)*	H=54.75%		36.66 (36.54-36.72)*	I=54.75%	
		2	1	7.06	E=50%		100.91	E=50%		17.58	C=50%	
		3	1	0.505	B=25%		10.67	B=25%		2.47	B=25%	
	Total	1	50	19.80 (19.57-20.01)*	L=56.09%		183.2 (180.16-184.85)*	H=56.09%		36.81 (36.54-36.99)*	I=56.09%	
		2	2	7.11	E=50%		101.96	E=50%		17.7	C=50%	
		3	2	0.5	B=25%		10.79	B=25%		2.43	B=25%	
Age 50-60 yrs	M	1	-									
		2	-									
		3	1	0.6125	D=25%		11.63	D=25%		2.59	B=25%	
	F	1	-									
		2	-									
		3	1	0.61	D=25%		11.29	C=25%		2.73	B=25%	
	Total	1	-									
		2	-									
		3	2	0.612	D=25%		11.39	C and D=25%		2.66	B=25%	
Age 60-70 yrs	M	1	-	-								
		2	1	7.91	F=50%		109.58	F=50%		19.17	E=50%	
		3										
	F	1										
		2	1	7.61	F=50%		108	F=50%		19.05	E=50%	
		3										
	Total	1	-									
		2	2	7.76	F=50%		108.78	F=50%		19.11	E=50%	
		3		-								

Please refer the table legend in the following pages i.e pg s.129-130

2.12 A. <u>Mining occupation</u> based prevalence distribution of:

(i b) Serum based tubular function biochemical markers

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter					
MINING									
				Serum bicarbonates			Serum phosphate		
				mean in mEq/L (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)
Age 20-30 yrs	M	1	1	8.39	C=2.12%	A=8.1-8.2	1.17	C=2.12%	A=0.9-1.0
		2	-	-	-	B=8.2-8.3	-	-	B=1.0-1.1
		3	-	-	-	C=8.3-8.4	-	-	C=1.1-1.2
	F	1	1	8.34	C=2.12%	D=24-25	1.14	C=2.12%	D=3.9-4.0
		2	-	-	-	E=25-26	-	-	E=4.0-4.1
		3	-	-	-	F=26-27	-	-	F=4.1-4.2
	Total	1	2	8.36	C=2.12%	G=27-28	1.13	C=2.12%	G=4.2-4.3
		2	-	-	-	H=28-29	-	-	H=4.3-4.4
		3	-	-	-	I=29-30	-	-	I=4.4-4.5
Age 30-40 yrs	M	1	19	8.29 (8.25-8.30)	B=40.42%		1.09 (1.06-1.10)	B=40.42%	J=4.5-4.6
		2	-	-	-		-	-	
		3	2	29.45 (28.89-30.15)	H=25%; I=25%		4.6 (4.58-4.63)	I=25%; J=25%	
	F	1	16	8.25 (8.23-8.28)	B=40%		1.05 (1.03-1.08)	B=40%	
		2	-	-	-		-	-	
		3	1	29.88	I=23%		4.55	J=23%	
	Total	1	35	8.27 (8.23-8.30)	B=40.21%		1.07 (1.03-1.10)	B=40.21%	
		2	-	-	-		-	-	
		3	3	29.66 (28.89-30.15)	H=25%; I=24%		4.58 (4.55-4.63)	I=25%; J=24%	
Age 40-50 yrs	M	1	27	8.19 (8.15-8.20)*	A=57.44%		0.985 (0.93-0.99)*	A=57.44%	
		2	1	26.78	F=48%		4.25	G=48%	
		3	1	28.49	H=25%		4.5	I=25%	
	F	1	23	8.14 (8.12-8.18)*	A=54.75%		0.944 (0.91-0.97)*	A=54.75%	
		2	1	26.14	F=48%		4.23	G=48%	
		3	1	28.74	H=25%		4.45	I=25%	
	Total	1	50	8.16 (8.33-8.40)*	A=56.09%		0.96 (0.1-0.99)*	A=56.09%	
		2	2	26.46	F=48%		4.24	G=48%	
		3	2	28.61	H=25%		4.48	I=25%	
Age 50-60 yrs	M	1	-	-	-		-	-	
		2	-	-	-		-	-	
		3	1	27.36	G=25%		4.42	H=25%	
	F	1	-	-	-		-	-	
		2	-	-	-		-	-	
		3	1	27.8	G=25%		4.38	H=25%	
	Total	1	-	-	-		-	-	
		2	-	-	-		-	-	
		3	2	27.58	G=25%		4.4	H=25%	
Age 60-70 yrs	M	1	-	-	-		-	-	
		2	1	25	D=50%		3.99	D=50%	
		3	-	-	-		-	-	
	F	1	-	-	-		-	-	
		2	1	24.3	D=50%		3.93	D=50%	
		3	-	-	-		-	-	
	Total	1	-	-	-		-	-	
		2	2	24.7	D=50%		3.96	D=50%	
		3	-	-	-		-	-	

Please refer the table legend in the following pages i.e pgs.129-130

2.12 A. <u>Mining occupation</u> based prevalence distribution of:

(ii a) Glomerular damage specific selectivity indices

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter											
MINING															
				uPCR			uACR			uAPR			uAlb/b2M ratio		
				mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels	mean (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels
Age 20-30 yrs	M	1	1	214.54	E=2.12%	A=24-25	33.96	E=2.12%	A=2-3.0	0.1585	F=2.12%	A=0.10-0.12	3.634	B=2.12%	A=3.57-3.59
		2	-	-	-	B=25-26			B=3-4.0			B=0.12-0.14			B=3.63-3.65
		3	-	-	-	C=26-27			C=4-5.0			C=0.14-0.16			C=3.65-3.67
	F	1	1	214.08	E=2.12%	D=212-214	32.68	D=2.12%	D=32-33	0.1535	D=2.12%	D=0.153-0.154	3.578	A=2.12%	D=3.87-3.89
		2	-	-	-	E-214-216			E=33-34			E=0.154-0.155			E=40-50
		3	-	-	-	F=216-218			F=36-37			F=0.158-0.159			F=50-60
	Total	1	2	214.31	E=2.08%	G=218-220	33.37	D and E=2.12%	G=702-705	0.156	D a nd F=2.12%	G=0.159-0.160	3.606	A and B=2.12%	G=60-70
		2	-	-	-	H=220-222			H=705-708			H=0.163-0.164			H=5860-5910
		3	-	-	-	I=1055-1060			I=708-711			I=0.660-0.662			I=5910-5960
Age 30-40 yrs	M	1	19	216.81 (216.63-217.92)	F=40.42%	J=1060-1065	34.00 (33.7-34.01)	E=40.42%	J=714-717	0.1587 (0.1585-0.1592)	D=34.04%; F=6.38%	J=0.662-0.664	3.642 (3.636-3.649)	B=40.42%	J=5960-6010
		2	-			K=1065-1070						K=0.664-0.666			
		3	2	25.42 (24.93-25.78)	A=25%; B=25%		2.84 (2.82-3.08)	A=25%; B=25%		0.122 (0.114-0.137)	A=25%; B=25%	L=0.670-0.672	45.87 (44.15-51.29)	E=25%; F=25%	
	F	1	16	216.26 (216.18-217.72)	F=40%		32.94 (32.70-33.01)	D=40%		0.1541 (1536-0.1542)	D=35%; F=5%		3.635 (3.634-3.642)	B=40%	
		2	-												
		3	1	24.63	A=23%		2.8	A=23%		0.117	A=23%		44.17	E=23%	
	Total	1	35	216.53 (216.18-217.92)	F=40.21%		33.47 (32.70-34.01)	D =40.%; E=40.42%		0.1564 (0.1536-0.1592)	D=34.52%; F=5.69%		3.6343 (3.634-3.649)	B=40.21%	
		2	-												
		3	3	25.03 (24.63-25.78)	A=24%; B=25%		2.82 (2.8-2.93)	A=24%; B=25%		0.119 (0.117-0.137)	A=24%; B=25%		45.02 (44.15-51.29)	E=24%; F=25%	
Age 40-50 yrs	M	1	27	219.14 (218.43-222.78)	G=55.31%; H=2.12%		36.16 (36.04-36.9)	F=57.44%		0.1596 (0.1593-0.1643)	G=55.31%; H=2.12%		3.673 (3.654-3.893)	C=55.31%; D=2.12%	
		2	1	1055.96	I=50%		708.15	I=50%		0.671	L=48%		5959.5	I=48%	
		3	1	25.52	B=25%		3.29	B=25%		0.141	B=25%		54.92	F=25%	
	F	1	23	218.64 (218.06-218.68)	G=54.75%		33.95 (33.17-34.01)	E=54.75%		0.1552 (0.1544-0.158)	G=52.95%; H=1.8%		3.661 (3.653-3.871)	C=52.95%; D=1.8%	
		2	1	1060.12	I=50%		702.24	G=50%		0.661	K=48%		5869.71	H=485	
		3	1	25.13	B=25%		3.14	B=25%		0.138	B=25%		51.16	F=25%	
	Total	1	50	219.19 (218.06-222.78)	G=55.03%; H=2.12%		35.06 (33.17-36.9)	E =54.75%; F=57.44%		0.1573 (0.1544-0.1643)	G=54.12%; H=1.96%		3.667 (3.653-3.893)	C=54.12%; D=1.96%	
		2	2	1060.04	I=50%		705.19	G and I=50%		0.667	K and L=48%		5914.6	H and I=48%	
		3	2	25.33	B=25%		3.22	B=25%		0.139	B=25%		53.04	F=25%	
Age 50-60 yrs	M	1	-												
		2	-												
		3	1	25.95	B=25%		4.22	C=25%		0.157	C=25%		64.49	G=25%	
	F	1	-												
		2	-												
		3	1	25.34	B=25%		3.92	B=25%		0.152	C=25%		62.39	G=25%	
	Total	1	-												
		2	-												
		3	2	25.64	B=25%		4.07	B and C=25%		0.154	C=25%		63.44	G=25%	
Age 60-70 yrs	M	1	-												
		2	1	1065.6*	K=50%		710.71*	I=50%		0.6733*	L=48.2%; M=1.8%		5960.9*	I=50%	
		3													
	F	1													
		2	1	1065.35*	K=50%		708.74*	I=50%		0.6651*	K=50%		5917.5*	I=50%	
		3													
	Total	1	-												
		2	2	1065.47*	K=50%		70.9.73*	I=50%		0.6691*	K=50%; L=48.2%; M=1.8%		5940.7*	I=50%	
		3													

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 A. Mining occupation based prevalence distribution of:

(ii b) Tubular nephropathy specific selectivity indices

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter					
MINING									
				uBCR			uNCR		
				mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in U/mmol (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in U/ mmol creatinine
Age 20-30 yrs	M	1	1	9.27	J=2.12%	A=0.0620-0.0625	2.31	J=2.12%	A=0.014-0.015
		2	-			B=0.0625-0.0630			B=0.015-0.016
		3	-			C=0.0630-0.0635			C=0.016-0.017
	F	1	1	9.18	I=2.12%	D=0.0635-0.0640	1.94	I=2.12%	D=0.017-0.018
		2	-			E=0.0640-0.0645			E=0.030-0.031
		3	-			F=0.10-0.11			F=0.031-0.032
	Total	1	2	9.22	I and J=2.12%	G=0.11-0.12	2.12	J and I=2.12%	G=0.032-0.033
		2	-			H=0.13-0.141			H=0.033-0.034
		3	-			I=9.12-9.22			I=1.-2
Age 30-40 yrs	M	1	19	9.33 (9.30-9.36)	J=40.42%	J=9.22-9.32	2.43 (2.33-2.49)	J=40.42%	J=2-3.0
		2	-			K=9.32-9.42			
		3	2	0.0641 (0.0640-0.0645)	D=25%; E=25%		0.0168 (0.01647-0.01704)	C=25%; D=25%	
	F	1	16	9.24 (9.22-9.25)	I=40%		2.09 (1.99-2.11)	J=40%	
		2	-						
		3	1	0.0628	B=23%		0.0153	B=23%	
	Total	1	35	9.28 (9.22-9.36)	I=40%; J=40.42%		2.26 (1.99-2.49)	J= 40.21%	
		2	-						
		3	3	0.0634 (0.0628-0.0645)	B-23%; D and E=25%		0.0160 (0.0153-0.0173)	B=23%; C and D=25%	
Age 40-50 yrs	M	1	27	9.42 (9.37-9.45)*	K=57.44%		2.89 (2.83-2.91)*	J=57.44%	
		2	1	0.123	G=50%		0.033	G=50%	
		3	1	0.064	D=25%		0.017	D=25%	
	F	1	23	9.27 (9.25-9.30)*	J=54.75%		2.21 (2.17-2.22)*	J=54.75%	
		2	1	0.111	F=50%		0.031	F=50%	
		3	1	0.0631	B=25%		0.0163	C=25%;	
	Total	1	50	9.34 (9.25-9.45)*	J=54.75%; K=57.44%		2.55 (2.17-2.91)*	J=56.069%	
		2	2	0.117			0.032	F and G=50%	
		3	2	0.0636	B and D=25%		0.0166	C and D=25%	
Age 50-60 yrs	M	1	-						
		2	-						
		3	1	0.0645	E=25%		0.0175	D=25%	
	F	1	-						
		2	-						
		3	1	0.0636	D=25%		0.0171	D=25%	
	Total	1	-						
		2	-						
		3	2	0.0642	C and D=25%		0.0173	D=25%	
Age 60-70 yrs	M	1	-						
		2	1	0.135	H=50%		0.0335	H=50%	
		3							
	F	1							
		2	1	0.112	G=50%		0.0332	H=50%	
		3							
	Total	1	-						
		2	2	0.1235	G and H=50%		0.0333	H=50%	
		3							

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 B. Farming occupation based prevalence distribution of:-

(i a) Serum based general renal function biochemical markers

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter								
FARMING												
				Serum creatinine			Serum urea			Serum uric acid		
				mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)
Age 20-30 yrs	M	1	1	17.97	I=20%	A=0.45-0.5	178.76	G=20%	A=10-10.5	33.83	F=20%	A=1.0-2
		2	-	-		B=0.5-0.55			B=10.5-11			B=2.0-3.0
		3	-	-		C=0.55-0.6			C-11-11.5			C=17-18
	F	1	1	17.62	I=22%	D=0.6-0.65	177.02	G=22%	D=11.5-12	33.41	F=22%	D=18-19
		2	-	-		E=7-7.5			E=100-105			E=19-20
		3	-	-		F=7.5-8			F=105-110			F=33-34
	Total	1	2	17.76	I=21%	G=16.5-17	177.89	G=21%	G=175-180	33.62	F=21%	G=34-35
		2	-	-		H=17-17.5			H=180-185			H=35-36
		3	-	-		I=17.5-18						I=36-37
Age 30-40 yrs	M	1	2	18.45	J=40%	J=18-18.5	179.55 (178.43-180.22)	G=38.2%; H=1.8%		3.4.88 (34.41-35.32)	G=39%; H=1%	
		2	-			K=18.5-19						
		3	10	0.48 (0.46-0.52)	A=31.3%; B=2.0%		10.83 (10.47-10.9)	A=30.3%; B=3.0%		1.86 (1.81-2.01)	A=29.3%; B=4.0%	
	F	1	2	18.32 (18.0-18.67)	J=42%	L=19.5-20	178.04 (176.68-180.11)	G=40.6%; H=1.4%		34.56 (34.28-35.16)	G=40%; H=2%	
		2	-			M=19.5-20						
		3	11	0.45(0.44-0.51)	A=35.4%; B=1.2%		10.71 (10.33-10.86)	A=34.4%; B=22%		1.94 (1.87-2.13)	A=33.6%; B-3.0%	
	Total	1	4	18.33 (18.0-18.78)	J=41%		178.79 (176.68-180.22)	G=39.4%; H=1.6%		34.72 (34.28-35.32)	G=39.5%; H=1.5%	
		2	-									
		3	21	0.47 (0.46-0.52)	A=33.5%; B=1.6%		10.77 (10.33-10.90)	A=32.5%; B=2.6%		1.90 (1.81-2.13)	A=31.45%; B=3.5%	
Age40-50 yrs	M	1	3	19.90 (19.52-20.0)*	L=60%		181.94 (178.1-182.81)*	H=60%		36.89 (36.50-36.94)*	I=60%	
		2	-	-	-		103	-		-	-	
		3	14	0.485 (0.48-0.56)	A=45.0%; B=3.6%		10.88 (10.64-10.90)	B=48.6%		2.33 (1.89-2.39)	A=46.6%; B-2.0%	
	F	1	2	19.64 (19.52-19.63)*	L=58.2%		180.35 (178.13-180.70)*	H=58.2%		36.60 (36.48-36.67)*	I=58.2%	
		2	-	-	-		100.31	-		-	-	
		3	14	0.47 (0.46-0.53)	A=47.5%; B=2.5%		10.62 (10.50-10.84)	B=50%		2.41 (1.92-2.47)	A=48%;B-2.0%	
	Total	1	5	19.76 (19.52-20.0)*	L=59.1%		181.12 (178.13-18.2.81)*	H=59.1%		36.75 (36.48-36.94)*	I=59.71%	
		2	-	-	-		102.65	-		-	-	
		3	28	0.478 (0.46-0.56)	A=46.35%; B=3.1%		10.75 (10.50-10.90)	B=49.3%		2.37 (1.89-2.47)	A=47.3%; B=2%	
Age 50-60 yrs	M	1	-	-				-				
		2	2	7.33 (7.22-7.47)	E=22.2%		104.88 (103.58-104.90)	E=22.2%		18.20 (17.85-18.45)	C=19.2%; D=3.0%	
		3	3	0.59 (0.54-0.60)	B=8.5; C=2.2%		11.58 (11.50-11.62)	D=10.7%		2.55 (2.50-2.57)	B=10.7%	
	F	1	-									
		2	1	7.26 (7.10-7.38)	E=21.1%		103.95 (102.15-104.84)	E=40%		18.09 (17.68-18.38)	C=18.6%; D=2.5%	
		3	3	0.56 (0.52-0.58)	B=8.1%; C=1.9%		11.24 (11.04-11.27)	C=10%		2.68 (2.55-2.71)	B=10%	
	Total	1	-							-		
		2	3	7.30 (7.10-7.47)	E=21.65%		104.42 (102.15-104.90)	E=21.65%		18.15 (17.85-18.45)	C=18.9%; D=2.75%	
		3	6	0.58 (0.52-0.60)	B=8.3%; C=2.1%		11.34 (11.04-11.62)	C=10.7%; D=10%		2.61 (2.50-2.71)	B=10.35%	
Age 60-70 yrs	M	1	-	-				-		-		
		2	2	7.88 (7.60-7.92)	F=25%		109.53 (107.78-109.68)	F=25%		19.11 (18.26-19.53)	D=2.5%; E=22.5%	
		3	-							-		
	F	1	-							-		
		2	2	7.58 (7.50-7.68)	F=22%		107.95 (106.70-108.89)	F=22%		18.99 (18.13-19.39)	D=2%; E=20%	
		3								-		
	Total	1	-							-		
		2	4	7.73 (7.50-7.92)	F=23.5%		108.74 (106.70-109.68)	F=23.5%		19.07 (18.13-19.53)	D=2.25%; E=21.25%	
		3	-	-						-		

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 B. Farming occupation based prevalence distribution of:

(i b) Serum based tubular function biochemical markers

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter					
FARMING									
				Serum bicarbonates			Serum phosphate		
				mean in mEq/L (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)
Age 20-30 yrs	M	1	1	8.36	C=20%	A=8.1-8.2	1.15	C=20%	A=0.9-1.0
		2	-	-	-	B=8.2-8.3	-	-	B=1.0-1.1
		3	-	-	-	C=8.3-8.4	-	-	C=1.1-1.2
	F	1	1	8.31	C=20%	D=24-25	1.12	C=20%	D=3.9-4.0
		2	-			E=25-26			E=4.0-4.1
		3	-			F=26-27			F=4.1-4.2
	Total	1	2	8.33	C=20%	G=27-28	1.135	C=20%	G=4.2-4.3
		2	-			H=28-29			H=4.3-4.4
		3	-			I=29-30			I=4.4-4.5
Age 30-40 yrs	M	1	2	8.25 (8.21-8.27)	B=40%		1.05 (1.02-1.08)	B=40%	J=4.5-4.6
		2	-						
		3	10	29.40 (29.22-29.76)	I=33.3%		4.58 (4.52-4.60)	J=33.3%	
	F	1	2	8.21 (8.20-8.24)	B=40%		1.03 (1.0-1.06)	B=40%	
		2	-						
		3	11	29.83 (29.43-30.0)	I=33.3%		4.52 (4.50-4.55)	J=36.6%	
	Total	1	4	8.23 (8.20-8.27)	B=40%		1.05 (1.0-1.10)	B=40%	
		2	-						
		3	21	29.61 (29.22-30.0)	I=33.3%		4.55 (4.50-4.60)	J=34.9%	
Age 40-50 yrs	M	1	3	8.15 (8.12-8.18)*	A=60%		0.978 (0.92-0.99)*	A=60%	
		2	-	-	-		-	-	
		3	14	28.45 (28.26-29.17)	H=46%; I=2%		4.47 (4.43-4.56)	I=47.0%; J=1.6%	
	F	1	2	8.1 (8.1-8.15)*	A=58.2%		0.938 (0.91-0.96)*	A=60%	
		2	-	-	-		-	-	
		3	14	26.70 (28.49-29.23)	H=48%; I=2%		4.41 (4.4-4.48)	I=50%	
	Total	1	5	8.13 (8.1-8.18)*	A=59.71%		0.958 (0.91-0.96)*	A=60%	
		2	-	-	-		-	-	
		3	28	28.57 (28.26-29.23)	H=47%; I=2%		4.44 (4.40-4.56)	I=48.5%; J=1.6%	
Age 50-60 yrs	M	1	-		-				
		2	2	26.73 (25.31-26.90)	E=2.5%; F=37.5%		4.21 (4.17-4.26)	F=38.1%; G=1.9%	
		3	3	27.32 (27.05-28.11)	G=8.6%; H=1.4%		4.38 (4.35-4.44)	H=7.9%; I=2.1%	
	F	1	-						
		2	1	26.09	E=2.5%; F=37.5%		4.19	F=40%	
		3	3	27.76 (27.63-28.34)	G=8.9%; H=1.1%		4.34 (4.32-4.40)	H-8.2%; I=1.8%	
	Total	1	-						
		2	3	26.41 (25.31-26.90)	E=2.5%; F=37.5%		4.20 (4.17-4.26)	F=39.05%; G=1.9%	
		3	6	27.54 (27.05-28.34)	G=8.6%; H=1.4%		4.36 (4.32-4.44)	H=8.1%; I=1.9%	
Age 60-70 yrs	M	1	-				-		
		2	2	25.70 (24.89-25.92)	D=2.5%; E=22.5%		4.07 (4.02-4.13)	E=1.5%; F=23.5%	
		3	-						
	F	1	-						
		2	2	25.22 (24.60-25.47)	D=2.0%; E=23%		4.03 (4.0-4.10)	E=2.0%; F=22.5%	
		3							
	Total	1	-						
		2	4	25.46 (24.60-25.92)	D=2.24%; E=23.75%		4.05 (4.0-4.13)	E=1.75%; F=23%	
		3	-						

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 B. Farming occupation based prevalence distribution of:

(ii a) Glomerular damage specific selectivity indices

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter											
FARMING															
				uPCR			uACR			uAPR			uAlb/b2M ratio		
				mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels (in mg/g creatinine)	mean (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels	mean (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels
Age 20-30 yrs	M	1	1	212.49	D=20%	A=24-25	33.92	E=20%	A=2-3.0	0.1581	F=20%	A=0.10-0.12	3.63	B=20%	A=3.57-3.59
		2	-			B=25-26			B=3-4.0	-		B=0.12-0.14		-	B=3.63-3.65
		3	-			C=26-27			C=4-5.0	-		C=0.14-0.16		-	C=3.65-3.67
	F	1	1	211.01	D=20%; E=2%	D=212-214	32.64	D=22%	D=32-33	0.1531	D=20%	D=0.153-0.154	3.571	A=20%	D=3.87-3.89
		2	-			E-214-216			E=33-34			E=0.154-0.155			E=40-50
		3	-			F=216-218			F=36-37			F=0.158-0.159			F=50-60
	Total	1	2	213.75	D-20%; E-2%	G=218-220	33.32	D=20%; E=22%	G=702-705	0.1556	D and F=20%	G=0.159-0.160	3.602	A and B=20%	G=60-70
		2	-			H=220-222			H=705-708			H=0.163-0.164			H=5860-5910
		3	-			I=1055-1060			I=708-711			I=0.660-0.662			I=5910-5960
Age 30-40 yrs	M	1	2	216.74 (216.55-217.84)	F=40%	J=1060-1065	33.96 (33.91-33.98)	E=40%	J=714-717	0.1583 (0.1581-0.1588)	F=40%	J=0.662-0.664	3.634 (3.631-3.644)	B=40%	J=5960-6010
		2	-			K=1065-1070						K=0.664-0.666			
		3	10	25.35 (25.23-25.72)	B=33.3%		2.78 (2.76-2.87)	A=33.3%		0.116 (0.109-0.132)	A=30.3%; B=3.0%	L=0.670-0.672	45.82 (44.11-51.24)	E=31.3%; F=2.0%	
	F	1	2	216.19 (216.11-217.65)	F=42%		32.87 (32.65-32.97)	D=40%		0.1537 (0.1531-0.1538)	D=40%		3.630 (3.630-3.637)	B=40%	
		2	-							-					
		3	11	24.55 (24.47-24.89)	A=3.6%.; B=30.0%		2.74 (2.71-2.80)	A=36.6%		0.112 (0.108-0.130)	A=34.6%; B=2.0		44.11 (43.88-50.26)	E=33.3%; F=3.3%.	
	Total	1	4	216.46 (216.11-217.84)	F=41%		33.42 (32.65-33.98)	D and E=40%		0.1560 (0.1531-0.1588)	D and F=40%		3.633 (3.630-3.644)	B=40%	
		2	-												
		3	21	24.95 (24.47-25.72)	A=3.6%; B=32.5%		2.76 (2.71-2.87)	A=34.9%		0.114 (0.108-0.132)	A=32.45%; B=2.5%		44.96 (43.88-51.24)	E=32.3%; F=2.65%	
Age 40-50 yrs	M	1	3	219.08 (218.35-222.71)	G=57.5%; H=2.5%		36.12 (36.0-36.59)	F=60%		0.1592 (0.1590-0.163)	G=59%; H=1%		3.669 (3.650-3.889)	C=58.31%; D=1.7%	
		2	-	-	-		-	-		-	-		-	-	
		3	14	25.44 (25.30-25.87)	B=48.6%		3.23 (3.21-3.87)	B=48.6%		0.137 (0.126-0.152)	B=40.6%; C=8%		54.88 (52.77-69.31)	F=46.6%; G=2.0%	
	F	1	2	218.58 (21.0-219.62)	G=58.2%		33.90 (33.12-33.96)	E-58.2%		0.1548 (0.1540-0.1549)	E=58.2%		3.657 (3.649-3.659)	C=56.6%; D=1.8%	
		2	-	-	-		-	-		-	-		-	-	
		3	14	25.06 (25.0-25.11)	B=50%		3.09 (3.03-3.66)	B=50%		0.134 (0.122-0.144)	B=42%; C=8%		51.12 (50.13-67.57)	F=47.4%; G=2.6%	
	Total	1	5	219.13 (218.0-222.71)	G=57.85%; H=2.5%		35.01 (33.12-36.5)	E–58.2%; F=60%		0.1569 (0.1540-0.163)	E=58.2%; G=59%; H=1%		3.663 (3.649-3.889)	C=57.45%; D=1.75%	
		2	-	-	-		-	-		-	-		-	-	
		3	28	25.25 (25.0-25.87)	B=49.3%		3.16 (3.03-3.87)	B=49.3%		0.135 (0.122-0.152)	B=41.3%; C=8%		53.0 (50.13-69.31)	F=47%; G=2.3%	
Age 50-60 yrs	M	1	-	-											
		2	2	1061.35 (1061.06-1064.37)	J-40%		708.52 (708.22-709.08)	I=40%		0.06717 (0.670-0.06719)	L=40%		5958.14 (5957.29-5959.9)	I=40%	
		3	3	25.88 (25.46-26.60)	B=9.7%; C=1.0%		4.17 (4.03-4.43)	C=10%		0.152 (0.150-0.159)	C=10%		64.45 (63.49-68.27)	G=10%	
	F	1	-												
		2	1	1061.17 (1060.0-1064.14)	J-40%		705.30 (705.10-707.35)	H=40%		0.6628 (0.6620-0.6638)	J=40%		5895.19 (5868.19-5909.29)	H=40%	
		3	3	25.27 (25.08-25.62)	B=10%		3.88 (3.55-3.98)	B=10%		0.147 (0.141-0.149)	C=10%		62.35 (661.11-63.12)	G=10%	
	Total	1	-												
		2	3	1061.26 (1061.0-1064.37)	J-40%		706.91 (705.10-709.08)	H and I=40%		0.06672 (0.06619-0.6719)	L and J=40%		5926.66 (5868.19-5959.9)	H and I=40%	
		3	6	25.57 (25.08-26.62)	B=9.85%; C=1%		4.03 (3.58-4.43)	B and C=10%		0.149 (0.141-0.159)	C=10%		63.40 (61.11-68.25)	G=10%	
Age 60-70 yrs	M	1	-												
		2	2	1065.53 (1065.11-1067.40)*	K=25%		710.66 (710.15-718.42)*	I=22.5%; J=2.5%		0.6728 (0.6721-0.6729)*	M=25%		5960.89 (5958.12-5967.39)*	I=23%; J=2%	
		3	-												
	F	1	-												
		2	2	1065.28 (1065.07-1067.03)*	K=22%		708.68 (705.22-709.73)*	H=1.8%; I=20.2%		0.6647 (0.6639-0.6649)*	J=2.0%; K=20%		5914.52 (5903.59-5917.12)*	H=2%; I=23%	
		3													
	Total	1	-												
		2	4	1065.41 (1065.07-1067.40)*	K=23.5%		709.67 (705.22-718.42)*	H=1.8%; I=21.65%; J=2.5%		0.6687 (0.6639-0.6729)*	J=2.0%; K=20%; M=25%		5937.09 (5903.59-5967.39)*	H=2%; I=23%; J=2%	
		3	-												

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 B. <u>Farming occupation</u> based prevalence distribution of:

(ii b) Tubular nephropathy specific selectivity indices

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter					
FARMING									
				uBCR			uNCR		
				mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in U/mmol (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in U/ mmol creatinine)
Age 20-30 yrs	M	1	1	9.22	I=20%	A=0.0620-0.0625	2.27	J=20%	A=0.014-0.015
		2	-		-	B=0.0625-0.0630	-	-	B=0.015-0.016
		3	-		-	C=0.0630-0.0635	-	-	C=0.016-0.017
	F	1	1	9.13	I=20%	D=0.0635-0.0640	1.9	I=20%	D=0.017-0.018
		2	-			E=0.0640-0.0645			E=0.030-0.031
		3	-			F=0.10-0.11			F=0.031-0.032
	Total	1	2	9.17	I=20%	G=0.11-0.12	2.08	I and J=20%	G=0.032-0.033
		2	-		-	H=0.13-0.141			H=0.033-0.034
		3	-		-	I=9.12-9.22			I=1.-2
Age 30-40 yrs	M	1	2	9.27 (9.23-9.29)	J=40%	J=9.22-9.32	2.38 (2.28-2.45)	J=40%	J=2-3.0
		2	-			K=9.32-9.42			
		3	10	0.0635 (0.0634-0.06385)	C=30.3%; D=3.0%		0.0164 (0.0163-0.0170)	C=33.3%	
	F	1	2	9.18 (9.16-9.19)	I=40%		2.04 (1.94-2.06)	I=1.8%; J=38.2%	
		2	-						
		3	11	0.0622 (0.0620-0.0624)	A=36.6%		0.0149 (0.0146-0.0152)	A=4.6%; B=32.0%	
	Total	1	4	9.20 (9.16-9.29)	I and J=40%		2.21 (1.94-2.45)	I=1.8%; J=39.1%	
		2	-						
		3	21	0.06289 (0.0620-0.06385)	A=36.6%; C=30.3%; D=3.0%		0.0156 (0.0146-0.0169)	A=2.6%; B=32%; C=33.3%	
Age 40-50 yrs	M	1	3	9.37 (9.32-9.40)*	K=60%		2.85 (2.79-2.87)*	J=60%	
		2	-	-	-		-	-	
		3	14	0.0637 (0.0636-0.0640)	D=48.6%		0.0166 (0.0165-0.0171)	C=46.6%; D=2.0%	
	F	1	2	9.22 (9.20-9.25)*	I=1.8%; J=56.4%;		2.16 (2.13-2.18)*	J=58.2%	
		2	-	-	-		-	-	
		3	14	0.0626 (0.0624-0.0630)	A=2.5%; B=47.5%		0.159 (0.152-0.161)	B=2.6%; C=47.4%	
	Total	1	5	9.29 (9.20-9.40)*	I=1.8%; J=56.4%; K=60%		2.49 (2.13-2.87)*	J=59.71%	
		2	-	-			-	-	
		3	28	0.0631 (0.0624-0.06407)	A=2.7%; B=47.5%; D=48.6%		0.162 (0.152-0.171)	B=2.6%; C=46%; D=2%	
Age 50-60 yrs	M	1	-						
		2	2	0.1184 (0.1181-0.1198)	G=40%		0.0328 (0.0320-0.0337)	G=39%; H=2%	
		3	3	0.06395 (0.0639-0.0640)	D=10%		0.0172 (0.0171-0.0175)	D=10%	
	F	1	-						
		2	1	0.1070 (0.1062-0.1096)	F=40%		0.0306 (0.0300-0.0318)	E=3%; F=37%	
		3	3	0.0630 (0.029-0.0631)	C=10%		0.0167 (0.0165-0.0169)	C=10%	
	Total	1	-						
		2	3	0.1127 (0.1062-0.1198)	F and G=40%		0.0317 (0.0300-0.0337)	E=3%; F=37%; G=39%; H=2%	
		3	6	0.0635 (0.0629-0.0640)	C and D=10%		0.0169 (0.0165-0.0175)	C and D=10%	
Age 60-70 yrs	M	1	-						
		2	2	0.1344 (0.1190-0.1409)	G=23.5%; H=1.5%		0.0329 (0.0324-0.0338)	G=22.5%; H=2.5%	
		3	-						
	F	1	-						
		2	2	0.114 (0.1092-0.1179)	F=2.0%; G=20%		0.0326 (0.0316-0.0328)	F=2.0%; G=20.0%	
		3							
	Total	1	-						
		2	4	0.1229 (0.1092-0.1409)	F=2.0%; G=21.75%; H=1.5%		0.0327 (000316-0.0338)	F=2.0%; G=21.75%; H=2.5%	
		3	-						

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 C. <u>Fishing occupation</u> based prevalence distribution of:-

(i a) Serum based general renal function biochemical markers

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter								
FISHING												
				Serum creatinine			Serum urea			Serum uric acid		
				mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)
Age 20-30 yrs	M	1	1	17.95	I=20%	A=0.45-0.5	178.67	G=20%	A=10-10.5	33.81	F=20%	A=1.0-2
		2	-	-		B=0.5-0.55			B=10.5-11			B=2.0-3.0
		3	-	-		C=0.55-0.6			C-11-11.5			C=17-18
	F	1	1	17.6	I=15%	D=0.6-0.65	176.7	G=15%	D=11.5-12	33.37	F=20%	D=18-19
		2	-	-		E=7-7.5			E=100-105			E=19-20
		3	-	-		F=7.5-8			F=105-110			F=33-34
	Total	1	2	17.77	I=17.5%	G=16.5-17	177.49	G=17.5%	G=175-180	33.63	F=20%	G=34-35
		2	-	-		H=17-17.5			H=180-185			H=35-36
		3	-	-		I=17.5-18						I=36-37
Age 30-40 yrs	M	1	1	18.43	J=20%	J=18-18.5	179.53	G=20%		34.85	G=20%	
		2	-			K=18.5-19						
		3	14	0.46 (0.44-0.50)	A=45%		10.8 (10.45-10.88)	A=25%; B=20%		1.85 (1.80-2.0)	A=45%	
	F	1	4	18.3 (17.98-18.65)	J=26%	L=19.5-20	178.01 (176.66-180.09)	G=26%		34.54 (34.25-35.13)	G=26%	
		2	-			M=19.5-20						
		3	14	0.43 (0.42-0.49)	A=45%		10.76 (10.30-10.83)	A=25%; B=20%		1.93 (1.86-2.12)	A=30%; B=15%	
	Total	1	5	18.31 (17.98-18.65)	J=23%		178.77 (176.66-180.09)	G=23%		34.69 (34.25-35.13)	G=23%	
		2	-									
		3	28	0.45 (0.44-0.50)	A=45%		10.75 (10.31-10.88)	A=25%; B=20%		1.89 (1.80-2.12)	A=37.5%; B=15%	
Age 40-50 yrs	M	1	3	19.89 (19.50-19.99)*	L=60%		182.94 (180.1-183.81)*	H=60%		36.88 (36.49-36.93)*	I=60%	
		2	-	-	-		103	-		-	-	
		3	11	0.465 (0.46-0.54)	A=25%; B=9.6%		10.86 (10.62-10.88)	B=34%		2.31 (1.87-2.37)	A=25%; B=9.6%	
	F	1	6	19.64 (19.52-19.63)*	L=57.45%		181.35 (179.13-181.70)*	H=57.45%		36.58 (36.46-36.65)*	I=57.45%	
		2	-	-	-		100.31	-		-	-	
		3	11	0.45 (0.44-0.51)	A=25%; B=9.6%		10.60 (10.50-10.82)	B=34%		2.39 (1.90-2.45)	A=25%; B=9.6%	
	Total	1	9	19.74 (19.50-19.99)*	L=58.73%		182.15 (180.1-183.81)*	H=58.73%		36.74 (36.46-36.93)*	I=58.73%	
		2	-	-	-		102.65	-		-	-	
		3	22	0.468 (0.44-0.54)	A=25%; B=9.6%		10.73 (10.50-10.88)	B=34%		2.35 (1.87-2.45)	A=25%; B=9.6%	
Age 50-60 yrs	M	1	-	-				-				
		2	4	7.31 (7.20-7.45)	E=40%		104.85 (103.55-104.57)	E=40%		18.18 (17.83-18.43)	D=40%	
		3	6	0.57 (0.52-0.58)	B=12.9%; C=7.1%		11.55 (11.47-11.59)	C=15.9%; D=4.1%		2.53(2.48-2.55)	B=19%	
	F	1	-									
		2	4	7.24 (7.08-7.36)	E=40%		103.92 (102.14-104.81)	E=40%		18.07 (17.66-18.36)	D=40%	
		3	6	0.54 (0.50-0.56)	B=12.9%; C=7.1%		11.21 (11.01-11.24)	C=19%		2.65 (2.53-2.69)	B=19%	
	Total	1	-							-		
		2	8	7.28 (7.08-7.45)	E=40%		104.39 (102.12-104.87)	E=40%		18.13 (17.66-18.43)	D=40%	
		3	12	0.57 (0.50-0.58)	B=12.9%; C=7.1%		11.35 (11.01-11.59)	C=17.45; D=4.1%		2.59 (2.48-2.69)	B=19%	
Age 60-70 yrs	M	1	-	-				-		-		
		2	5	7.86 (7.58-7.90)	F=52.5%		109.50 (107.75-109.65)	F=52.5%		19.09 (18.25-19.52)	D=35.3%; E=17.2%	
		3	-							-		
	F	1	-							-		
		2	4	7.56 (7.48-7.66)	F=50%		107.92 (106.67-108.86)	F=50%		18.97 (18.12-19.38)	D=34.1%; E=15.9%	
		3								-		
	Total	1	-							-		
		2	9	7.70 (7.48-7.90)	F=51.25%		108.71 (106.67-109.65)	F=51.25%		19.05 (18.12-19.52)	D=34.7%; E=16.5%	
		3	-	-						-		

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 C. <u>Fishing occupation</u> based prevalence distribution of:

(i b) Serum based tubular function biochemical markers

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter					
FISHING									
				Serum bicarbonates			Serum phosphate		
				mean in mEq/L (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)	mean in mg/dL (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/dL)
Age 20-30 yrs	M	1	1	8.34	C=20%	A=8.1-8.2	1.12	C=20%	A=0.9-1.0
		2	-	-	-	B=8.2-8.3	-	-	B=1.0-1.1
		3	-	-	-	C=8.3-8.4	-	-	C=1.1-1.2
	F	1	1	8.3	C=20%	D=24-25	1.105	C=20%	D=3.9-4.0
		2	-	-	-	E=25-26	-	-	E=4.0-4.1
		3	-	-	-	F=26-27	-	-	F=4.1-4.2
	Total	1	2	8.31	C=20%	G=27-28	1.112	C=20%	G=4.2-4.3
		2	-	-	-	H=28-29	-	-	H=4.3-4.4
		3	-	-	-	I=29-30	-	-	I=4.4-4.5
Age 30-40 yrs	M	1	1	8.24	B=20%		1.03	B=20%	J=4.5-4.6
		2	-	-	-		-	-	
		3	14	29.38 (29.20-29.74)	I=45%		4.56 (4.50-4.58)	J=45%	
	F	1	4	8.20 (8.18-8.23)	B-26%		1.01 (1.00-1.03)	B=26%	
		2	-	-	-		-	-	
		3	14	29.81 (29.41-29.98)	I=45%		4.51 (4.48-4.53)	J=42.8%; I=2.2%	
	Total	1	5	8.22 (8.18-8.24)	B=23%		1.03 (1.00-1.07)	B=23%	
		2	-	-	-		-	-	
		3	28	29.59 (29.20-29.98)	I=45%		4.53 (4.51-4.58)	J=43.9%; I=2.2%	
Age 40-50 yrs	M	1	3	8.14 (8.12-8.18)*	A=60%		0.976 (0.95-0.99)*	A=60%	
		2	-	-	-		-	-	
		3	11	28.43 (28.24-29.15)	H=31.9%; I=2.1%		4.45 (4.41-4.54)	I=30.6%; J=3.8%	
	F	1	6	8.12 (8.10-8.15)*	A=57.45%		0.936 (0.91-0.96)*	A=57.45%	
		2	-	-	-		-	-	
		3	11	28.68 (28.47-29.21)	H=31.9%; I=2.1%		4.41 (4.4-4.48)	I=34%	
	Total	1	9	8.13 (8.10-8.18)*	A=58.73%		0.957 (0.91-0.99)*	A=58.73%	
		2	-	-	-		-	-	
		3	22	28.55 (28.24-29.21)	H=31.9%; I=2.1%		4.42 (4.38-4.54)	I=32.3%; J=3.8%	
Age 50-60 yrs	M	1	-	-	-		-	-	
		2	4	26.71 (25.29-26.88)	F=37.4%; E=2.6%		4.19 (4.15-4.24)	F=38.9%; G=1.1%	
		3	6	27.30 (27.03-28.09)	G=17.9%; H=1.1%		4.35 (4.32-4.40)	H=19%	
	F	1	-	-	-		-	-	
		2	4	26.08 (25.09-26.38)	F=36.1%; E=3.9%		4.17 (4.10-4.21)	F=37.1%: G=2.9%	
		3	6	27.74 (27.61-28.32)	G=17.2%; H=1.8%		4.31 (4.30-4.37)	H=19%	
	Total	1	-	-	-		-	-	
		2	8	26.39 (25.09-26.88)	F=36.75%; E=3%		4.18 (4.10-4.24)	F=38%; G=2%	
		3	12	27.52 (27.03-28.32)	G=17.6%; H=1.5%		4.36 (4.30-4.40)	H=19%	
Age 60-70 yrs	M	1	-	-	-		-	-	
		2	5	25.68 (24.87-25.90)	E=45.7%; D=6.8%		4.05 (4.0-4.11)	E=50.9%; F=1.6%	
		3	-	-	-		-	-	
	F	1	-	-	-		-	-	
		2	4	25.20 (24.58-25.45	E=44.2%; D=5.8%		4.01 (4.0-4.11)	E=48.7%; F=1.3%	
		3		-	-		-	-	
	Total	1	-	-	-		-	-	
		2	9	25.44 (24.58-25.90)	E=44.95%; D=6.3%		4.03 (4.0-4.11)	E=49.8%; F=1.45%	
		3	-	-	-		-	-	

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 C. Fishing occupation based prevalence distribution of:

(ii a) Glomerular damage specific selectivity indices

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter											
FISHING															
				uPCR			uACR			uAPR			uAlb/b2M ratio		
				mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels (in mg/g creatinine)	mean (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels	mean (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annotation for the ranges of the biomarker levels
Age 20-30 yrs	M	1	1	213.49	E=20%	A=24-25	33.9	E=20%	A=2-3.0	0.158	F=20%	A=0.10-0.12	3.63	B=20%	A=3.57-3.59
		2	-	-	-	B=25-26	-	-	B=3-4.0	-	-	B=0.12-0.14	-	-	B=3.63-3.65
		3	-	-	-	C=26-27	-	-	C=4-5.0	-	-	C=0.14-0.16	-	-	C=3.65-3.67
	F	1	1	212.01	E=20%	D=212-214	32.63	D=20%	D=32-33	0.1532	D=20%	D=0.153-0.154	3.572	A=20%	D=3.87-3.89
		2	-	-	-	E-214-216	-	-	E=33-34	-	-	E=0.154-0.155	-	-	E=40-50
		3	-	-	-	F=216-218	-	-	F=36-37	-	-	F=0.158-0.159	-	-	F=50-60
	Total	1	2	212.75	E=20%	G=218-220	33.3	D and E=20%	G=702-705	0.1557	D and F=20%	G=0.159-0.160	3.6	A and B=20%	G=60-70
		2	-	-	-	H=220-222	-	-	H=705-708	-	-	H=0.163-0.164	-	-	H=5860-5910
		3	-	-	-	I=1055-1060	-	-	I=708-711	-	-	I=0.660-0.662	-	-	I=5910-5960
Age 30-40 yrs	M	1	1	216.71 (216.52-217.81)	F=20%	J=1060-1065	33.95	E=20%	J=714-717	0.1584	F=20%	J=0.662-0.664	3.635	B=20%	J=5960-6010
		2	-	-	-	K=1065-1070	-	-		-	-	K=0.664-0.666	-	-	
		3	14	25.32 (25.20-25.69)	B=45%		2.76 (2.74-2.85)	A=42.5%; B=2.5%		0.115 (0.107-0.130)	A=40.8%; B=4.2%	L=0.670-0.672	45.80 (44.09-50.04)	E=45%	
	F	1	4	216.17 (216.08-217.62)	F=26%		32.85 (32.63-32.94)	D=26%		0.1535 (0.1529-0.1536)	D=26%		3.63 (3.627-3.635)	B=26%	
		2	-	-	-		-	-		-	-		-	-	
		3	14	24.52 (24.44-24.86)	A=15%; B=30%		2.72 (2.69-2.78)	A=45%		0.110 (0.106-0.128)	A=42.8%; B=2.2%		44.09 (43.86-50.02)	E=45%	
	Total	1	5	216.53 (216.18-217.92)	F=23%		33.40 (32.63-33.96)	D=26%; E=20%		0.1558 (0.1529-0.1584)	D=26%; F=20%		3.631 (3.627-3.635)	B=23%	
		2	-	-	-		-	-		-	-		-	-	
		3	28	24.92 (24.44-25.69)	A=15% B=37.5%;		2.74 (2.69-2.85)	A=41.25%; B-2.5%		0.116 (0.106-0.130)	A=41.4%; B=3.2%		45.94 (43.86-50.04)	E=45%	
Age 40-50 yrs	M	1	3	219.06 (218.33-222.69)	G=56.31%; H=4.12%		36.10 (35.98-36.57)	F=60%		0.1590 (0.1587-0.1637)	F=58.31%; H=2.12%		3.667 (3.650-3.887)	C=58.31%; D=1.7%%	
		2	-	-	-		-	-		-	-		-	-	
		3	11	25.41 (25.28-25.85)	B=34%		3.22 (3.20-3.85)	B=34%		0.135 (0.124-0.150)	B=25%; C=9.6%		54.86 (52.75-69.29)	F=25%; G=9.6%	
	F	1	6	218.56 (218.18-218.60)	G=57.45%		33.89 (33.09-33.94)	E=57.45%		0.1546 (0.1538-0.1552)	D=55.65%; E=1.8%		3.655 (3.650-3.657)	C=55.65%; D=1.8%	
		2	-	-	-		-	-		-	-		-	-	
		3	11	25.05 (24.98-25.12)	B=34%		3.08 (3.02-3.65)	B=34%		0.132 (0.120-0.142)	B=25%; C=9.6%		51.10 (50.11-67.55)	F=24.8%; G=9.8%	
	Total	1	9	219.11 (217.98-222.69)	G=56.17%; H=4.12%		35.0 (33.09-36.57)	E =57.45%; F=60%		0.154 (0.1538-0.1637)	D=52.95%; E=1.8%; F=58.31%; H=2.12%		3.661 (3.62-3.86)	C=56.95%; D=1.75%	
		2	-	-	-		-	-		-	-		-	-	
		3	22	25.30 (24.98-25.56)	B=34%		3.15 (3.02-3.85)	B=34%		0.133 (0.120-0.150)	B=25%; C=9.6%		52.98 (50.11-69.29)	F=24.9%; G=9.7%	
Age 50-60 yrs	M	1	-	-	-		-	-		-	-		-	-	
		2	4	1061.33 (1061.04-1064.35)	J-40%		708.50 (708.20-709.06)	I=40%		0.6715 (0.6707-0.6717)	L=40%		5958.12 (5957.27-5959.7)	I=40%	
		3	6	25.86 (25.44-26.58)	B=12.9%; C=7.1%		4.15 (4.01-4.41)	C=19%		0.150 (0.148-0.157)	C=19%		64.43 (63.47-68.23)	G=19%	
	F	1	-	-	-		-	-		-	-		-	-	
		2	4	1061.15 (1059.98-1064.12)	J-40%		705.28 (705.08-707.33)	H=40%		0.6626 (0.6617-0.6636)	J-40%		5895.17 (5868.17-5909.27)	H=40%	
		3	6	25.25 (25.06-25.60)	B=19%		3.86 (3.53-3.96)	B=19%		0.145 (0.139-0.147)	C=19%		62.33 (61.09-63.10)	G=19%	
	Total	1	-	-	-		-	-		-	-		-	-	
		2	8	1061.24 (1059.98-1064.35)	J-40%		706.89 (705.08-708.06)	I and H=40%		0.6670 (0.6617-0.6717)	L and J=40%		5926.64 (5868.17-5959.70)	H and I=40%	
		3	12	25.55 (25.06-26.58)	B=15.95%; C=7.1%		4.01 (3.53-4.41)	B and C=19%		0.147 (0.139-0.157)	C=19%		63.38 (61.09-68.23)	G=19%	
Age 60-70 yrs	M	1	-	-	-		-	-		-	-		-	-	
		2	5	1065.51 (1065.09-1067.38)*	K=52.5%		710.64 (710.13-718.40)*	I=47.5%; J=5%		0.6726 (0.6719-0.6727)*	L=51%; M=1.5%		5960.87 (5958.10-5967.37)*	I=50.5%; J=2.5%	
		3	-	-	-		-	-		-	-		-	-	
	F	1	-	-	-		-	-		-	-		-	-	
		2	4	1065.26 (1065.05-1067.01)*	K=50%		708.66 (705.20-709.71)*	I=50%		0.6645 (0.6637-0.6647)*	K=50%		5914.50 (5903.57-5917.10)*	H=1.8%; I=48.2%	
		3	-	-	-		-	-		-	-		-	-	
	Total	1	-	-	-		-	-		-	-		-	-	
		2	9	1065.39 (1065.05-1067.38)*	K=51.25%		709.65 (705.20-718.40)*	I=48.75%; J=5%		0.6685 (0.6637-0.6727)*	K=50%; L=51%; M=1.5%		5937.68 (5903.57-5967.37)*	H=49.35%; J=2.5%	
		3	-	-	-		-	-		-	-		-	-	

Please refer the table legend in the following pages i.e. pgs. 129-130

2.12 C. Fishing occupation based prevalence distribution of:

(ii b) Tubular nephropathy specific selectivity indices

	Sex	Study group	Number of subjects in each occupational category for both genders of each age category	Parameter					
FISHING									
				uBCR			uNCR		
				mean in mg/g (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in mg/g creatinine)	mean in U/mmol (min-max)	Prevalence (from the total number of subjects in each occupational category)	Annnotation for the ranges of the biomarker levels (in U/ mmol creatinine)
Age 20-30 yrs	M	1	1	9.2	J=20%	A=0.0620-0.0625	2.25	J=20%	A=0.014-0.015
		2	-	-	-	B=0.0625-0.0630	-	-	B=0.015-0.016
		3	-	-	-	C=0.0630-0.0635	-	-	C=0.016-0.017
	F	1	1	9.11	I=20%	D=0.0635-0.0640	1.89	I=20%	D=0.017-0.018
		2	-	-	-	E=0.0640-0.0645	-	-	E=0.030-0.031
		3	-	-	-	F=0.10-0.11	-	-	F=0.031-0.032
	Total	1	2	9.15	I and J=20%	G=0.11-0.12	2.06	I and J=20%	G=0.032-0.033
		2	-		-	H=0.13-0.141	-	-	H=0.033-0.034
		3	-		-	I=9.12-9.22	-	-	I=1.-2
Age 30-40 yrs	M	1	1	9.25	J=20%	J=9.22-9.32	2.36	J=20%	J=2-3.0
		2	-	-	-	K=9.32-9.42	-	-	
		3	14	0.0633 (0.0633-0.0637)	C=35%; D=10%		0.0162 (0.0161-0.0167)	C=45%	
	F	1	4	9.16 (9.14-9.17)	I=26%		2.02 (1.92-2.04)	I=3.8%; J=22.2%	
		2	-	-	-		-	-	
		3	14	0.0621 (0.0620-0.0623)	A=45%		0.0147 (0.0144-0.0150)	A=8.5%; B=36.5%	
	Total	1	5	9.18 (9.14-9.27)	I=20%; J=26%		2.19 (1.92-2.36)	I=3.8%; J=21.1%	
		2	-	-	-		-	-	
		3	28	0.0626 (0.0618-00636)	A=45%; C=35%; D=10%		0.0154 (0.0144-0.0167)	A=8.5%; B=36.5%; C=45%	
Age 40-50 yrs	M	1	3	9.35 (9.30-9.38)*	K=60%		2.83 (2.78-2.85)*	J=60%	
		2	-	-	-		-	-	
		3	11	0.0635 (00634-0.0637)	D=34%		0.0164 (0.0163-0.0169)	D=34%	
	F	1	6	9.20 (9.18-9.23)*	I=1.8%; J=55.65%;		2.14 (2.11-2.16)*	J=57.45%	
		2	-	-	F=50%		-	-	
		3	11	0.0624 (0.0622-0.0628)	A=2.7%; B=31.3%		0.0157 (0.0150-0.0158)	C=34%	
	Total	1	9	9.27 (9.18-9.38)*	I=1.8%; J=57.45%; K=60%		2.48 (2.11-2.85)*	J=58.73%	
		2	-	-	-		-	-	
		3	22	0.0629 (0.0622-0.0637)	A=2.7%; B=31.3%; D=34%		0.0160 (0.0150-0.0169)	C and D=34%	
Age 50-60 yrs	M	1	-	-	-		-	-	
		2	4	0.1182 (0.1179-0.1196)	G=40%		0.0326 (0.0318-0.0335)	G=40%	
		3	6	0.06375 (0.0637-0.0638)	D=19%		0.0168 (0.0167-0.0170)	D=19%	
	F	1	-	-	-		-	-	
		2	4	0.1068 (0.1060-0.1094)	F=40%		0.0304 (0.0.300-0.0310)	E=40%	
		3	6	0.0628 (00628-0.0629)	C=19%		0.0163 (0.0161-0.0165)	D=19%	
	Total	1	-	-	-		-	-	
		2	8	0.1125 (0.1060-0.1196)	F and G=40%		0.0315 (0.0298-0.0335)	G and E=40%	
		3	12	0.0633 (0.0630-0.0638)	C and D=19%		0.0165 (0.0161-0.0170)	D=19%	
Age 60-70 yrs	M	1	-	-	-		-	-	
		2	5	0.1342 (0.1187-0.1407)	G=40.7%; H=11.8%		0.0328 (0.0323-0.0330)	H=52.5%	
		3	-	-	-		-	-	
	F	1	-	-	-		-	-	
		2	4	0.1112 (0.1090-0.1177)	F=8.1%; G=41.9%		0.0325 (0.0315-0.0329)	H=50%	
		3	-	-	-		-	-	
	Total	1	-	-	-		-	-	
		2	9	0.1227 (0.1090-0.1407)	F=8.1%; G=41.3%		0.0326 (0.0315-0.0330)	H=51.25%	
		3	-	-	-		-	-	

Please refer the table legend below

Table 2.12:- Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio; M-male; F-female.

A)represents mining occupation based prevalence distribution of various general renal function biochemical markers (ia) , tubular function biochemical markers (ib), glomerular injury specific selectivity indices (iia) and tubular nephropathy specific selectivity indices (iib) for different age categories and sexes in all the three study cohorts.
B) represents fishing occupation based prevalence distribution of various general renal function biochemical markers(ia) , tubular function biochemical markers (ib), glomerular injury specific selectivity indices (iia) and tubular nephropathy specific selectivity indices (iib) for different age categories and sexes in all the three study cohorts.
C) represents farming occupation based prevalence distribution of various general renal function biochemical markers (ia) , tubular function biochemical markers (ib), glomerular injury specific selectivity indices (iia) and tubular nephropathy specific selectivity indices (iib) for different age categories and sexes in all the three study cohorts.
Values are represented as mean with the range of minimum to maximum levels of each serum based biochemical marker (general renal [ia] and tubular function [ib]) and urinary based nephropathy specific selectivity index (glomerular [iia] or tubular [iib]) for each occupational category viz. mining (A), fishing(B) and farming(C) depicted as well. The prevalence of the serum based biochemical marker (general renal [ia] and tubular function [ib]) and urinary based nephropathy specific selectivity indices (glomerular [iia] or tubular [iib]) for each occupational category viz. mining (A), fishing(B) and farming(C) in various ranges of their levels for each age and sex category of the three study groups are represented by prevalence % and annotated by uppercase letters(i.e. A to M) for convenience. The annotations for each estimated biochemical marker or selectivity index varies and have been independently described. The prevalence % denotes the proportion of subjects from the total number of a particular occupational category (i.e. mining/farming/fishing) for each of the respective sexes of a particular age groups bearing the serum based biochemical marker or urinary based nephropathy specific selectivity index at a given range of their levels. The total number of subjects involved in the mining occupation(i.e. males/females); farming(males/females) and fishing(males and females) for study group 1 are 47/40; 6/5 and 5/11 respectively. For study group 2, the total number of subjects involved in the mining occupation(i.e. males/females); farming(males/females) and fishing(males and females) 2/2;4/3 and 9/8 respectively. For study group 3, the total number of subjects involved in the mining occupation(i.e. males/females); farming(males/females) and fishing(males and females) are 4/3; 27/28 and 30/28 respectively. For example in the case of biochemical markers, the prevalence % annotation of 'A' in the case of serum creatinine denotes the percentage of the total number of subjects involved in a particular occupation for the respective genders (i.e. the total number in each occupation being separate for males and females) of a particular age group possessing levels in the range of 0.45-0.5 mg/dL. Whereas in the example of selectivity indices, the prevalence % annotation of 'A' in the case of PCR denotes the percentage of the total number of subjects involved in a particular occupation for the respective genders (i.e. the total number in each occupation being separate for males and females) of a particular age group possessing levels in the range of 24-25 mg/g creatinine. Differences in a particular study cohort at *p<0.05 were considered to be statistically significant as compared to the other two cohorts (wherever applicable) and also relative to the lower age categories (in a given study group).No occupational bias in serum based tubular function markers'(viz.bicarbonates & phosphates) and urinary based tubular nephropathy specific markers'(viz.uBCR,uNCR) expression in study group 1 and glomerular injury markers' viz. uPCR, uACR**,** uAPR and uAlb/b2M expression in study group 2 were noted, which was evident from similar levels of the respective markers and its prevalence in comparable concentration ranges being observed in all occupational categories (viz.mining,fishing and farming) in all study groups.Moreover, levels of tubular injury markers in study group 1 and glomerular nephropathy specific markers in study group 2 elevated with increasing age with significant (*p<0.05) prevalence of their levels at higher ranges being noted in higher age groups (i.e.40-50yrs in study group 1 and 60-70yrs in study group 2). This elevated levels of tubular injury markers being noted at the higher age group(i.e.40-50 yrs) in study group 1 was attributed to the bioaccumulative potencies of toxins that were deemed responsible for CKDu causation which caused accumulation and aggravation of tubular damage effects with increased exposure (i.e.age),that was evident from the increase in expression of tubular injury markers with rising age. Moreover, the escalated levels of glomerular injury markers being noted at the higher age group(i.e.40-50 yrs) in study group 2 was attributed to the inherent or natural degeneration and compromise in the glomerular function with age(i.e.beyond 50yrs) which increases the susceptibility of glomerulus to damage by major risk factors(diabetes,hypertension), visible from increase in glomerular damage markers' levels with age. For detailed units of each biochemical parameter and selectivity index, refer Tables 2.7 and 2.8.

As indicated in **Table 2.12**, the tubular and glomerular markers of dysfunction and nephropathy specific selectivity indices were present at significantly elevated and comparable levels in all the occupation types [i.e. mining (Table 2.12 A), farming (Table 2.12 B) and fishing (Table 2.12 C)] for both the sexes and various age categories in the respective study cohorts 1 and 2.Herein also values of the respective functional biomarkers(glomerular and tubular) [Table 2.12 A (i 'a' and 'b'), Table 2.12B (i 'a' and 'b') and Table 2.12C (i 'a' and 'b')] and glomerular or tubular nephropathy specific selectivity indices) [Table 2.12 A (ii 'a' and 'b'), Table 2.12B (ii 'a' and 'b') and Table 2.12C (ii 'a' and 'b')] significantly ($p<0.05$) increased with increasing age irrespective of the type of occupation carried out or sex with the highest range of value being prevalent at the highest age category of each of the study cohorts. In study group 1, the abnormally highest range of levels of each tubular dysfunction biomarker and nephropathy specific selectivity indices [Table 2.12A (i 'a' and 'b'; ii 'b'), Table 2.12B (i 'a' and 'b'; ii 'b') and Table 2.12C (i 'a' and 'b'; ii 'b')] were prevalent at roughly 55% and 45% in the age groups of 40-50 yrs. and 30-40 yrs. respectively at a

comparable extent in both the sexes and occupation type. Moreover, in study cohort 2 the maximum level range of various glomerular injury markers were prevalent at approximately 58% and 42% in the age categories of 60-70 yrs and 50-60 yrs. respectively to a similar degree in all the occupational and gender groups [Table 2.12A (ii 'a'), Table 2.12B (ii 'a') and Table 2.12C (ii 'a')]. Thereby negating the role of occupational risk factor in CKDu development in Canacona as it was observed to distress all occupational categories to a comparable extent irrespective of the gender.Thus suggesting that etiology of CKDu in Canacona is plausibly linked to an environmental factor(s) mostly associated with drinking water as elimination of occupational risk negates the exposure to nephrotoxins via air (Abiola, 2017; Gifford et al., 2017; Ratnayake et al., 2017; Weaver et al., 2015).

This was backed by the non-functionality of the granite mine present in the vicinity of the CKDu hit area for the past 15 years that reduces the predisposition of exposure to occupational toxins via air among the highly prevalent previous miners of study group 1 (The Hindu, 2017). This was further justified by diverse occupational constitution of this taluka's CKDu affected study group 1 comprising of previous miners, farmers and fishermen(as established from our demographic survey), suggesting that this mixed occupational cohort was exposed to a common environmental source (like drinking groundwater) contaminated by nephrotoxins(Jayasumana et al.,2015b;Khandare et al.,2015; Reddy and Gunasekar,2013; Siriwardhana et al.,2018) that was possibly responsible for the manifestation of CKDu in the taluka. Moreover the findings from this occupation based analysis also provided supporting evidence to the tendency of CKDu to develop at earlier stages in life (i.e. 3^{rd} to 5^{th} decade) (Lebov et al.,2015; Orantes-Navarro et al.,2016; Raines et al., 2014; Wanigasuriya,2012).

Overall, these findings were contradictory to a majority of the previously reported observations of CKDu analysis in Central America which stated that CKDu possesses a high tendency to affect agricultural workers or the farming community owing to its high risk of exposure to various renal damage inducing causal factors like dehydration, heat stress & very few nephrotoxic pesticides (López-Marín et al.,2014; Orantes-Navarro et al., 2017).

However, our results were also found to be in accordance with the findings from other related studies of the CKDu scenario in India specifically the Udannam region of Andhra Pradesh, (Ganguli, 2016; Khandare et al., 2015; Tatapudi et al., 2018); Egypt (Emad and Osama, 2010; El Minshawy, 2011 a) and some parts of Central America (Peraza et al., 2012) which supported a non-occupational risk in the disease etiology. Therefore suggesting that despite CKDu being an environmentally induced disease, its etiological origin (i.e. causal factors) will inevitably possess regional differences owing to variations in geography, geology,

occupational constitution and lifestyle habits in these regions (Gifford et al., 2017). Furthermore, the results from this occupational based prevalence distribution of biomarkers refuted the involvement of an occupational peril in development of glomerular dysfunction based CKD in the non-endemic study group 2 of the taluka as well. These findings paralleled the study of effect of various occupations on pathologies of diabetic & hypertensive glomerular nephropathies by Smith et al (2011).Thus providing confirmatory evidence to role of traditional causative factors (i.e. diabetes,hypertension) in induction of CKD in this area.

2.3.5.2 Demographic risk factors associated with the pattern of disease presentation specifically the renal function biomarker profile of the study population

In an epidemiological study, analysis of the association of various demographic variable with biochemical and anthropometric characteristics of the study population enables suitable prediction of the pattern of biomarkers that will exceed reference limits which helps in identification of risk groups for disease development (Ratnayake et al.,2017;Smith et al., 2011).In the current study, relationship between demographic risk-factors, anthropometric variables and major biochemical selectivity indices(renal function biomarkers profile) of the entire study population were analysed via a simple linear regression analysis using SPSS statistical software. Herein, levels of biomarkers and anthropometric measurements were retained as continuous variables and categorical variable(demographic characteristics like presence of diabetes,hypertension, drinking untreated groundwater etc.) were recoded as binary variable/(0-1 indicator variable) in which respondents of the effect were coded as 1 & non-respondents as 0(Nanayakkara et al.,2012a and b; Smith et al.,2011) with results presented in Table 2.13.As indicated in Table 2.13, anthropometric parameter viz. eGFR & demographic exposure variables like age, drinking untreated well water, presence of mine in the vicinity of affected region, history of skeletal disorders, chronic consumption of NSAIDs were significant ($p<0.05$) and independent associates (evident from higher and significantly different regression coefficients) with increase in each of the tubular dysfunction biomarkers) (i.e. uBCR, uNCR) in study group 1. eGFR was inversely associated with increase in each of these tubular dysfunction biomarkers indicating that as tubular damage progresses (as reflected by elevated levels of its markers, renal function proportionately decreases (manifested from reduced GFR), highlighting the existence of severe tubular injuries in this group. These findings were consistent with results of CKDu study in Sri Lanka which reported that decrease in renal function (indicated by reduced GFR) among CKDu subjects was strongly associated with increase in tubular damage (Nanayakkara et al.,2012a and b).

Table 2.13: Linear regression analysis of association between demographic risk factors & anthropometric cum biochemical characteristics of the study population using the former features (i.e. demographic & anthropometric) as dependent variables for prediction of susceptible risk groups of CKDu development.

	uPCR			uACR			uBCR			uNCR			uAPR			uAlb/b2M ratio		
Arthropometric & Demographic features/ Study group	**1 (n=114)**	**2 (n=28)**	**3 (n=124)**	**1 (n=114)**	**2 (n=28)**	**3 (n=124)**	**1 (n=114)**	**2 (n=28)**	**3 (n=124)**	**1 (n=114)**	**2 (n=28)**	**3 (n=124)**	**1 (n=114)**	**2 (n=28)**	**3 (n=124)**	**1 (n=114)**	**2 (n=28)**	**3 (n=124)**
Male	0.13	0.15	0.14	0.09	0.1	0.11	0.07	0.04	0.03	0.13	0.09	0.06	0.07	0.09	0.08	0.1	0.12	0.13
Female	0.141	0.16	0.15	0.06	0.12	0.09	0.09	0.06	0.02	0.07	0.04	0.01	0.05	0.1	0.08	0.07	0.15	0.1
Age	**25.3 (0.02)**	**27.8 (0.018)**	1.5	1.1	**15.9 (0.023)**	0.9	**10.8 (0.031)**	0.9	0.5	**12.6(0.041)**	0.5	0.1	1.3	**16.8 (0.027)**	0.7	2	**18.1 (0.045)**	0.7
Systolic BP(mm of Hg)	0.05	**55.9 (0.021)**	0.03	0.09	**45.7 (0.016)**	0.06	0.04	0.03	0.01	0.07	0.05	0.03	0.06	**48.3 (0.025)**	0.04	0.11	**46.3 (0.015)**	0.07
Diastolic BP(mm of Hg)	0.08	**50.9 (0.034)**	0.05	0.07	**49.1 (0.019)**	0.01	0.02	0.02	0.03	0.06	0.03	0.05	0.04	**49.8 (0.012)**	0.03	0.1	**47.1 (0.018)**	0.05
Fasting blood glucose (mg/dL)	0.09	**80.9 (0.026)**	0.07	0.1	**96.4 (0.015)**	0.12	0.16	0.04	0.06	0.09	0.07	0.03	0.08	**92.3 (0.041)**	0.12	0.09	**94.3 (0.025)**	0.16
Glycosylated Hb (Hb1Ac)(%)	0.05	**95.4 (0.03)**	0.1	0.02	**92.7 (0.031)**	0.03	0.05	0.09	0.05	0.08	0.014	0.03	0.04	**94.6 (0.035)**	0.05	0.02	**95.1 (0.023)**	0.04
eGFR (ml/min/1.73 m^2)	**-49.2 (0.012)**	**-87.9 (0.013)**	0.25	-11.3	**-75 .3 (0.017)**	0.07	**-21.3 (0.047)**	-8.3	0.15	**-19.6 (0.035)**	-7.8	0.17	-10.2	**-78.9 (0.012)**	0.05	-9.1	**-76.4 (0.025)**	0.09
Occurrence of diabetes-	0.16	**78.6 (0.024)**	0.69	0.11	**90.8 (0.021)**	0.04	0.09	0.05	0.06	0.04	0.03	0.02	0.13	**89.3 (0.014)**	0.06	0.15	**92.3 (0.03)**	0.07
Occurrence of hypertension-	0.1	**69.8 (0.041)**	0.89	0.07	**81.6 (0.03)**	0.03	0.1	0.06	0.02	0.09	0.07	0.03	0.06	**85.6 (0.02)**	0.04	0.09	**83.4 (0.021)**	0.05
Occupation																		
Mining	1.69	1.12	0.56	0.09	0.05	0.03	0.91	0.23	0.08	0.85	0.19	0.07	0.11	0.08	0.03	0.1	0.04	0.02
Farming	1.05	1.18	0.69	0.08	0.04	0.05	0.21	0.14	0.06	0.26	0.18	0.03	0.06	0.04	0.02	0.15	0.06	0.06
Fishing	1.26	1.09	0.57	0.06	0.02	0.03	0.35	0.18	0.04	0.39	0.14	0.36	0.08	0.05	0.03	0.04	0.02	0.03
Use and exposure to chemical agrochemicals (pesticides)	0.09	0.23	0.15	0.07	0.29	0.02	0.15	0.09	0.05	0.12	0.07	0.03	0.09	0.25	0.04	0.1	0.23	0.05
Presence of mines in the vicinity	**11.2 (0.018)**	0.68	0.14	0.08	0.16	0.05	**89.6 (0.035)**	0.58	0.12	**92.3 (0.03)**	0.69	0.15	0.1	0.16	0.06	0.04	0.11	0.07
Source of drinking water																		
Well (groundwater)	**9.6 (0.032)**	1.79	1.03	0.05	0.11	0.03	**91.3 (0.018)**	0.25	0.11	**93.6 (0.016)**	0.29	0.13	0.08	0.15	0.05	0.04	0.15	0.07
Municipality treated public supply (surface water)	0.57	0.45	0.12	0.02	0.08	0.03	0.2	0.14	0.08	0.15	0.11	0.06	0.04	0.07	0.04	0.08	0.1	0.09
Family history of CKD	0.009	0.012	0.007	0.004	0.008	0.003	0.008	0.01	0.004	0.006	0.009	0.002	0.006	0.009	0.004	0.008	0.006	0.006
Use of long-term alternate/ ayurvedic medicine	0.003	0.009	0.004	0.002	0.005	0.001	0.003	0.006	0.002	0.005	0.003	0.001	0.004	0.006	0.002	0.007	0.009	0.004
History of skeletal disorders	**3.9 (0.041)**	1.14	0.69	0.07	0.06	0.03	**35.9 (0.012)**	1.23	0.56	**33.6 (0.016)**	1.13	0.59	0.09	0.08	0.04	0.05	0.03	0.04
History of NSAIDs consumption	**2.6 (0.025)**	0.74	0.51	0.06	0.03	0.04	**32.3 (0.025)**	1.09	0.36	**32.4 (0.025)**	0.99	0.21	0.09	0.06	0.04	0.04	0.05	0.03
Prolonged duration of NSAIDs consumption(for > 5 yrs)	**4.2 (0.045)**	0.96	0.89	0.06	0.02	0.01	**39.6 (0.014)**	1.05	0.21	**38.1 (0.036)**	0.89	0.19	0.07	0.05	0.03	0.09	0.07	0.06

Abbreviations: R-regression coefficient; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; eGFR-estimated glomerular filtration rate; uACR- urinary to albumin ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; 'n' represents the number of subjects of which the demographic and biochemical characteristics were assessed; NSAIDs-non-steroidal anti-inflammatory drugs; uPCR-urinary protein to creatinine ratio. The levels of the biomarkers and the anthropometric measurements were retained as continuous variables and the categorical variables (i.e. demographic characteristics) were recoded as binary variables in which respondents of the effect were coded as 1 and non-respondents as 0. Values are represented as regression coefficients (R) with only significant p values(i.e. $p<0.05$) mentioned in parenthesis. Increasing numbers of coefficients denotes the rising strength of association. Regression coefficients(R) with values greater than 1 and lesser than 0 (negative values) indicates positive and negative association respectively between given two variables.Coefficient with values unusually larger than 1 and significantly different(p value<0.05) from controls indicates that exposure to these risk factors enhanced danger of developing either glomerular or tubular nephropathy wherever applicable. Demographic risk factors that are independently & significantly($p<0.05$) associated with elevation in tubular dysfunction biochemical markers & related development of tubular pathology are represented by enhanced regression coefficient values which are highlighted in bold red. Whereas demographic risk factors significantly ($p<0.05$) linked to the increase in glomerular nephropathic markers & associated manifestation of glomerular nephropathy are also depicted by elevated coefficient values and highlighted in bold orange.As indicated increasing age, drinking untreated well water, presence of mine in the vicinity,history of skeletal disorders, chronic consumption of NSAIDs were confirmed to be significant risk factors that were remarkably associated in causing an increase in tubular injury development among CKDu affected subjects which was evident from strong associations (i.e significantly higher regression coefficient) of these risk factors with the tubular injury markers viz. uBCR and uNCR. Concurrently,diabetes & hypertension were also confirmed as risk factors for CKD development in study group 2 evident from significant higher associations(i.e. elevated regression coefficient) of the same with glomerular injury markers(viz.uACR,uPCR,uAPR ,uAlb/b2M ratio.

Our results also highlighted **(Table 2.13)** that these tubular injuries tend to become more pronounced with advancing age as evident from the strong relationship observed between increasing age and rising levels of tubular dysfunction biomarkers in the current study. These findings were consistent with the observations of the age based susceptibility for tubular dysfunction associated with CKDu in other similarly affected regions of Central America and Sri Lanka (Herrera et al., 2014; Ramírez-Rubio et al., 2015; Selvarajah et al., 2016). This was attributed to the increased time taken by the tubular injury inducing causal agents' i.e. environmental nephrotoxins to induce significant reno-toxic effects. It has been previously reported that a prolonged exposure to nephrotoxins for more than 25 yrs. is a prerequisite for severe induction of tubular dysfunction that can manifest in renal failure. This explains the severe induction of tubular dysfunction with increasing age in this study group as age can be approximately translated into extent of exposure, wherein older an individual gets, higher is the period of exposure (Abiola, 2017; Sabath and Robles-Osorio, 2012).

The risk of exposure to environmental nephrotoxins capable of inducing tubular dysfunction typical of CKDu pathology can be severely enhanced (evident from enhance tubular injury biomarkers), when exposed to certain demographic and geographic variables like consumption of untreated groundwater, engaging in mining or being located in the close proximity of a mining site (Khandare et al., 2015; Ramírez-Rubio et al., 2015; Selvarajah et al., 2016; Suwazono et al., 2015; Tatapudi et al., 2018). Groundwater can serve as a hub for various environmental toxins like heavy metals etc. when disturbed by anthropogenic activities like mining. The susceptibility of groundwater contamination by nephrotoxins is significantly enhanced when the aquifer is located in the vicinity of an operational or non-operational mine. This contamination is majorly attributed to the acid mine drainage from the open pits of the mines into the proximal aquifer as a result of continuous precipitation induced dissolution of the ubiquitous sulfide minerals present in these open pits that decrease the pH of the groundwater. This reduced pH excessively leaches out the nephrotoxins from the aquifer which thereby increases the risk of exposure on prolonged consumption of untreated groundwater (well-water) (Gifford et al., 2017; Khandare et al., 2015; Reddy and Gunasekar, 2013; Weaver et al., 2015; Wimalawansa, 2014). These established findings explained the higher risk of CKDu development in the study group 1 of the Canacona taluka owing to the strong and significant association of this susceptible population with the demographic risk factors like presence of mines in the vicinity and consumption of untreated groundwater (Table 2.13). Furthermore our results were in accordance with the studies of CKDu conducted in Sri Lanka which established long term nephrotoxin exposure of

cadmium and arsenic via consumption of mining contaminated groundwater to be responsible for the CKDu development in these regions (Correa-Rotter et al., 2014; Rajapakse et al., 2016; Levine et al., 2016; Wasana et al., 2016).

Generally, the involvement in mining inevitably increases the risk of occupational exposure to renal toxins like heavy metals via air (Weaver et al., 2015; Wimalawansa and Wimalawansa, 2016). In the current study as well, mining was observed to be a risk-factor for CKDu development in the study group 1 of the taluka. However this association is not completely justifiable due to the non-functionality of the mine for the past 15 yrs in this taluka due to which the affected subjects are not currently involved in mining (The Hindu, 2017). The higher association was solely obtained as a result of larger proportions of the CKDu subjects being reported to be previously involved in mining. This was further supported by the similar display of tubular dysfunction biomarkers at their highest range in all of the occupational categories of the study group 1(Table 2.12), indicating that this mixed occupational cohort (comprising of farmers, previous miners and fishermen) share a common source of nephrotoxin exposure which in large probability is environmentally related (El Minshawy, 2011 b; Khandare et al., 2015).

Therefore these findings negated the role of mining and other occupations as occupational risk factors for CKDu development in the Canacona taluka and strongly highlighted the environmental etiology of this disease to be possibly related to nephrotoxin exposure (like heavy metals) via consumption of untreated groundwater (Levine et al., 2016). Our findings were contradictory to some of the studies of CKDu in Central America which stated the etiology to be occupational related with a specific link to the farming community due to their high risk of exposure to heat stress and dehydration (Orantes-Navarro et al., 2017). However, our results were also noted to be at accord with the observations of CKDu studies in India, Egypt and some parts of Central America which stated the absence of an occupational linkage and advocated an environmental origin of this disease (Emad &Osama, 2010; El Minshawy, 2011 a; Ganguli, 2016; Khandare et al., 2015; Peraza et al., 2012; Tatapudi et al., 2018).

Moreover, previous history of musculo-skeletal problems and associated long-term consumption of NSAIDs for alleviation of these pains have been strongly reported in the causation of analgesic based nephropathy whose major pathological presentation is tubular dysfunction. The widespread prevalence of this causal CKDu risk factors have been reported to be significantly ($p<0.05$) associated with the enhanced induction of tubular dysfunction markers as noted in some of the similarly affected CKDu endemic regions of Central America and India (specifically the state of Andhra Pradesh) (Brooks, 2009; Khandare et al.,

2015; Vela Parada et al., 2014; Wesseling et al., 2013). Thus provides supporting evidence to the possible association of NSAIDs with the etiological development of CKDu in the study group 1 as noted in the current study **(Table 2.13)**.

In totality, these findings from our current study were consistent with the previously described results of the analysis of various demographic predictors for development of CKDu in the study group 1 by risk-ratio estimations (see section 2.3.3.2).Moreover our results of the predictive effects of various demographic risk factors on the pattern of disease presentation of CKDu (i.e. tubular dysfunction biomarker profile) in the Canacona taluka were also found to be in accordance with the observations from various studies related to the demographic risk assessment of the CKDu disease in the endemic regions of India(specifically Andhra Pradesh), Central America, Sri Lanka and Egypt as cited above.

Thereby justifying the high predisposition of certain segments of the population of Canacona taluka to develop tubulointerstitial nephropathies, characteristic of CKDu pathology, when subjected to these independently associated demographic risk-factors. As per our current study, these demographic risk factors were established to be advancing age in younger adults, presence of mine in proximity of CKDu affected area, long-term consumption of untreated groundwater, past history of skeletal disorders and prolonged NSAIDs consumption.

These established demographic risk factors of CKDu development in study group 1 were completely lacking in study group 2 comprising of typical CKD cases suggestive of a non-tubular (i.e. specifically glomerular) renal pathology in this study cohort (Smith et al., 2011).

As indicated in **Table 2.13**, anthropometric variable i.e. GFR and demographic exposure variables like age, presence of diabetes and hypertension, elevated levels of glycosylated hemoglobin, higher fasting blood glucose and blood pressure were found to be significantly and strongly associated with the enhancement in the levels of each of the biomarkers of glomerular injury (i.e. uACR, uPCR, uAlb/b2M, uAPR) in study group 2. The strong association of these biomarkers with age is attributed to the intrinsic tendency of aging to modify and disturb the glomerular architecture and selectivity with time that causes an increased urinary excretion of glomerular filtration precluded proteins majorly comprising of albumin (DeFina et al., 2016). This explained the strong relationship observed between age and the glomerular dysfunction markers in this study group 2 (Fisher et al., 2013; Smith et al., 2011; Unnikrishnan et al., 2018). This glomerular damage was further amplified by the presence of pre-existing medical conditions like diabetes and hypertension indicated by greater associations of the glomerular injury markers with elevated blood glucose levels, increased glycosylated hemoglobin levels and higher blood pressure in the study cohort 2

(Smith et al., 2011; Solini et al., 2014; Warady et al., 2015; Yang et al., 2017). Uncontrolled and prolonged diabetes and hypertension have been well reported to be major culprits involved in the induction of renal dysfunction via disruption of the glomerular structural and functional integrity. The major pathogenic mechanism associated with these glomerular disturbances is believed to be that excess glucose and hypertension causes severe glycation of the renal proteins constituting the endothelial capillaries of the glomerular filtration apparatus resulting in scarring and lesions in the same that eventually disrupts the glomerular capillaries structure causing hyper-filtration associated with endothelial capillary dysfunction. This enhanced hyper-filtration ultimately results in the increased elimination of high molecular weight proteins from the plasma viz. albumin that under normal health conditions is restricted from filtration via the glomerulus due to its large size (Gnudi et al., 2016). Thus justifying the strong relationship noted between presence of diabetes and hypertension with the widespread prevalence of glomerular dysfunction in the study group 2 (as indicated by increased markers of glomerular injuries) **(Table 2.13).**These results were also found to be in agreement with the previously reported studies which stated that the occurrence of diabetes and hypertension as risk factors in susceptible populations were strongly and significant related to the enhancement in not only proteinuria and albuminuria (reflected from increased uACR, uPCR and uAlb/b2M ratio) but simultaneously led to elevations in the uAPR ratio as well, which cumulatively manifested in glomerular dysfunction among these individuals (De Nicola et al., 2013; Fisher et al., 2013; Smith et al., 2011; Warady et al., 2015). Thus affirming a high prevalence of glomerular dysfunction induced CKD among the subjects of study group 2 which possessed a known etiology related to typical causals of diabetes and hypertension.

Overall, our results of the analysis of various demographic risk factors for suitable prediction of renal disease presentation pattern in 2 diverse risk-groups confirmed origin of renal in the study group 1 (comprising of CKDu cases) and study group 2 (comprising of CKD cases) to be renal tubular dysfunction and glomerular injuries respectively. Thus providing supporting evidence to the fact that the CKDu manifested in the Canacona taluka is of tubulointerstitial pathology, which was consistent with the histopathological presentation of CKDu in other developing countries of the world like Sri Lanka and Central America (Gifford et al., 2017; López-Marín et al., 2014; Lusco et al., 2017 Wijetunge et al., 2015).

2.4 Conclusion

This study attempted to establish CKDu prevalence,geographic distribution cum demographic stratification, various risk factors or population groups susceptible at developing CKDu and pattern of CKDu disease presentation in Canacona owing to lack of information on the same.

Prevalence analysis depicted that 80% of CKD cases in Canacona were of unidentifiable etiology (i.e. unlinked to traditional causals-diabetes and hypertension), hence characterized as CKDu and remaining 20% linked to a diabetes or hypertensive etiology. The CKDu cases were restricted to 2 villages namely Ponsulem and Chaudi. On the demographic front,CKDu was found to affect adults in age group of 30-50 yrs and did not display gender or occupational bias. An increased prevalence (55%) of skeletal disorders and chronic consumption of NSAID (nephrotoxin) for pain alleviation were noted among CKDu subjects. Thus highlighting possible role of NSAID's in renal damage aggravation in the taluka. Moreover, CKDu risk factors were identified by statistical analysis of demographic characteristics of study population by significant risk-ratios determination.Chronic consumption of untreated well water, skeletal discomfort incidence,previous history of NSAID consumption,chronic NSAID intake,presence of unoperational mine in the proximity were identified as prominent risk factors in CKDu development in Canacona.

Despite the rising incidence of CKDu in the taluka, the biochemical based pattern of disease presentation crucial for quick detection and management has not been defined till date. Hence the current study illuminated the same by measurement of biochemical markers distinctive of two varied origins of renal pathological damage viz. tubular and glomerular origin in biological matrices (blood, urine) of CKDu, diabetes/hypertensive CKD and healthy control populations, for comparison of disease presentation patterns in CKDu and CKD pathology. Tubular damage is mainly assessed by measurement of tubular dysfunction biomarkers which include uBCR (urinary b2M to creatinine ratio) and uNCR (urinary NAG to creatinine ratio) which denote levels of LMV proteins (β2-microglobulin, b2M) and tubular localized enzyme (N-acetyl glucosaminidase, NAG) respectively,that remarkably rise in the urine on nephrotoxin induced tubular damage due to compromise in intrinsic tubular reabsorption of these proteins into plasma. Whereas glomerular damage is analysed by measurement of glomerular dysfunction biomarkers that include uPCR (urinary protein to creatinine ratio), uACR (urinary albumin to creatinine ratio), uAPR (urinary albumin to protein ratio) and uAlb/b2M ratio (urinary albumin to b2M ratio) which denotes protein levels and its major component i.e. albumin, that remarkably rises in the urine owing to diabetes/ hypertension

induced glomerular injury which under good-health conditions precludes glomerular filtration into the urine due to their high molecular weight. Reported evidence on biochemical presentation have reported that since CKDu pathology was observed to be of tubular origin (i.e. CTN) owing to nephrotoxin's tendency to target proximal tubule, a significant trend of increase in tubular dysfunction biomarkers will be obtained. In line with existing evidence, a tubular proteinuric pattern (comprising of increased urinary excretion of tubular injury specific proteins viz. b2M and NAG) and increased urinary elimination of electrolytes-bicarbonates and phosphates (that are reabsorbed by proximal tubules) were noted in CKDu pathological presentation of the taluka as well. These observations were further backed by existence of normal glomerular dysfunction markers levels in CKDu subjects with higher levels of the same noted in diabetes/hypertensive CKD cases signifying CKDu and CKD in corresponding endemic and non-endemic regions of the taluka to be of tubular and glomerular pathological origin respectively. Moreover, predicted CKDu risk factors were noted to be positively cum significantly ($p<0.05$) and inversely cum insignificantly associated (determined via regression analysis) with tubular and glomerular injury markers respectively indicating significant role of these risk factors in tubular pathological manifestation associated with CKDu development. Overall these results were consistent with demographic and biochemical findings of CKDu analysis in homologously affected regions of Sri Lanka, Andhra Pradesh, Central America and Egypt. Hence our results provided justifying evidence to CKDu in the taluka to be of tubular pathological manifestation which emphasized the etiological role of environmental nephrotoxins in disease development, therefore was extensively explored in the following chapters.

Chapter 3

Monitoring the role of various environmental nephrotoxins in the causation of CKDu

Section 3.1

Environmental monitoring of nephrotoxins in the 'groundwater (drinking water)' exposure matrix and assessment of their contribution in CKDu manifestation

3.1.1 Introduction

Previous CKDu analysis in developing countries have stated environmental nephrotoxins to be responsible for CKDu causation, hence nephrotoxin(s) role in CKDu etiology of Canacona was explored. Human exposure to nephrotoxin can be assessed by toxins analysis in its major environmental exposure routes viz. groundwater &food (Lusco et al., 2017). This section stresses on nephrotoxins anlaysis in groundwater (well-water) used for drinking in this taluka. Previous CKDu studies in developing countries and current demographic analysis of Canacona CKDu cases have stated this disease to affect rural communities that heavily rely on groundwater exploited through infiltration wells for drinking thus making groundwater a potential exposure source of nephrotoxins in rural Canacona as well (Giri and Singh, 2017; Indiastat, 2017; Khandare et al., 2015; Statistical Handbook of Goa, 2016).Since groundwater is entrapped in the earth's crust [that is ubiquitously enriched in nephrotoxins bound to other organic and inorganic elements (metals, non-metals) that under ideal conditions restrict its bioavailability to human exposure]; it can serve as a hub for these nephrotoxins when disturbed by human activities like mining or industrial discharges of metal laden effluent into the earth that subsequently leaches these toxins into water table (Flora et al., 2012; Noli and Tsamos, 2016; Sankhla et al., 2016; Singh et al., 2012). However in this study, presence of an unoperational granite mine in proximity of CKDu affected villages of Canacona was suspected as the aquifer's prime contamination source via acid mine drainage (AMD). AMD can cause increased acidification of groundwater in the mining area and neighbouring aquifers (via inherent drainage of waterways) by large production of sulphuric acid due to rapid oxidation and dissolution of exposed ubiquitous sulphide minerals present in the mine's opened pits on continuous precipitation. AMD is a persistent environmental problem that occurs for several ages even after mining stops and can significantly decrease groundwater quality by leaching out nephrotoxins like heavy metals and trace geogenic elements viz.silica from aquifer's bedrock (ubiquitously containing varied toxins levels) into water whose availability is reportedly enhanced at acidic pH. This nephrotoxin contaminated groundwater on chronic intake can result in renal damage specifically tubular pathology (chronic tubulointerstitial nephritis) as previously reported in other CKDu hit countries (Galhardi and Bonotto, 2016; McCarthy, 2011; Khan et al., 2015; Sankhla et al., 2016; Xu et al., 2018).

Prevalent nephrotoxins in groundwater that are previously reported to be implicated in CKDu causation include heavy metals (like lead, cadmium, arsenic, mercury). Mechanism of heavy metal nephrotoxicity is well-reported and has been detailed in Chapter1, being mediated by

induction of oxidative damage to nephron's proximal tubule that manifests in CTN development, hallmark of CKDu(Gifford et al.,2017; Lentini et al.,2017;Weaver et al., 2015).
Recently, a trace geogenic element viz. silica is gaining attention as a nephrotoxin. Silica abundantly constitutes the earth's crust but possesses limited bioavailability, hence categorised as trace geogenic. It's availability can be enhanced on anthropogenic disturbances of aquifer's bedrock (by AMD) resulting in increased human exposure and associated induction of health-hazards. Epidemiological studies have mainly focused on induction of respiratory diseases on chronic exposure, negating possible induction of extra-pulmonary effects like renal damage (Sen et al., 2016). However, few animal silica toxicity studies and epidemiological analysis have highlighted a link between chronic exposure to silica through intake of silica contaminated water and tubular injuries development, deduced from resemblance of renal histopathological alterations(on biopsy analysis) and increased urinary excretion pattern of tubular injury markers(b2M,NAG) on silica exposure with that of CTN (Dobbie-Smith,1982;Kawanabe et al.,1992;Khandare et al.,2015;Radovanovic et al.,1991).
In lieu with these reported observations we hypothesised that CKDu affected subjects of Canacona taluka could have been chronically exposed to heavy metal(s) and silica via long-term consumption of groundwater contaminated with these nephrotoxins as a result of AMD from the non-operational granite mine located in the vicinity of this CKDu endemic region that could have excessively leached out these nephrotoxins from the granitic aquifer's bedrock [reported to be enriched in silica and lead (Fernandes & Widdowson, 2009)] into the groundwater resulting in enhanced exposure and subsequent development of CTN, the major pathological manifestation of CKDu. Thus in order to validate this hypothesis, the aims of the current study were set as to conduct a complete hydro-geochemical screening of CKDu-endemic region's groundwater for levels of various nephrotoxins (like silica, heavy-metals); analyze various physiochemical-factors affecting toxin bioavailability and subsequent nephrotoxicity; estimate nephrotoxin(s) intake and associated renal toxicity risk in CKDu affected population against matched healthy controls and relate nephrotoxin(s) intake with etiology of CKDu in Canacona by assessment of influence of prevalent toxin intake/exposure levels on pattern of expression of urinary tubular injury biomarkers via correlation analysis.

3.1.2 Materials and methods

3.1.2.1 Ethical approval and consideration

The entire study was approved by Government of Goa by obtaining necessary permission from government health safety regulatory body i.e. Directorate of Health-Services, Panaji,

Goa (Reference No:-DHS/Sp.Cell/Sect/8/1119).It was conducted in agreement with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Furthermore the ethical approval for this study was granted by two Institutional Ethics regulatory bodies of the host research-institute and the state head government hospital of Goa viz. Institutional Human-Ethics Committee of BITS-Pilani, India (Reference No:-IHEC/11M/2) and Institutional Ethics Committee of Goa Medical Hospital (Dated 20th July 2015) respectively.

3.1.2.2 Study region and design

In order to assess how the CKDu incidence in the Canacona taluka varies based on the presence of nephrotoxic contaminants in the ground water, appropriate study regions needs to be selected (Jayatilake et al., 2013). Hence our study regions were selected based on the criteria of locating regions in the taluka displaying the presence and absence of the CKDu disease. Our previous demographic study had already established the lack of registries or literature stating the exact prevalence of the CKDu disease in the taluka. Hence in order to gain an initial insight into the prevalence of CKDu in the Canacona taluka, we had approached two main hospitals in Goa which regularly conducts dialysis for the chronic renal failure patients of Canacona taluka, namely Apollo Victor hospital and Canacona Health Centre, to gain access to their medical records for appropriate selection of the study population and their respective regions of residence. Permission to gain access to hospital registries were obtained from the state government health regulatory body i.e. Directorate of Health Services followed by the Medical Superintendents/Directors of the selected hospitals.

After obtaining the necessary permissions from the hospital management, an exhaustive list of the taluka's CKD affected patients were obtained from both the hospitals. On analysis it was found that 142 from a combined total of 180 CKD affected patients were hailing from the Canacona taluka. From this 80% of the CKD affected patients (n=114) possessed no defined etiology hence the CKD among these subjects was recategorized as Chronic kidney disease of unknown etiology (CKDu).These CKDu affected subjects were residents of two villages namely Ponsulem and Chaudi, that were located in the vicinity of a non-operational granite mine. Hence for convenience the residing subjects in these two CKDu endemic villages were cumulatively grouped under one broad category i.e study-group 1 and their area of residence was clubbed as study-area 1/study region 1.The remaining 28 patients from the grand total of 142 patients possessed known etiology of CKD related to traditional causals of diabetes and hypertension. These CKD affected subjects were hailing from scattered villages of the taluka namely Cola, Poinguinim and Anvali and were collectively grouped under the

non-endemic study-group 2 and their area of residence categorized as study-area 2/study region 2. For selection of true-controls, a total of 124 volunteers with a 1: 1 sex ratio from two healthy villages of the taluka namely Molorem and Endrem (showing no prevalence of CKD whatsoever) were randomly chosen to match the age and sex distribution of the CKDu cases. These healthy volunteers from both non-CKD prevalent villages were clubbed together under study-group 3 with their area of residence grouped as study-area 3/study region 3. The details of 3 study-groups are presented in Table 2.1 of Chapter 2 of this thesis.

Overall, 3 different study regions were identified for the current study and were as follows: (1)-regions displaying high prevalence of the CKDu disease (endemic CKDu area, study region 1); (2)-regions displaying the low incidence of the CKD disease caused by traditional causals viz. diabetes and hypertension located in the non-endemic area (CKD non-endemic, study region 2) and (3)-regions showing absolutely no prevalence of CKDu or diabetic and hypertensive CKD (healthy region, study region 3). The sampling sites have been pictorially represented in **Figure 3.1.1** (not to scale). The study region 1 comprised of two main villages namely-Ponsulem and Chaudi located in Canacona taluka of south Goa, which showed high prevalence of CKDu as per our demographic study (Chapter 2). These villages are located in the vicinity of a non-operational granite mine bearing geographical-coordinates: 15°5′ 17″ N and 74°5′ 64″ E with an average elevation of 10 m above mean sea-level, at a distance of approximately 2-3 kms from the mine. The study region 2 comprised of Cola, Poinguinim and Anvali villages that were located at larger distances from each other and at an average of 10-12 kms from the non-operational granite mine. Moreover, study region 3 comprised of Molorem and Endrem villages that were located at opposite distances from each other, situated very close to tips of the taluka bearing considerable distances at an average of 20-23 kms from the non-operational granite mine (Fernandes and Widdowson,2009; Nadaf, 2009a). The climate in all 3 study regions of the taluka is uniform and is reported to be tropical with 3 seasons-winter (December-February), Summer (March-May) & Monsoon (June-September), mean temperature:27.3°C - 40.6°C and annual precipitation of 2995 mm (Nadaf, 2009c & d). However there are significant geological variations in these 3 study regions. The geological formation in aquifer's bedrock of Canacona taluka consists mainly of granite and metabasites wherein geological bedrock of this CKDu-endemic region (i.e. study region 1) was reported to comprise of older-trondhjemitic sodium-plagiogranitic gneiss, while flanking non-endemic CKD-region (study region 2) and healthy control region (study region 3) possesses greenschists-metabasites. This granitic-gneiss is intruded by younger granites exposed in Chaudi village (Fernandes and Widdowson, 2009; Nadaf, 2009a), hence served as a granite

mining site previously. The mineral wealth of Canacona is very poor with very low iron or manganese deposits found in this taluka unlike other talukas of South Goa district (Fernandes and Widdowson, 2009; Nadaf, 2009a). The geology of this taluka is has been pictorially shown in **Figure 3.1.1.** The drainage is dendritic consisting of west-flowing rivers draining into the Arabian Sea viz. Talpona (flowing through CKDu-endemic granitic-belt) and Galgibagh, Saleri river (flowing through metabasitic-formations of the CKD-non endemic and healthy regions) (Nadaf, 2009e)(**Figure 3.1.1).**

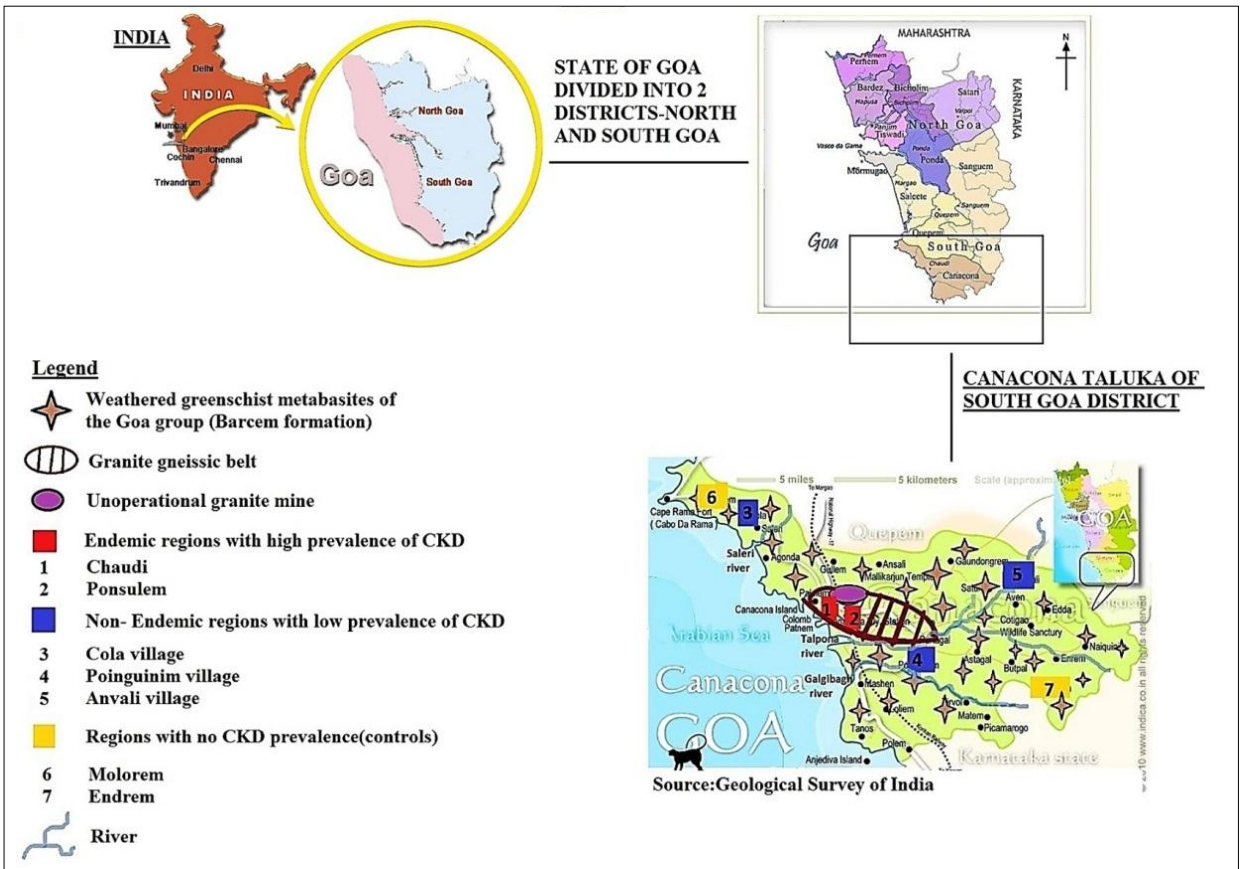


Figure 3.1.1. Sampling locations of the groundwater and the geology of the CKDu-affected Indian sub-district located in the state of Goa namely Canacona

The red squares denote the CKDu endemic regions from where the drinking groundwater samples were collected while the blue squares denote the non-endemic water sampling regions and the yellow squares indicates the water sampling regions with no CKD prevalence. The number inside the boxes corresponds to the name of the village of the three respective study areas. The main geological formation of the Indian sub-district (located in the state of Goa, namely Canacona) that is the granite gneissic belt and the metabasite cover are also shown. The three main rivers of Canacona sub- district-Talpona, Galgibagh and Saleri flowing through the area are also depicted. As shown, there is a non-operational granite mine located in the Chaudi named village of the Canacona sub-district which is located in the close proximity of the CKDu endemic region.

3.1.2.3 Sample collection

It has already been established that groundwater can serve as a exposure source to various nephrotoxins when invaded by anthropogenic activities like mining (Senapati et al.,2018). Since the major source of drinking water in all 3 three study region as determined from our demographic study was common and established to be groundwater which was exploited through bore wells connected to hand pumps; a full hydro-geochemical analysis of the

groundwater was carried out to analyse levels of various environmental nephrotoxins if present. The well water samples for analysis were collected by personally visiting each of the study group subject's residence based on address obtained from them during the interview. The wells have been used by participants for drinking through their lifetime that is around 35 yrs & more, hence were chosen for sampling. The samplings were carried out during 3 different seasons which are pre-monsoon period (PRM)(summer month-May 2016), monsoon period (MON) (July 2016) & post-monsoon periods (POM) (winter month-November 2015) to assess for seasonal variations (if any) in level of nephrotoxins in groundwater (Chandrajith et al., 2011; Ranasinghe et al., 2015). The groundwater samples were collected in previously acid cleaned (with trace metal grade 1N HNO_3 and rinsed with 18MΩ/cm deionized water) high density polypropylene bottles. The water was allowed to run for five minutes & bottle was rinsed 3 times with water to be collected before sampling. 500ml of the sample was collected in duplicates from each well, one of which was acidified with 10% concentrated nitric acid (Merck, Mumbai, India) for trace metal & heavy metal analysis. All the samples were tightly capped after collection and details of the sample at the time of collection (like date, time, season, place) were recorded and maintained simultaneously in the sample collection registry (Jayatilake et al., 2013; Nanayakkara et al., 2014).

Overall, a total of 114 samples were collected from the CKDu endemic region (study region 1), 28 samples from the diabetes and hypertensive CKD non-endemic region (study region 2) and 124 samples from the region with no CKD prevalence (study region 3). The sampling study regions and the approximate location of the abandoned granite mine and the geology of the areas under investigation (not to scale) have been pictorially represented in **Figure 3.1.1.**

3.1.2.4 Analytical Methods

3.1.2.4.1 Reagents/chemicals

Multi-element (1000 mg/L) [used for trace metal, geogenic elements, essential metal and heavy metal estimations] and Rhodium (1000 mg/L) and solution were obtained from PerkinElmer (Waltham, MA, USA). Sodium metasilicate (used for silica standard preparation) of weight 500g was procured from Thermo Fisher Scientific (Waltham, MA, USA). Essential metal cations [like sodium (Na^+), potassium (K^+), calcium (Ca^+), magnesium (Mg^{+2})] and contaminating anions [like nitrate (NO_3^-), phosphate (PO_4^{-3}), sulphate (SO_4^{-2}), chloride (Cl^-) and fluoride (F^-)], each of concentration 1000 mg/L for ion-chromatographic analysis were purchased from Sigma-Aldrich (Bangalore, India). All containers and glassware were cleaned by soaking into 20 percent nitric acid for at least 24 h and rinsed

three times with deionized water prior to use. Deionized water (18 MΩ/cm resistivity) prepared from Merck Millipore system (MilliQ IQ/7003 ultrapure and pure water system, MA, USA) was used for preparation of all working solutions and utilized for all spectrophotometric and ion chromatographic analysis. All the reagents and solutions used were of analytical grade and mainly procured from Sigma-Aldrich (Bangalore, India).

3.1.2.4.2 Glassware and instruments

All the glassware and plastic ware (i.e. polyethylene bottles and tubes) used for analysis were carefully cleaned and rinsed with HNO_3, to avoid trace metals, heavy metal, silica, and other potential toxin contamination. This was achieved by first washing the apparatus thoroughly with water and detergent followed by rinsing with tap water and deionized water. The cleaning was eventually terminated by the final soaking in dilute acid (i.e. 500 ml conc.HNO_3 + 4500 mL deionized water) for 2 hours followed by rinsing with the same dilute acid and 5 extensive washings in deionized water and ultimately drying in a class-100 laminar flow hood before use. A clean laboratory and laminar-flow hood capable of producing class 100 (Thomas Scientific, NJ, USA) were used for preparing solutions (Al-Saleh et al., 2017)**.** Inductively coupled plasma mass spectrometer (NexION 350 ICP-MS, PerkinElmer, Massachusetts, USA was used for the estimation of trace metals and heavy metals. UV-Vis-spectrophotometer (Shimadzu UV-1800, Tokyo, Japan) was used for silica measurements and other spectrophotometric estimations. Microwave oven (Multiwave 3000, Anton Paar, Bangalore, India) was used for sample digestion. Essential metal cations like sodium (Na^{+}), potassium (K^{+}), calcium (Ca^{+2}), magnesium (Mg^{+2}) & contaminating anion like nitrate (NO_3^{-}) , phosphate (PO_4^{-3}), sulphate (SO_4^{-2}), chloride (Cl^{-}) & fluoride (F^{-}) were measured by using an ion-chromatographic sytem (Dionex ICS-2000 Ion-chromatography system, California, USA). Programmable furnace (Lindberg/Blue, Thermoscientific, Waltham, MA, USA) was used for sample digestion with thermostat maintaining temperatures of 450±25ºC. Microwave oven (Multiwave 3000, Anton Paar, Bangalore, India) was used for sample digestion as and where applicable. Ashing was supported by use of hot plate with heating control up to 200ºC.

3.1.2.4.3 Physicochemical analysis of the groundwater of the study regions of Canacona

Physical and chemical characteristics of groundwater have been well reported in influencing bioavailability of various nephrotoxins in the groundwater; hence determination of the same is crucial for assessing the degree of nephrotoxin contamination in groundwater. Hence a complete physiochemical analysis of groundwater samples collected at 3 different time

points/seasons–PRM, MON and POM, from the 3 study regions was conducted to assess seasonal and spatial variation in the characteristic of groundwater that could affect the availability of nephrotoxins for human exposure (Ghazali et al.,2018; Noli and Tsamos, 2016). pH and electrical conductivity were measured in-situ using Thermo Scientific's multi-parameter portable meter(PC Testr 35). Physical parameters like Alkalinity, Total dissolved solids (TDS), Turbidity and Total Hardness, were measured according to A.P.H.A standard methods (Eaton et al.,2005a).The analyses of physical parameters forms basis for ascertaining factors on which chemical characteristics of water depend (Galhardi and Bonotto, 2016).

The chemical characteristics of water are mainly dependent on the major soluble cations like sodium (Na^{+}), potassium (K^{+}), calcium (Ca^{+}), & magnesium (Mg^{+2}) and anions like Nitrate (NO_3^{-}), phosphate (PO_4^{-3}), sulphate (SO_4^{-2}) , chloride (Cl^{-}) and fluoride (F^{-}). Both the cations and anions were measured by the ion chromatographic techniques. The ion analysis was performed by a Dionex ICS-2000 reagent-free ion chromatography (RF-IC) system equipped with an automated EG50 Eluent Generator Module and CD25 conductivity detector. The cations were separated using an Ion Pac CS17 (4 mm) column with Methane sulfonic acid (MSA) as the eluent at a flow rate of 1.0 ml/min, using the standard gradient method and an Ion Pac CG17 Guard column, with a CSRS-ULTRA Cation Self Regenerating Suppressor. The anions were separated on an Ion Pac AS11-HC (4 mm) column with Potassium Hydroxide (KOH) at 1.2 ml min^{-1} as eluent, using gradient method and an Ion Pac AG11-HC Guard column, with an ASRS-ULTRA (4 mm) Anion Self Regenerating Suppressor. The sample injection volume for the cation analysis was 100µL and anion analysis was 1000µL. Calibration was done using serial dilutions of IV (Inorganic Ventures) high-purity standard stock solutions separately for both cations and anions and were run every day prior to sample analysis. The appropriate blanks were run in between samples to assess baseline & contamination from previous samples. Detection limit was ≥1µg/L (Thamban et al., 2010).

3.1.2.4.4 Analysis of trace metals, nephrotoxic heavy metals and trace geogenic elements specifically silica nephrotoxin in the groundwater of study regions of Canacona taluka.

Trace metals as the name suggests are present at trace or minimal concentrations in the groundwater under ideal environmental conditions. Therefore an elevation in their levels significantly indicates the influence of anthropogenic invasion in the groundwater aquifer. Similarly trace geogenic element includes those group of elements that solely and distinctly originates solely from the earth's crust (hence the name geogenic) with increased levels of the same depicting increased interactions between the bedrock and the compromised poor quality

groundwater under the influence of anthropogenic activities (Förstner and Wittmann, 2012). It has been well reported that trace metals and geogenic elements on surpassing their respective WHO and BIS established permissible ranges can prove to be toxic to human beings and have been well known to induce various health hazards such as skeletal disorders. Some of them have also been associated with certain amount of renal damage, based mostly on the results from animal toxicity studies with no evidence from human beings (Nordberg and Nordberg , 2016; Prashanth et al., 2015). Moreover some of these trace metals have been well reported in widely influencing the bioavailability of certain nephrotoxins specifically silica to human exposure. Hence analysis of the trace metals and geogenic elements are critical for assessment of the confounding factors affecting the distribution of the nephrotoxins in the groundwater and the ability to inflict other potential health perils (such as skeletal disorders) (Förstner and Wittmann, 2012; Noli and Tsamos, 2016).Thus the analysis of trace metal and geogenic elements (except for the silica nephrotoxin) in groundwater collected during 3 different seasons and from three varied study regions was conducted in the current study by Inductively coupled Plasma Mass-spectroscopy (ICP-MS) to assess seasonal and spatial variation in these trace metals and geogenic elements (Jayatilake et al., 2013).

Similarly, well-known nephrotoxic heavy metals like lead, cadmium, mercury and arsenic were also analyzed in the same samples of the present study over seasonal and spatial trends by ICP-MS to analyze the contributing role of any potential nephrotoxin exposure in CKDu development in Canacona. The ICP-MS method used for analysis of all of the 12 trace metals (i.e. Al, V, Fe, Mn, Ni, Cu, Zn, Ba, Co, Mo, Ag and Cr), four nephrotoxic heavy metals (i.e. Pb, Cd, As and Hg) and four trace geogenic elements (except for silica viz. Li, B, Sr) was common and adopted from a previously reported procedure by Al-Badaii et al (2016) with a few modifications. For this, multi-element stock solutions (NIST certified) containing 100 mg/L or 100 ppm of each element obtained from Perkin-Elmer (Waltham, MA, USA) was used for calibration. Analytical calibration standards were prepared daily in 25ml volumetric flasks over the range of 0-100 µg/L for nephrotoxic heavy metals (i.e. Pb, Cd, As and Hg) and trace metals (i.e. Al, V, Fe, Mn, Ni, Cu, Zn, Ba, Co, Mo, Ag and Cr) and at a range of 0-50 mg/L for the remaining geogenic elements (i.e. Li, B and Sr) by suitable serial dilutions of multi-element stock solution in deionized water (with resistivity of 18MΩ) containing 1% (v/v) HPLC grade nitric acid (Sigma-Aldrich, Bangalore, India) to prepare a calibration curve for obtaining the unknown concentrations. Rhodium was used as internal standard at the concentration 10 µg/L Rh prepared from a Rhodium stock solution (of 1 g/L), that was obtained from PerkinElmer (Waltham, MA, USA). Appropriate matrix matched reagent

blanks were analyzed in addition to the samples, and the corrections wherever deemed necessary were incorporated. The RSD (relative standard deviation) was observed to be < 6% in all analysed samples indicating a good precision for analysis. The detection limit for all analytes was 0.1µg/L.For the ICP-MS analysis, a PerkinElmer NexION-350 ICP-MS instrument (ELAN DRC II, Massachusetts, USA) with high-purity argon (99.99%,Sigma-Aldrich, Bangalore, India), which utilized a Meinhard concentric nebulizer (PerkinElmer, Waltham, Massachusetts, USA) connected to a cyclonic spray chamber was used. A radiofrequency of 1100W power was selected in pulse mode with autolens one and sample uptake rate was set at 1.1 ml/min. Sample data were acquired by using 20 sweeps/reading,1 reading/replicate, and a dwell time of 60 ms. Argon nebulizer gas flow, auxillary gas flow and plasma gas flow rate were optimized daily from 0.5-0.9L/min,1.5-1.85 L/min and 12-15 L/min respectively. Data were acquired in counts per second. For verification, absorbance of reagent blank, working multi-element standards of each of the analyzed heavy metals, trace metals and geogenic elements (except silica) & sample solutions were recorded at respective metal specific wavelength, after which corresponding heavy metals, trace metals & geogenic element content were calculated from their standard calibration curves by interpolation.

Silica was measured by the ammonium-molybdate standard spectrophotometric (Shimadzu UV-1800, Tokyo, Japan) method recommended by the APHA (4500-SiO_2.C) (Eaton et al., 2005b). For this, the primary silica stock solution of concentration 1000 mg/L was prepared by dissolution of 4.73 g of sodium metasilicate (Thermo Fisher Scientific, Waltham, MA, USA) in deionized water (with resistivity of 18MΩ).Analytical calibration working standards were freshly prepared in 50ml volumetric flask over the range of 0-200 mg/L (to cover the expected concentration range) by appropriate serial dilution of the stock solution in deionized water (18MΩ) to prepare a calibration curve for obtaining the unknown concentrations. Appropriate reagent blanks were successively analyzed in addition to the samples and the corrections wherever deemed necessary were incorporated. The relative standard deviation (RSD) was observed to be less than 5% in all analysed samples indicating a good precision for analysis. For the spectrophotometric analytical procedure, the standards or the test sample was initially treated with 1 ml of 1:1HCl and 2ml of ammonium molybdate followed by incubation for 10 min to form a yellow coloured heteropoly acid (i.e. molybdoreactive silicic acid complex). Following this, 2 ml of oxalic acid was added for a 5min incubation period to reduce the interference from inhibiting phosphates and the absorbance of the formed yellow coloured product was quantified at a ƛ max of 410 nm. A standard calibration curve was plotted from the absorbance values of the working standards at 410 nm versus their respective

concentration in mg/L and the unknown concentration in the test samples were calculated by interpolation. The accuracy of the method was frequently verified by measurement of standards for every 20 test samples, wherein a good reproducibility between various sets of measured values of a given standard at different time points was exhibited.

3.1.2.5 Nephrotoxicity risk assessment

Environmental nephrotoxicity monitoring studies in populations prone to nephrotoxin exposure frequently utilize various measures for characterizing risk of developing renal damage in this population at exposure levels prevalent in the targeted regions. 2 of such measures that are commonly used for nephrotoxicity risk assessment are estimated daily intake and target hazard quotient (Bandara et al., 2010a; Chandrajith et al., 2011).

3.1.2.5.1 Estimated daily intake (EDI)

Estimated daily intake is a reliable indicator of the rate of nephrotoxin transfer from the exposure source (groundwater or food) into the human beings to accurately predict the risk of developing renal toxicity in susceptible populations. The higher rate of contamination of the exposure source (in this case groundwater) with nephrotoxins does not necessarily indicate that human beings will be exposed to such high nephrotoxin levels unless rate of exposure to the given nephrotoxin is suitably determined which can be established by determination of estimated daily intake of nephrotoxin (EDI) (Chandrajith et al., 2011; Jayatilake et al., 2013). Thus daily intake of nephrotoxins is highly dependent on nephrotoxin concentration in the exposure source and daily rate of water consumption. Hence, EDI concept was formulated to take into consideration all these factors. The EDI (in µg/ day kg bw for heavy metals & in mg/day kg bw for trace geogenic element viz. silica) was calculated by following formula:

$$EDI = \frac{C \times Cons}{Bw} \quad \textit{equation no. (1)}$$

Where ‘C’ is the concentration of the nephrotoxin (µg/L for heavy metals and mg/L for silica) in the contaminated groundwater sample; ‘Cons’ stands for the average daily consumption of water (in L/day) and ‘Bw’ represents the body weight (in kg) of an individual (Ma et al., 2016b; Wongsasuluk et al., 2014).

The average daily consumption of water by the study subjects as established during the personal interview conducted during sample collection was found to be 3.7 L being comparable in all of the three study regions This consumption rate was found to be homologous to the values established by the water management legislative authority of Goa viz. Department of water resources (Statistical Handbook of Goa, 2016). Thus in order to

retain uniformity in intake calculations, the state's department of water resource established average daily water consumption rates of 3.5L/day were utilized for EDI calculations. Moreover the average body weight of the subjects belonging to all the three study regions was set to 60 kg which is the general body weight considered for a studied individual) in the present study for consistency in calculations (Giri and Singh , 2015; Wu et al., 2009).

3.1.2.5.2 Target hazard quotient (THQ)

THQ is another method of statistically estimating the risk of developing renal damage on oral exposure(via consumption of water or food) to a nephrotoxin and appropriately depicts the fractional contribution of the EDI of a given nephrotoxin to its oral reference dose (RfD), which is strongly indicative of the probability of renal toxicity manifestation (Bandara et al., 2008; Chandrajith et al., 2011; Chotpantarat et al., 2014).To support the assessment of the role of nephrotoxins contamination in the exposure source(i.e. groundwater in this case) in inflicting severe renal damage, the WHO has set a specific oral reference dose for each of these toxins. The oral reference dose is defined by WHO "as an estimation of maximum permissible risk on human population through daily exposure to nephrotoxin (or intake of toxin) during the lifetime via chronic water consumption taking into consideration a sensitive or a susceptible group." The oral reference dose is specific and different for exposure (or consumption) via the water & food routes (Giri and Singh, 2015; Wanigasuriya et al., 2011).

The THQ was calculated based on the following equation (JECFA, 2000):

$$THQ=\frac{EDI}{RfD} \quad \textit{equation no. (2)}$$

where 'EDI' stands for estimated daily intake and 'RfD' stands for oral reference dose with both possessing units of µg/ day kg bw for heavy metals or mg/day kg bw for silica.

The WHO established RfDs for Pb, Cd, As and Hg are 0.6, 0.3, 0.5, 0.4, µg/day kg bw respectively and for silica is 2mg/ day kg bw (as established from previous animal and a few epidemiological studies) (Nanayakkara et al., 2014; Wongsasuluk et al., 2014).

If THQ value is less than 1, then the exposed population is anticipated not to manifest any nephrotoxicity. The THQ equation was conceptualized on the basis that oral intake dose equals absorbed toxin dose, wherein cooking or boiling has no influence on toxin levels and average Bw of an adult for EDI estimation was considered as 60 kg (He et al., 2018; Nanayakkara et al., 2014). It has been well noted that exposure to multiple nephrotoxins at any given point in time can result in additive and/or interactive effects. Thus, in the present study the combined nephrotoxic risk was assessed by the summation of the individual THQ

values of each nephrotoxin for a particular exposure route (i.e. groundwater consumption in this case) and expressing it as the total THQ (TTHQ) (also branded as the hazard index, HI). The formula for *HI/ TTHQ is = THQ (toxin 1) + THQ (toxin 2) +. . . . + THQ (toxin n)*
This formula is conceptualized on the assumption that the extent of the renal damaging effect is directly proportional to the additive effects of various multiple nephrotoxin exposure which is a direct upshot of the homology in the toxicological attack mechanism of these nephrotoxins on the target organ (i.e. kidney in this case). For HI also, the safe threshold set is 1, similar to THQ. Higher the HI value, greater is the danger level. The study population is considered to be safe if the HI is lower than 1.The HI value that surpasses 1, generally depicts the significantly elevated risk of developing renal toxicity emerging from the combined action of various nephrotoxins, therefore highlights the urgent necessity for adoption of the remedial and preventive measure to avert the future rise in the incidence of CKDu (He et al., 2018; Jayatilake et al., 2013; Karim, 2011; Wongsasuluk et al., 2014).

3.1.2.6 Statistical analysis

The analysis of physicochemical characteristics, nephrotoxin, trace metals, & trace geogenic elements in groundwater samples were duplicated for better data reproducibility. All the statistical analyses were performed with SPSS (Version 20.0) for Windows. The data for each continuous variable were expressed as a mean±SE (standard error) with a range of minimum and maximum values provided of two independent experiments. The calculation of the values were conducted independently for categories distributed temporally (i.e. mean values with the minimum to maximum range measured for a variable at three different time points i.e. PRM, MON, POM in each group); spatially (i.e. mean values with the minimum to maximum range of a variable compared between the three study regions) and demographically (i.e. mean values compared between various sub-categories of major demographic variables like occupation).The statistical differences in the continuous variables between the three study groups (spatial), the three different time points (seasons) and the demographically stratified sub-groups (occupational categories) were independently and separately compared using one-way analysis of variance test and the significance of the differences within these three individual comparison groups (i.e. temporally, spatially and demographically) were recognized post-hoc using Dunnet's test. The association between various analysed nephrotoxins in the groundwater were investigated via calculation of Pearson's correlation coefficients (r) with their respective p-values to ascertain the significantly ($p<0.05$) common source of origin (if any) of these nephrotoxins in the exposure source, permitting the

implementation of necessary remedial measures to eradicate the contamination in the exposure source which could ultimately assist in the decrease of the CKDu incidence in the taluka. Moreover the role of the oral daily intake of these nephrotoxins in the manifestation of the tubular or glomerular pathology in the taluka was evaluated by calculation of the Pearson's correlation coefficients (r) with their respective p-values between the estimated daily intake of these nephrotoxins and the urinary selectivity indices specific for tubular or glomerular damage. Differences at *p-values<0.05 were considered to be statistically significant in the entire sets of assessments (i.e. analysis of the continuous variables between the three study groups and correlation assessments) (Chandrajith et al., 2011; He et al., 2018; Rango et al., 2015; Jayatilake et al., 2013; McClean et al., 2012; Wongsasuluk et al., 2014).

3.1.3 Results and discussion

3.1.3.1 Physicochemical characteristics of the groundwater of the three study regions of Canacona taluka

The physiochemical characteristics (i.e. physical & chemical characteristics) of the water are the major determinants of the extent of groundwater contamination with various environmental nephrotoxins and the associated bioavailability of these toxins to human beings. These characteristics are in turn influenced by the weathering of the aquifer's bedrock (which is dependent on the temperature and precipitation), mineral composition of the bedrock, the season and the level of anthropogenic activities like mining, industrial related and agricultural activities etc. (Hemond and Fechner, 2014; National Research Council, 2014). Hence determination of the physical and chemical characteristics of the groundwater is crucial and mandatory for the assessment of the degree of nephrotoxin contamination in the groundwater and predicts the probable extent of human exposure to the nephrotoxins and the induction of renal damaging effects, thus was conducted in the present study (Chandrajith et al., 2011; Noli, and Tsamos, 2016). The physiochemical profile of the groundwater during the three different seasons -Pre monsoon (PRM), Monsoon (MON) and post monsoon (POM) for the three study areas of investigation are presented **in Figures 3.1.2 and Table 3.1.1 & Figure 3.1.3. and Table 3.1.2**. As observed in **Figures 3.1.2 and Table 3.1.1 & Figure 3.1.3. and Table 3.1.2**, all of the physiochemical parameters were well within the WHO and BIS established permissible limits (WHO, 2011) except for the physical parameter viz. pH **(Figure 3.1.2 and Table 3.1.1)**. Temporal and spatial variations were noted in all the measured characteristics.

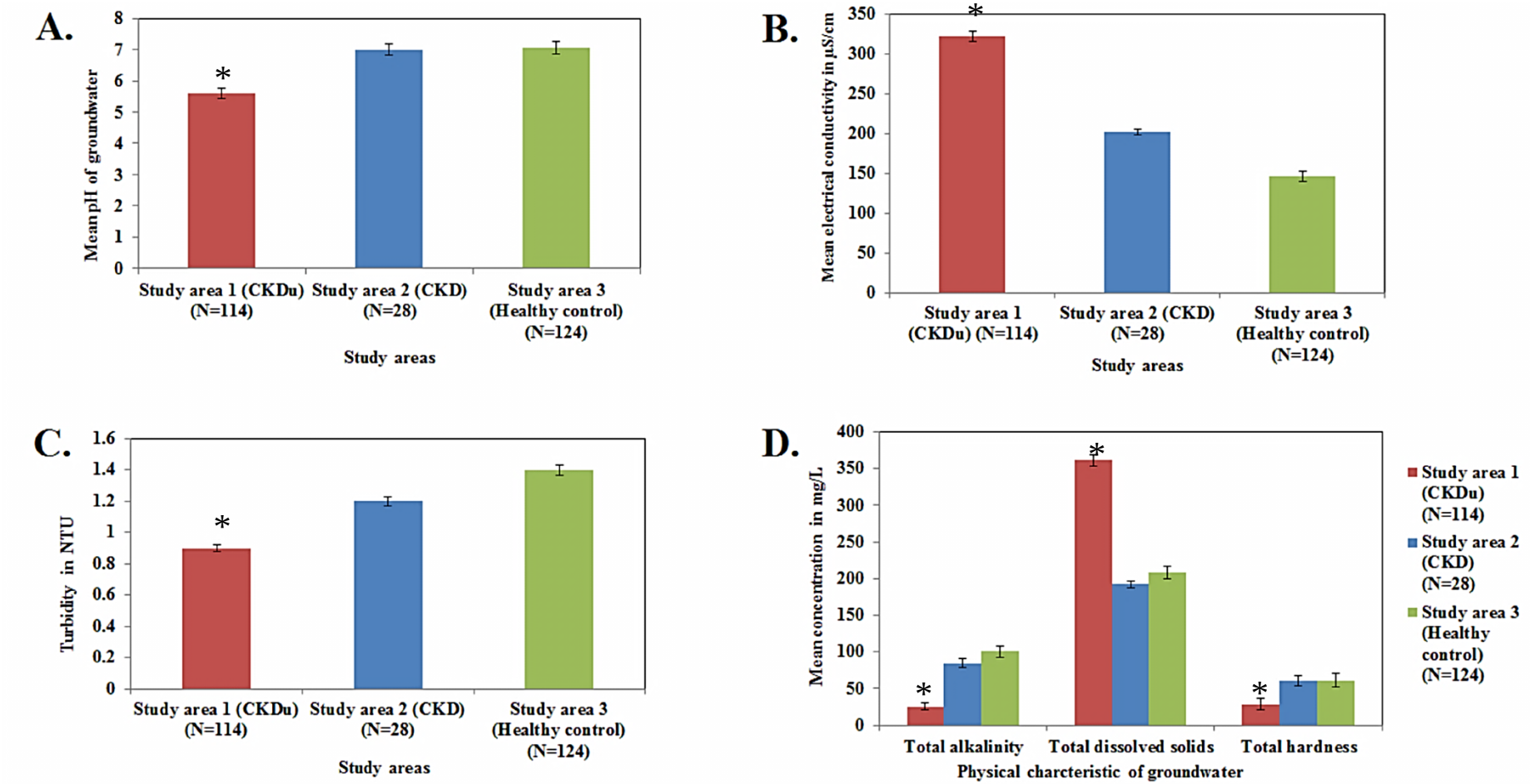


Figure 3.1.2 Physical properties of the groundwater in the three study areas viz.CKDu endemic ,CKD prevalent and healthy control areas during three different time points or seasons.
Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology. Data are derived from two independent experiments with the levels of each parameter in an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which the groundwater samples were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons Pre -Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal variations in the physical characteristics of groundwater.
(A) pH of the groundwater of the three areas; (B) Electrical conductivity of the groundwater;(C) Turbidity of the groundwater and (D) Total alkalinity, dissolved solids and hardness of the groundwater. Values are represented as mean ± SE (standard error) of the levels of a particular parameter analysed over three different seasons/time-points i.e. PRM,MON and POM in all of the three study areas. All the values are in mg L^{-1} except for pH, turbidity (NTU) and Electrical conductivity (EC):µS cm^{-1}. Differences at *p<0.05 were considered to be significant.The WHO established permissible limits for pH=6.5-8.5; EC=400 µS cm^{-1} ;Turbidity=10 NTU; Total alkalinity=200 mg/L; Total dissolved solids=500 mg/L; Total hardness= 300 mg/L.All of the physical characteristics of groundwater displayed spatial variations with significantly higher levels of Total dissolved solids and EC and remarkably lower levels of turbidity, total alkalinity and hardness noted in the study area 1 (i.e. CKDu endemic region) as compared to study area 2(i.e. diabetes or hypertensive CKD prevalent region) and study area 3(healthy controls).A significantly acidic pH (that was beyond the WHO permissible limit) was noted in the groundwater of the CKDu endemic region(study area 1) which was attributed to acid mine drainage from a non-operational granite mine in the vicinity of the CKDu region as opposed to neutral pH noted in study area's 2 and 3.The details on the seasonal distribution of the parameter and the minimum to maximum range of values of each parameter are listed in **Table 3.1.1**.

Table 3.1.1: Physical properties of the groundwater in the three study areas during the three different seasons in reference to the WHO permissible limits and Bureau of Indian Standard (BIS) specifications of drinking water.

CKD status	CKDu Endemic				CKD Non-endemic				No CKD prevalence				WHO permissible limit (mg L^{-1})	BIS standards (IS 10500)	
Sampling site and period/ Parameter (mg L^{-1})	Study area/ group 1 (n=114)				Study area/ group 2 (n=28)				Study area/ group 3 (n=124)					Desirable limit (mg L^{-1})	Maximum limit (mg L^{-1})
	PRM	MON	POM	MEAN [a]	PRM	MON	POM	MEAN [a]	PRM	MON	POM	MEAN [a]			
pH	**5.7±0.02***	**5.5±0.04***	**5.8±0.01***	**5.6±0.02*[b]**	7.1±0.04*	6.8±0.03*	7.3±0.03*	7.0±0.04*	7.1±0.05*	6.9±0.01*	7.2±0.02*	7.05±0.05*	6.5-8.5	6.5-8.5	No relaxation
	(5.5-5.8)	**(5.3-5.6)**	**(5.6-5.9)**	**(5.3-5.9)**	(6.8-7.2)	(6.7-7.0)	(6.9-7.4)	(6.7-7.4)	(6.9-7.3)	(6.8-7.2)	(7.0-7.5)	(6.8-7.5)			
Total alkalinity	33.5±0.1	15.7±0.6	28.3±0.3	25.83±0.2	85.3±0.2	78.4±0.1	89.7±0.3	84.4±0.5	118.4±0.2	83.7±0.1	99.1±0.3	100.4±0.5	-	200	600
	(32.4-36.9)	(14.6-16.7)	(25.3-29.6)	(14.5-36.9)	(83.6-87.9)	(76.6-80.3)	(87.6-92.3)	(76.6-92.3)	(115.6-122.1)	(81.5-86.9)	(97.6-103.4)	(81.5-122.1)			
EC (µS cm^{-1})	345±0.6*	250±0.3*	371±0.2*	322.2±0.03*[c]	205±0.2*	180±0.5*	223.4±0.4*	202±0.2*	151±0.1*	123±0.3*	163±0.6*	146.1±0.3*	-	400	1000
	(340-349)	(247-254)	(368-375)	(247-375)	(196-211)	(172-189)	(213-230.6)	(172-230.6)	(147-156)	(118-129)	(155-175)	(118-175)			
TDS	361±0.2*	324±0.4*	398±0.1*	361.5±0.2*[c]	195±0.3*	155±0.1*	225.4±0.5*	192±0.2*	210±0.1*	193±0.5*	222±0.3*	208±0.1*	500	500	2000
	(355-367)	(321-334)	(389-410)	(321-410)	(187-203)	(145-162)	(217-232)	(145-203)	(201-215)	(185-204)	(215-236)	(185-236)			
Turbidity (NTU)	0.8±0.01	1.3±0.04	0.7±0.02	0.9±0.02	1.2±0.02	1.5±0.1	1.1±0.02	1.2±0.03	1.3±0.02	1.7±0.01	1.4±0.02	1.4±0.03	10	10	25
	(0.6-1.4)	(1.0-1.9)	(0.4-1.2)	(0.6-1.9)	(0.7-1.5)	(1.2-2.2)	(0.9-1.8)	(0.7-2.2)	(0.9-1.7)	(1.3-2.9)	(1.2-2.6)	(0.9-2.9)			
Total hardness	30.4±0.2	21.5±0.1	33.6±0.5	28.5±0.3	62.9±0.2	53.8±0.6	65.3±0.2	60.6±0.1	61.5±0.4	56.4±0.2	66.8±0.7	61.5±0.6	-	300	600
`	(25.6-35.6)	(17.5-26.9)	(30.3-37.9)	(17.5-37.9)	(58.9-68.9)	(49.3-57.6)	(62.5-73.7)	(49.3-73.7)	(57.6-68.9)	(50.3-60.4)	(63.1-74.6)	(50.3-74.6)			

Abbreviations: BIS-Bureau of Indian standards; CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; EC-electrical conductivity; TDS:-total dissolved solids; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. Data are derived from two independent experiments with the levels of each parameter in an individual experiment measured in triplicates. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected.

a Values are represented as mean ± SE (standard error) of a particular parameter, estimated for all the samples(n) collected recurrently during three different sampling time points-Pre -Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) in the three study regions. The range of minimum to maximum values obtained for a given parameter have been represented in parenthesis individually for each sampling season (i.e. PRM, MON and POM) and cumulatively for the average of all the seasons combined. (i.e. mean of all seasons) . All the values are in mg L^{-1} except for pH, turbidity (NTU) and Electrical conductivity (EC):µS cm^{-1}.Differences at *$p<0.05$ were considered to be significant.

b The values (highlighted in bold) denotes levels which were significantly beyond the WHO and BIS specifications for drinking-water, and observed only in the CKDu endemic-region(study-area 1).

c The values indicate levels which are a matter of concern. These values were higher only in the CKDu endemic region as compared to the CKD non-endemic and healthy control regions and were approaching the WHO's upper permissible limits for drinking water.

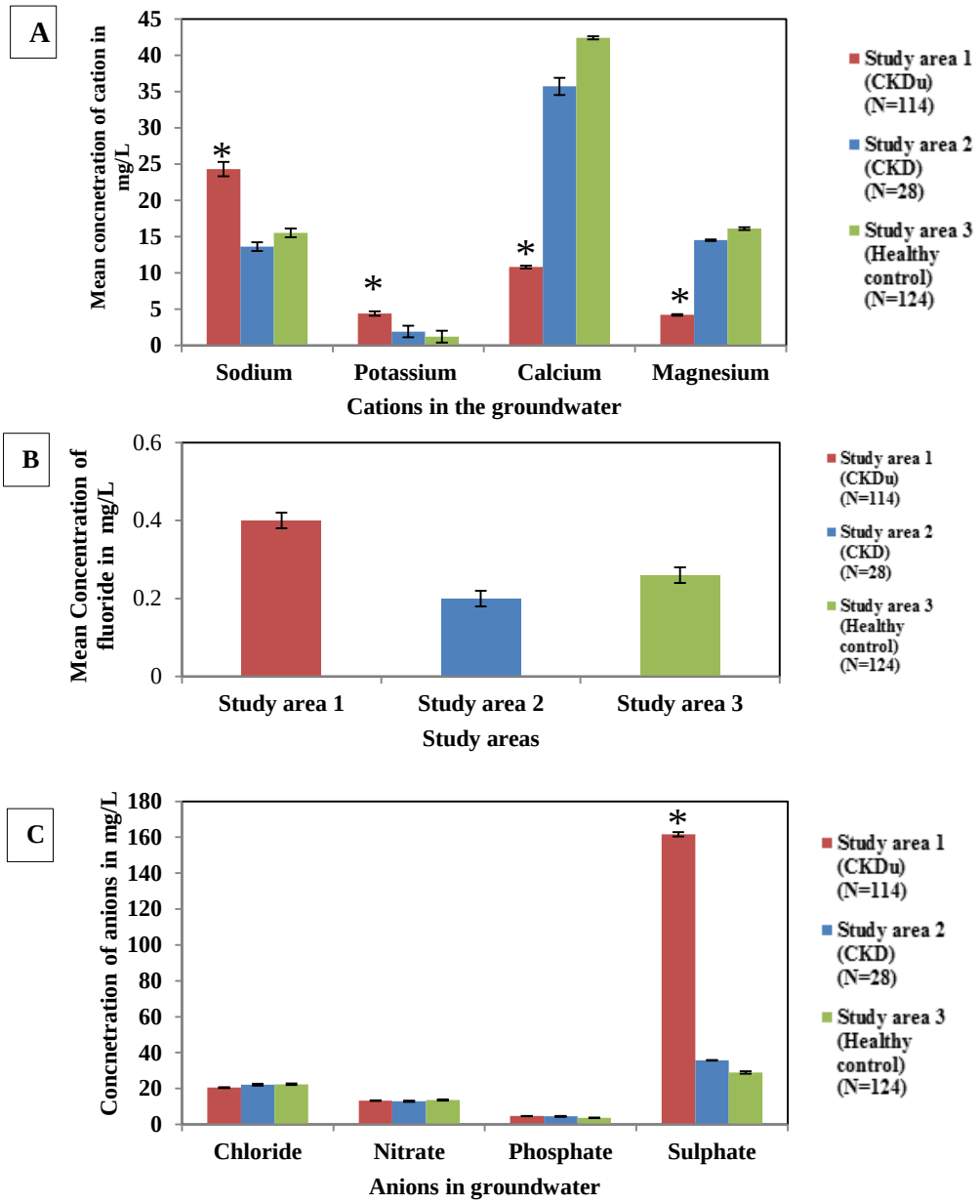


Figure 3.1.3 Chemical characteristics of the groundwater in the three study areas during the three different seasons

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology. Data are derived from two independent experiments with the levels of each parameter in an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which the groundwater samples were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons Pre -Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal variations in the physical characteristics of groundwater.

(A)Concentration of cations viz. sodium, potassium, calcium and magnesium in the groundwater of the three study areas; (B) Concentration of fluoride anion in the groundwater and (C) Concentration of anions viz.chloride, nitrate and sulphate in the groundwater.Values are represented as mean ± SE (standard error) of the levels of a particular parameter analysed over three different seasons/time-points i.e. PRM,MON and POM in all of the three study areas. All the values are in mg L^{-1}.Differences at *$p<0.05$ were considered to be significant. The WHO established permissible limits in potable groundwater for sodium= 50mg/L; potassium= 10mg/L; calcium=75mg/L;magnesium=50mg/L; fluoride=1 mg/L; chloride=50mg/L; nitrate=0.1 mg/L and sulphate=200 mg/L. Significantly higher concentrations of cations viz. sodium and potassium were noted in the CKDu endemic region's groundwater(i.e. study area 1) as compared to study area 2(i.e. diabetes or hypertensive CKD prevalent region) and study area 3(healthy controls) which could be attributed to this region's aquifer's bedrock being made of sodium plagiogranitic gneiss (rich in sodium plagioclase/tonalite mineral) intruded by potassic granites. Hence the bedrock is rich in sodium oxide(Na_2O) and potassium oxide(K_2O) and deficient in calcium oxide(CaO) and magnesium oxide(MgO) which explains the significantly lowered levels of calcium and magnesium in the CKDu region's groundwater as compared to study area's 2 and 3.Ca and Mg cations have been well reported to act as buffering agents and restrict the availability of nephtotoxins like silica and lead in the groundwater by forming inert metal complexes, hence deficiency of the same in the CKDu region's groundwater possibly contributed to increased acidity and availability of these toxins in this region's groundwater.Moreover significantly elevated levels of sulphate anion(approaching the WHO limit) was noted in the CKDu region's groundwater,which supported the possibility of acid mine drainage in this region which contributed to the acidification of the groundwater. The details on the seasonal distribution of the parameter; the minimum to maximum range of values of each parameter and the prevalence distribution (%) of the values of the parameter in various concentration ranges are listed in **Table 3.1.2**.

Table 3.1.2: General chemical characteristics of the groundwater in the three study areas during the three different seasons in reference to the WHO permissible limits and Bureau of Indian Standard (BIS) specifications of drinking water.

CKD status	CKDu Endemic					CKD Non-endemic					No CKD prevalence					Annnotation for the prevalence % of the concentration ranges (in mg L^{-1}) of the resepctive parameters[a]	WHO permissible limit (mg L^{-1})	BIS standards (IS 10500)	
Sampling site and period/ Parameter (mg L^{-1})	Study area/ group 1 (n=114)					Study area/ group 2 (n=28)					Study area/ group 3 (n=124)							Desirable limit (mg L^{-1})	Maximum limit (mg L^{-1})
	PRM	MON	POM	MEAN [a]	Prevalence %[a]	PRM	MON	POM	MEAN [a]	Prevalence %[a]	PRM	MON	POM	MEAN [a]	Prevalence %[a]				
Sodium	26.4±1.0	13.6±0.4	32.8±0.5	24.3±1.0	A=85.1%; B=14.9%	13.5±0.6	10.6±0.4	16.8±0.5	13.6±0.6	A=100%	15.5±0.4	11.3±0.3	19.8±0.7	15.5±0.6	A=100%	A=10-30; B=30-50	50		
	(18.6-29.9)	(10.3-20.4)	(30.3-39.6)	(10.3-39.6)		(10.7-22.3)	(10.0-18.7)	(12.6-22.9)	(10.0-26.9)		(11.6-20.9)	(10.5-17.3)	(13.9-26.4)	(10.5-26.4)					
Potassium	5.3±0.01	1.8±0.02	6.2±0.05	4.4±0.03	A=80.3%; B=19.7%	2.1±0.08	0.8±0.01	2.8±0.09	1.9±0.08	A=100%	1.5±0.05	0.6±0.03	1.6±0.09	1.2±0.08	A=100%	A=0.0-5.5; B=5.5-9.5	10		
	(4.1-5.5)	(1.6-2.1)	(5.9-6.7)	(1.6-6.7)		(1.5-3.3)	(0.5-1.1)	(2.0-5.1)	(0.5-5.1)		(0.7-3.6)	(0.2-0.9)	(1.3-4.9)	(0.2-4.9)					
Calcium	11.1±0.3	7.8±0.2	13.5±0.4	10.8±0.2	A=95.5%; B=4.5%	36.8±0.8	22.3±0.7	48.1±1.3	35.7±1.2	C=30.5%; D=42.5%; E=28.5%	43.5±0.6	35.6±0.2	48.3±0.5	42.4±0.2	D=65.3%; E=34.7%	A=7.5-11.5; B=11.5-15.5; C=19.5-23.5; D=35.5-39.5; E=47.5-51.5	75	75	200
	(9.6-11.5)	(7.5-9.0)	(11.9-14.6)	(7.5-14.6)		(35.5-43.3)	(20.3-36.9)	(35.9-51.1)	(20.3-51.1)		(37.3-45.9)	(35.3-39.5)	(39.5-51.3)	(39.5-45.9)					
Magnesium	5.5±0.2	1.1±0.05	6.2±0.03	4.2±0.1	A=6.6%; B=93.4%	15.8±0.1	10.4±0.1	17.5±0.2	14.5±0.1	C=12.5%; D=68.9%; E=18.6%	18.1±0.3	13.4±0.4	16.8±0.4	16.1±0.2	D=29.6%; E=70.4%	A=1-4; B=4-7; C=10-13; D=13-16; E=16-19	50	30	100
	(1.5-6.3)	(1.0-2.9)	(5.6-6.9)	(1.0-6.9)		(10.6-17.5)	(10.1-13.5)	(15.6-18.9)	(10.1-18.9)		(15.5-18.5)	(13.0-17.3)	(14.3-17.9)	(13.0-18.5)					
Flouride	0.4±0.02	0.26±0.02	0.68±0.03	0.4±0.02	A=98.3%; B=1.7%	0.2±0.02	0.1±0.05	0.26±0.03	0.2±0.02	A=100%	0.27±0.02	0.21±0.03	0.3±0.02	0.26±0.02	A=100%	A=0.1-0.5; B=0.5-0.8	1	0.6-1.2	1.5
	(0.2-0.45)	(0.21-0.36)	(0.38-0.70)	(0.21-0.70)		(0.15-0.35)	(0.10-0.15)	(0.20-0.42)	(0.10-0.42)		(0.15-0.36)	(0.18-0.25)	(0.26-0.48)	(0.18-0.48)					
Chloride	19.5±0.3	13.6±0.1	28.5±0.3	20.5±0.3	A=90.3%; B=9.75%	24.6±0.3	12.3±0.5	29.4±0.1	22.1±0.5	A=10.3%; B=89.7%	23.6±0.2	11.2±0.5	31.4±0.3	22.4±0.4	A=8.9%; B=91.1%	A=11-22; B=22-32	50	45	100
	(15.6-21.9)	(12.3-16.9)	(19.8-30.3)	(15.6-30.3)		(16.7-29.3)	(12.0-25.5)	(22.9-32.0)	(12.0-32.0)		(15.6-27.3)	(11.0-23.6)	(25.9-31.9)	(11.0-31.9)					
Nitrate	8.4±0.1	13.6±0.1	18.1±0.3	13.3±0.2	A=85.6%; B=14.4%	7.1±0.2	11.0±0.3	20.3±0.2	12.9±0.3	A=88.1%; B=11.9%	8.8±0.2	12.3±0.6	19.6±0.2	13.6±0.4	A=87.3%; B=12.7%	A=7-14; B=14-21	0.1	-	-
	(7.5-9.9)	(11.6-14.0)	(13.1-19.6)	(7.5-19.6)		(7.0-8.5)	(8.3-13.5)	(12.5-21.0)	(7.0-21.0)		(7.7-9.8)	(9.3-13.9)	(13.6-20.9)	(7.7-20.9)					
Phosphate	3.6±0.1	4.8±0.2	5.9±0.1	4.7±0.1	A=92.6%; B=7.3%	2.9±0.1	4.5±0.2	6.1±0.3	4.5±0.3	A=95.6%; B=4.4%	1.8±0.06	3.4±0.22	5.8±0.21	3.7±0.23	A=97.9%; B=2.1%	A=1-5; B=5-9			
	(1.5-5.2)	(2.8-6.3)	(3.9-8.1)	(1.5-8.1)		(2.3-6.5)	(3.4-7.7)	(4.5-8.9)	(2.3-8.9)		(1.0-5.0)	(2.2-5.8)	(2.7-7.5)	(1.0-7.5)					
Sulphate	**132±1.6***	**163±1.3***	**189±1.3***	**161.6±1.2*[b]**	B=5.5%; C=94.5%	33.2±0.2*	35±0.2*	38.6±0.4*	35.7±0.2*	A=100%	26.4±0.2*	28±0.7*	31±0.4*	28.9±0.7*	A=100%	A=0-50; B=100-150; C=150-200	-	200	400
	(125.6-155.3)	**(133.7-186.9)**	**(159.9-195.6)**	**(125.6-195.6)**		(27.5-38.9)	(30.3-40.6)	(33.6-41.2)	(27.5-41.2)		(22.7-32.3)	(25.6-35.7)	(29.7-38.9)	(22.7-38.9)					

Abbreviations: BIS-Bureau of Indian standards; CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. Data are derived from two independent experiments with the levels of each parameter in an individual experiment measured in triplicates. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected.

a Values are represented as mean ± SE of a particular parameter, measured in all the samples(n) collected repeatedly during three different sampling time points- Pre -Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) in the three study regions. The range of minimum to maximum values attained for a given parameter have been denoted in parenthesis individually for each sampling time-point (i.e. PRM, MON and POM) and cumulatively for the average of all the seasons combined. (i.e. mean of all seasons) . All the values are in mg L^{-1}.Differences at *$p<0.05$ were considered to be significant.The prevalence of the mean concentration of each parameter(averaged over all the sampling seasons combined) in various ranges of their levels in the groundwater of the three study regions have been represented by prevalence % and annotated by uppercase letters(i.e. A to E) for convenience. The annotations for each estimated parameter varies and have been independently described. The prevalence % denotes the proportion of samples from the total number in particular study region bearing the parameter at the stated concentration range. For example, the prevalence % annotation of 'A' in the case of sodium levels denotes the percentage of the total number of samples analysed in a particular study region possessing sodium concentrations at the range of 10-30 mg/L.

b The values(highlighted in bold) denoted levels which are a matter of concern. These values were reported to be higher only in the CKDu endemic region as compared to the CKD non-endemic and healthy control regions and were nearing the WHO established upper permissible limit for drinking water**.**

All the characteristics exhibited a significant seasonal variation, wherein the levels were higher POM due to rain water recharge of the aquifer resulting in excessive leaching of various elements (i.e. toxic metals; non-toxic/trace metals; major cations and anions) into the groundwater. The levels of these elements immediately dropped during MON due to dilution effect from continuous and prolonged precipitation and consequently increased PRM (summer) due to increased temperature associated higher rates of evaporation resulting in enhanced residence time and concentrating effect on these elements (Han et al., 2010; Kumar & Kumar, 2013; Noli & Tsamos, 2016; Wongsasuluk et al., 2014) **(Tables 3.1.1 and 3.1.2).**

Some of the physical characteristics of groundwater **(Figure 3.1.2 & Table 3.1.1)** like pH, alkalinity, total dissolved solids (TDS) and electrical conductivity (EC) showed significant ($p<0.05$) spatial variations as well. The most significant spatial heterogeneity was seen in groudwater's pH in the 3 study areas. Herein an acidic pH with a mean level of 5.6±0.02 (range=5.3 to 5.9) was noted in CKDu endemic study area 1 with around 95% of samples being prevalent in lower pH range of 5.25-5.75 and remaining 5% in the pH range of 5.75-5.9.Overall 100% of the samples were present in the pH range that was well below the lower normal limits of WHO and BIS permissible standards for drinking water as compared to other 2 study areas (which recorded a neutral pH) making the groundwater of study region1 unsuitable for consumption. This increased acidity could be explained by possibility of acid mine drainage (AMD) taking place in open quarries/pits of the abandoned granite mine located in Chaudi named village of Canacona (Nordstrom,2011; Udayabhanu and Prasad,2010). AMD is a chronic environmental problem that occurs for ages even after mining stops and is witnessed in hard rock mines (like granite in this case) and more severe in tropical areas with moderate rainfall like Canacona. AMD produces large amounts of sulphuric acid (sulphate and hydrogen ions) due to rapid oxidation and dissolution(by precipitation) of exposed ubiquitous sulphide minerals present in open pits and overburden thus decreasing the acidity of groundwater present in its immediate vicinity (Galhardi and Bonotto, 2016; Jacobs et al., 2014). Through the assistance of dendritic drainage as prominently observed in Canacona (Nadaf, 2009e), the acidic groundwater from abandoned mining area could have made its way to neighboring aquifers of CKDu hit endemic villages-Ponsulem and Chaudi (located in close proximity of this mine) resulting in acidic pH in these areas as well (Kumari et al., 2010; Simate and Ndlovu, 2014). This possibility of AMD was also supported by dominance of sulphate anion (mean= 161.6±0.02 mg/L; min-max=125.6-195.6 mg/L) in groundwater of this region with 95% of samples being prevalent in their higher concentration range (150-200 mg/L) as compared to other 2 study regions **(Figure**

3.1.3 & Table3.1.2). It has been reported that excess sulfate causes respiratory discomfort and cathartic effect on digestive tracts (Galhardi and Bonotto, 2016; McCarthy, 2011). As indicated in **Figure 3.1.3 and Table3.1.2,** sulfate levels were 4.5 fold higher than non-endemic study area 2 and 5.5 fold higher than areas with no CKD incidence (study area 3).

Like other physical parameters, pH also showed significant seasonal variation where it decreased to 5.5 during MON due to continuous precipitation that caused increased dissolution and oxidation of ubiquitous sulfide minerals present in open pits of the abandoned mine causing increased production of sulfuric acid, that drained into neighboring CKDu hit region's aquifer (study area1),thus further decreasing groundwater acidity(Galhardi and Bonotto,2016; Halim,et al.,2013; Jacobs et al.,2014; Khandare et al.,2015; Machiraju et al., 2013). Neutral pH of areas 2 and 3 can be justified from lack of proximity of these villages to granite mine hence escaping vicious clutches of AMD (Khandare et al.,2015) **(Table 3.1.1).**

The other factors that contributed to increased acidity of CKDu endemic region's groundwater (study area 1) could be decreased availability of Calcium (Ca) [mean=10.8± 0.02 mg/L, range=7.5-14.6 mg/L] & Magnesium (Mg)[mean=4.2±0.03 mg/L, range=1.0-6.9 mg/L] as indicated by low levels found in these areas with 95 % &93% of the samples being prevalent in their corresponding lower ranges for Ca and Mg respectively **(Figure 3.1.3 and Table 3.1.2).** This can be attributed to the sodium plagiogranitic gneiss geological cover(rich in sodium plagioclase/tonalite mineral) intruded by potassic granites in CKDu-endemic region(study area1) (Fernandes & Widdowson,2009).Hence these regions are rich in sodium oxide (Na_2O) &potassium oxide (K_2O) & deficient in calcium oxide (CaO) & magnesium oxide (MgO) ultimately leading to slightly elevated levels of Na and K as compared to Ca & Mg **(Figure 3.1.3 & Table 3.1.2)** due to acidic pH intensified rock-water interaction (Galhardi and Bonotto, 2016; Machiraju et al., 2013;Srinivasamoorthy et al., 2008). Increase in Na levels could also be supported by reverse cationic exchange with Ca, wherein Ca has a strong tendency to adsorb to clay particle surfaces containing inherent Na, thus resulting in leaching out of Na into groundwater and decrease in Ca levels. Thus, due to lack of buffering agents like Ca and Mg in groundwater, pH could have decreased even further (Diop and Tijani, 2008;Halim et al., 2013 Machiraju et al., 2013;Kumar and Kumar, 2013;Kumari et al., 2010; Nordstrom, 2011; Subramani et al., 2010).Contrarily, comparatively higher Ca & Mg levels and neutral pH of groundwater was observed in study area 2 & 3(non-endemic CKD hit and healthy villages**) (Figure 3.1.3 & Table 3.1.2)** due to geological constitution of its aquifer's bedrock comprising of greenschists metabasitic which flanks the granitic belt of CKDu endemic region (Fernandes and Widdowson,2009).Greenschists metabasites contain

varying levels of silicate minerals like chlorite, epidote, actinolite which are enriched in Calcium (Ca), iron (Fe), manganese (Mn), zinc (Zn), magnesium (Mg) in mineral lattice as a result of substitution of silicon (Si) and aluminium (Al) with these elements during metamorphosis. Hence Ca and Mg will be enriched in these areas groundwater through natural rock-water interactions (Daae et al., 2015; Singh et al., 2012).

The acidic groundwater of study area 1 (CKDu endemic region) led to side effects like elevated TDS [mean= 361.5±0.02mg/L, min-max=321-410mg/L] and EC [mean=322.2±0.03, min-max= 247-315 mg/L] levels in this area **(Figure 3.1.2 & Table 3.1.1).** High EC and TDS indicates that the groundwater is contaminated with inorganic elements like heavy and trace metals due to the AMD associated leaching of these elements into the water table. It has been well documented that discharge of heavy metals into a water body raises the EC as metallic ions conduct electricity. Thus the low pH, High EC & TDS observed indicates a cause for concern for the possibility of induction of a grave health hazard in this region (i.e. nephrotoxicity in this case) (Giri, & Singh, 2015; Halim et al., 2013; Machiraju et al., 2013).

Some of the chemical parameters **(Figure 3.1.2 & Table 3.1.2)** like nitrate, phosphate & chloride were well within permissible limits &comparable between study areas; but showed a seasonal trend with an increase observed POM (Chidambaran et al., 2013).This is mainly due to anthropogenic influence like agricultural runoff from fields containing fertilizers (rich in nitrates, phoshates etc) or saltwater (enriched in fluoride and chloride) intrusion. However, their levels are not high enough to cause health problems developed from excess nitrate like goiter, hypertension, methemoglobinemia, birth malformations, gastric cancer, and from excess chloride like hyperacidity and hypertension; suggesting low agricultural impact on groundwater in these areas; supporting limited usage of chemical fertilizers in this region (as noted from demographic study) (Edition, 2011; Fronczyk et al., 2016; McCallum et al., 2015; Noack et al., 2013; Rahmati et al., 2015; Singaraja et al.,2015; Wongsanit et al., 2015). The fluoride levels were also well within the permissible limits suggesting skeletal disorders observed in endemic villages cannot be attributed to fluoride. Excess Fluoride (fluorosis) is known to interfere with calcium mineral metabolism and homeostasis of bone and teeth causing decalcification, ligaments deformation tendons mineralization resulting in skeletal disorders like osteomalacia & dental disorders like enamel destruction, gum mottling, yellow teeth discoloration, retinal disorder, nervous disorders (Chakraborti et al., 2016;Goodarzi et al., 2016; Mohammadi et al., 2017). Fluorosis was ruled out during demographic study itself due to absence of the visible signs and symptoms & safe levels of fluoride present in groundwater at similar extents in all 3 study regions.

3.1.3.2 Trace metal profile of the groundwater of the three study regions of Canacona

Trace metals includes group of elements that occurs in natural and perturbed environments in small amounts and when present in sufficient bioavailable concentrations are toxic to living organism. They are limited in their distribution in groundwater and hence present in trace amounts due to normal geochemical weathering processes as a result of bedrock-groundwater interactions. On escalation of their levels (surpassing WHO permissible limits) as a result of anthropogenic influence on that area's groundwater reservoirs by activities like mining, untreated effluent discharge into land and seepage into water table from industries(like steel processing, iron ore and coal extraction, electroplating, metal smelting, textile, tanning) can inflict toxic health effects (Förstner and Wittmann, 2012). For example, trace metals like iron and zinc overdose are known to cause direct injury or necrosis of gastrointestinal mucosa manifesting in vomiting, diarrhea etc. along with induction of metabolic acidosis and disruption of muscular coordination (Carson, 2018;Prashanth et al., 2015). Whereas trace metal-Mn poisoning has been reported in inducing neurological and erythropoietic disorders (Carson, 2018; Ghazali et al., 2018; Jaishankar et al., 2014; Kavcar et al., 2009; Kharim, 2011; Nordberg et al., 2014). Copper and aluminum overdose have been known to exhibit mucosal irritation, capillary damage, central nervous system and hepatic injuries. Cobalt and nickel toxicity have been reported in causing asthma, wheezing and various other pulmonary disorders. Molybdenum overdose causes systemic sclerosis development. Some of the trace metals at toxic doses like vanadium, chromium, silver, and molybdenum have been reported to cause acute disruption of renal function in animal models whose toxicity can be reversed on metal chelation therapy. However no such nephrotoxicity of these metals have been noted in human epidemiological studies (Carson, 2018; Ghazali, et al., 2018; Giri and Singh, 2015; Mozrzymas, 2018; Wongsasuluk et al., 2014). However in the current study, levels of all estimated trace metals were found to be well within the WHO acceptable range for drinking water **(Figure 3.1.4 & Table 3.1.3)** (Edition, 2011;WHO, 2011). Moreover trace metal profile showed no significant seasonal variations but exhibited significant spatial variation among the 3 study areas. Herein mean levels of all essential trace metals in CKDu affected region's groundwater (study region 1) were significantly ($p<0.05$) reduced & prevalent in their respective lower concentration ranges as compared to levels in CKD-non endemic (study region 2) & healthy control regions (study region 3) **(Figure 3.1.4 & Table 3.1.3).** These trace metals are crucial for various metabolic functions in humans, thus failure in preservation of optimal levels enhances danger of health hazards (Nordberg et al., 2014).

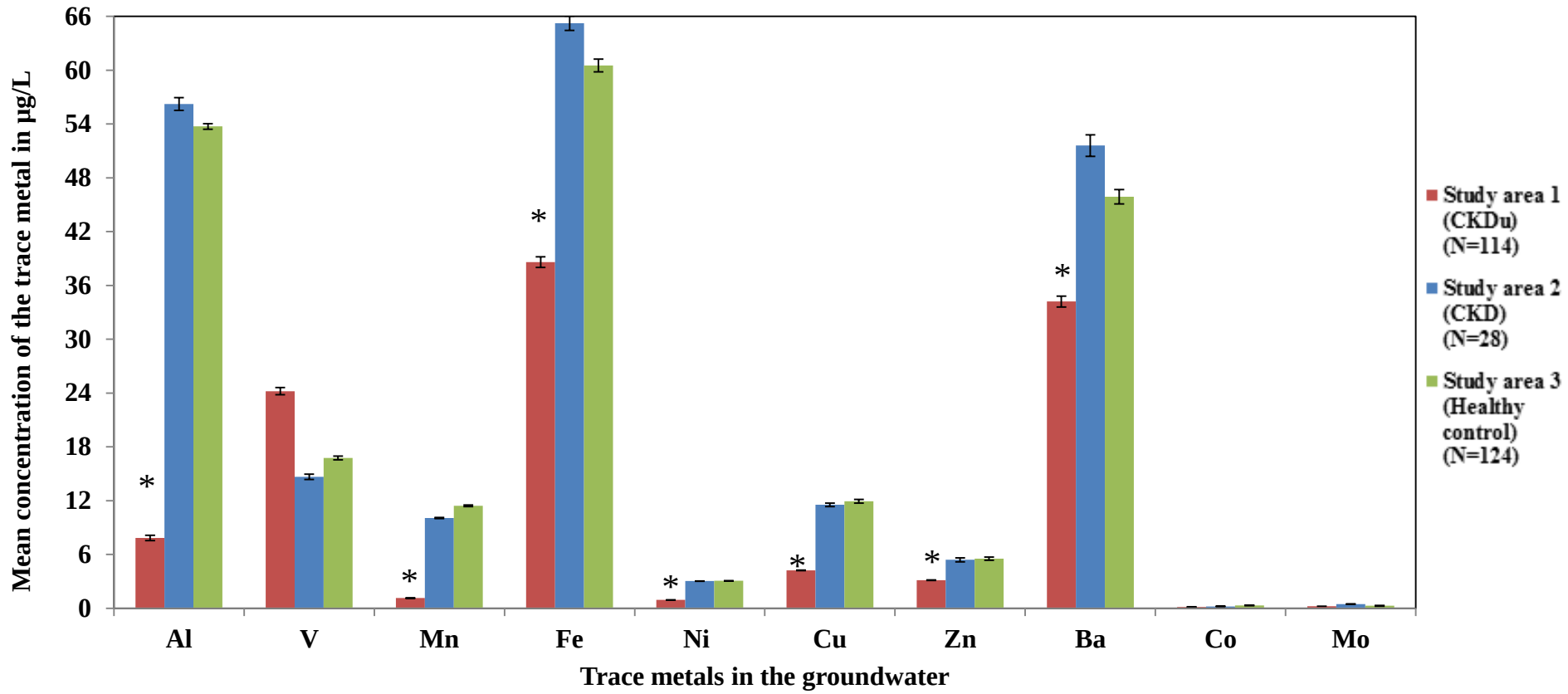


Figure 3.1.4:Trace metals profile of the groundwater of the three study areas during the three different seasons

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology. Data are derived from two independent experiments with the levels of each parameter in an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which the groundwater samples were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons Pre -Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal variations in the physical characteristics of groundwater. .Values are represented as mean ± SE (standard error) of the levels of a particular parameter analysed over three different seasons/time-points i.e. PRM,MON and POM in all of the three study regions. All the values are in µg L^{-1}. Differences at *p<0.05 were considered to be significant. The WHO established permissible limits in potable groundwater for Aluminium(Al)=200 µg L^{-1}; Vanadium(V) =50 µg L^{-1}; Manganese(Mn)= 50µg L^{-1}; Iron(Fe)=300µg L^{-1};Nickel(Ni)=70 µg L^{-1};Copper(Cu)= 2000µg L^{-1}; Zinc(Zn)=3000 µg L^{-1};Barium (Ba)=700 µg L^{-1};Cobalt=50 µg L^{-1} and Molybdenum(Mo)=70 µg L^{-1}. Silver(Ag) and Chromium(Cr) were present at below detectable levels in the groundwater of all three study areas, hence are not represented in the figure.Trace metals like Al,Mn,Fe,Cu,Zn,Ni and Ba have been previously reported in restricting the availability of nephrotoxins like heavy metals such as lead and silica by forming inert metal complexes with these toxins that prevents solubilisation of these toxins in the groundwater and hence reduces the availability of these toxins for uptake through water consumption,thus contributing to the aversion of nephrotoxic effects.However these trace metals were found to be present at significantly lowered levels in the groundwater of the CKDu endemic region(study area 1) as compared to the study area 2(i.e. diabetes or hypertensive CKD prevalent region) and study area 3(healthy controls); which could have possibly contributed to the increased availability of the nephrotoxins i.e.silica and lead in the groundwater of the CKDu region. The details on the seasonal distribution of the parameter; the minimum to maximum range of values of each parameter and the prevalence distribution(%) of the values of the parameter in various concentration ranges are listed in **Table 3.1.3**.

Table 3.1.3:Trace metals profile of the groundwater of the three study areas during the three different seasons with reference to the WHO standards.

CKD status	CKDu Endemic					CKD Non-endemic					No CKD prevalence					Annnotation for the prevalence % of the concentration ranges (in µg L^{-1}) of the resepctive parameters	WHO permissible limits (µg L^{-1})
Sampling site and period/ Parameter (µg L^{-1})	Study area/ group 1 (n=114)					Study area/ group 2 (n=28)					Study area/ group 3 (n=124)						
	PRM	MON	POM	MEAN [a]	Prevalence %	PRM	MON	POM	MEAN [a]	Prevalence %	PRM	MON	POM	MEAN [a]	Prevalence %		
Al	7.5±0.1*	7.8±0.4*	8.2±0.3*	7.83±0.3*	A=100%	56.6.±0.8*	53.4±0.1*	58.7±0.4*	56.23±0.7*	B=89.6%; C=10.4%	53.4±0.3*	51.7±0.2*	56.1±0.1*	53.73±0.3*	B=100%	A=3-11; B=50-58; C=58-64	200
	(5.5-9.6)	(6.9-10.3)	(7.5-10.8)	(5.5-10.8)		(52.6-62.4)	(50.3-59.3)	(52.6-63.4)	(50.3-63.4)		(51.3-56.4)	(50.5-53.6)	(54.3-57.9)	(50.5-57.9)			
V	25.2±0.6	23.1±0.1	24.3±0.3	24.2±0.4	C=100%	14.1±0.2	11.3±0.3	18.6±0.4	14.66±0.3	A=4.3%; B=85.7%	18.4±0.2	15.6±0.1	16.3±0.2	16.76±0.2	B=100%	A=8-14; B=14-20; C=20-26	50
	(22.6-26.0)	(20.6-24.9)	(22.3-25.5)	(20.6-26.0)		(12.3-17.9)	(9.5-16.5)	(13.6-20.0)	(9.5-20.0)		(16.5-19.9)	(12.5-17.4)	(14.1-18.9)	(12.5-19.9)			
Mn	1.1±0.01*	0.8±0.02*	1.5±0.03*	1.13±0.02*	A=100%	10.3±0.04*	8.6±0.05*	11.3±0.06*	10.06±0.06*	B=16.3%; C=84.7%	11.2±0.08*	9.5±0.08*	13.6±0.07*	11.43±0.08*	B=20.3%; C=77.3%; D=2.4%	A=0-2; B=8-10; C=10-12; D=12-14	50
	(0.5-1.7)	(0.3-1.2)	(1.1-1.9)	(0.2-1.9)		(8.5-11.2)	(8.0-10.5)	(9.2-12.0)	(8.0-12.0)		(9.2-13.6)	(8.5-12.9)	(9.4-14.0)	(8.5-14.0)			
Fe	35.3±0.7*	38.6±0.4*	41.9±0.6*	38.6±0.06*	A=98.7%; B=1.3%	68.8±1.01*	54.6±0.82*	72.3±0.61*	65.23±0.8*	C=15.5%; D=82.5%; E=2.0%	60.6±1.1*	51.7±0.72*	69.3±0.83*	60.53±0.7*	C=84.6%; D=15.4%	A=30-40; B=40-50; C=50-60; D=60-70; E=70-80	300
	(30.3-40.6)	(32.6-43.4)	(38.9-49.5)	(30.3-49.50)		(58.9-71.1)	(50.3-65.6)	(59.1-73.2)	(50.3-73.2)		(55.6-66.6)	(50.3-62.1)	(59.6-70.0)	(50.3-70.0)			
Ni	1.1±0.01	0.8±0.02	0.9±0.04	0.93±0.02	A=97.3%; B=2.7%	3.1±0.01	2.4±0.02	3.6±0.03	3.03±0.02	C=79.4%; D=20.6%	2.9±0.02	2.6±0.01	3.7±0.04	3.06±0.02	C=74.6%; D=25.4%	A=0-1; B=1-2; C=2-3; D=3-4	70
	(1.0-1.6)	(0.4-1.2)	(0.6-1.4)	(0.4-1.6)		(2.5-3.4)	(2.0-3.1)	(2.7-3.9)	(2.0-3.4)		(2.4-3.1)	(2.1-3.3)	(2.9-3.9)	(2.1-3.9)			
Cu	4.3±0.02	3.8±0.05	4.6±0.03	4.23±0.03	A=100%	10.5±0.1	9.5±0.2	14.6±0.1	11.53±0.2	B=3.7%; C=86.3.%	11.2±0.1	8.3±0.3	16.3±0.3	11.93±0.2	B=6.5%; C=91.3%; D=2.2%	A=0-5; B=5-10; C=10-15; D-15-20	2000
	(4.0-4.8)	(3.3-4.2)	(4.2-4.9)	(3.3-4.9)		(9.4-13.9)	(9.0-12.6)	(9.9-14.9)	(9.0-14.9)		(9.2-15.5)	(7.5-15.1)	(9.8-15.8)	(7.5-15.8)			
Zn	3.8±0.03	1.4±0.04	4.2±0.05	3.13±0.04	A=100%	6.8±0.23	2.1±0.11	9.3±0.24	5.4±0.23	A=2.7%; B=97.3%	5.4±0.15	1.8±0.13	9.4±0.24	5.53±0.18	A=1.5%; B=98.5%	A=0-5; B=5-10	3000
	(2.5-4.5)	(1.0-1.9)	(3.5-4.9)	(1.0-4.9)		(4.2-8.5)	(1.2-5.6)	(4.7-9.5)	(1.2-9.5)		(3.7-7.8)	(1.0-5.3)	(4.8-9.9)	(1.0-9.9)			
Ba	38.2±0.2	26.3±0.6	39.1±0.7	34.2±0.6	A=4.3%; B=95.7%	54.3±0.6	41.2±0.7	59.3±1.4	51.6±1.2	C=3.9%; D=96.1%	48.3±0.7	38.1±0.8	51.4±0.9	45.9±0.8	B=5.5%; C=92.3%; D=2.2%	A=20-30; B=30-40; C=40-50; D=50-60	700
	(28.9-39.6)	(24.3-35.3)	(29.3-40.0)	(24.3-40.0)		(48.6-55.6)	(40.3-52.3)	(49.1-60.0)	(40.3-60.0)		(35.6-51.2)	(36.6-50.3)	(39.4-53.9)	(35.6-53.9)			
Co	0.18±0.03	0.1±0.01	0.14±0.02	0.14±0.02	A=100%	0.3±0.04	0.1±0.03	0.2±0.01	0.2±0.01	A=5.6%; B=94.4%	0.3±0.03	0.2±0.04	0.4±0.02	0.3±0.03	A=4.1%; B=93.8%; C=2.1%	A=0-0.2; B=0.2-0.4; C=0.4-0.6	50
	(0.08-0.20)	(0.05-0.15)	(0.07-0.19)	(0.05-0.20)		(0.15-0.35)	(0.06-0.25)	(0.17-0.22)	(0.06-0.35)		(0.18-0.46)	(0.15-0.41)	(0.35-0.45)	(0.15-0.45)			
Mo	0.23±0.01	0.18±0.02	0.26±0.03	0.22±0.01	A=10.3%; B=89.7%	0.52±0.01	0.29±0.04	0.54±0.02	0.46±0.02	B=15.5%; C=84.5%	0.27±0.01	0.22±0.03	0.31±0.04	0.27±0.04	B=100%	A=0-0.2; B=0.2-0.4; C=0.4-0.6	70
	(0.15-0.35)	(0.12-0.32)	(0.19-0.39)	(0.12-0.39)		(0.37-0.58)	(0.25-0.45)	(0.39-0.60)	(0.25-0.60)		(0.22-0.34)	(0.20-0.25)	(0.27-0.37)	(0.20-0.37)			
Ag	BDL	BDL	BDL	BDL	-	BDL	BDL	BDL	BDL	-	BDL	BDL	BDL	BDL	-	-	100
	-	-	-	-		-	-	-	-		-	-	-	-			
Cr	BDL	BDL	BDL	BDL	-	BDL	BDL	BDL	BDL	-	BDL	BDL	BDL	BDL	-	-	50
	-	-	-	-		-	-	-	-		-	-	-	-			

Abbreviations: BDL-below detectable levels; CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. Data are derived from two independent experiments with the levels of each trace metal in an individual experiment measured in triplicates. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected.

a Values are represented as mean ± SE of each individual trace metal estimated for all the samples(n) collected recurrently during three different sampling time points-Pre-Monsoon(PRM), Monsoon(MON) and Post-Monsoon(POM) in the three respective study areas . The range of minimum to maximum values attained for a given trace metal have been denoted in parenthesis individually for each sampling time-point (i.e. PRM, MON and POM) and cumulatively for the average of all the seasons combined. (i.e. mean of all seasons). All the values are in (µg L^{-1}).Differences at*$p<0.05$ were considered to be significant.The prevalence of the mean concentration of each trace metal (averaged over all the sampling seasons combined) in various ranges of their levels in the groundwater of the three study regions have been represented by prevalence % and annotated by uppercase letters (i.e. A to E) for convenience. The annotations for each estimated parameter varies and have been independently described. The prevalence % denotes the proportion of samples from the total number in particular study region bearing the parameter at the stated concentration range. For example, the prevalence % annotation of 'A' in the case of aluminum levels denotes the percentage of the total number of samples analysed in a particular study region possessing concentrations at the range of 3-11 µg L^{-1} As indicated in the results, all of the trace metals analysed in the groundwater of the CKDu endemic region were noted to be highly prevalent in their respective lower concentration ranges as compared to the other two study regions(2 and 3) which depicted a larger prevalence in the higher concentration range. These lowered levels of the trace metals in the CKDu endemic region could have influenced the increased bioavailability of the nephrotoxins in the groundwater as these elements at higher concentrations have been well-reported in limiting the availability of the highly prevalent nephrotoxins (i.e. silica and lead) in this region.

Trace metals like Cu, Mn, Zn, Ni, Co, Fe, Al, Mo are necessary for bone metabolism as they serve as metal cofactors of various enzymes associated with the same. It has been well reported that trace metals- Zn, Mo, Co are metallo-cofactors for alkaline phosphatase enzyme involved in phosphate deposition in bone matrix for conferring structural strength. Cu, Ni are cofactors for enzymes involved in lysine oxidation and pyridinium cross-linking of elastin and collagen which constitutes the bone's organic matrix imparting structural tenacity and elasticity. Mn, Al, Ba, Fe are essential for glycosyl transferases enzymes crucial for glycosaminoglycan chondroitin sulfate production which also constitutes the bone's organic matrix necessary for bone growth and development (Angelova et al., 2011; Dermience et al., 2015; Frieden, 2012; Hirayama et al., 2011; Li et al., 2011; Mozrzymas, 2018; Pemmer et al., 2013; Prasad, 2013; Seo et al., 2010; Zheng et al., 2014; Zofková et al., 2013).

Studies on experimental animal models demonstrated that deficiencies in Cu, Zn, Mn, Fe, Al, Ni, Co have led to teratological bone abnormalities & osteoporotic bone conditions. Furthermore Cu, Al, Mn deficient rats have exhibited diminished osteoclast and osteoblast activity that are crucial for bone synthesis. Parallely, in a human epidemiological study, nutritional supplementation over 2-years in post-menopausal women consisting of Ca (800 mg/d), Zn (10 mg/d), Mn (4.0 mg/d), Cu (2.0 mg/d) led to a significant bone mineral density enhancement which averted future osteoporosis incidences in those women. Therefore, deficient intake of these abovesaid trace metals can hinder bone mineral metabolism and homeostasis resulting in increased calcium turnover and disruption of calcification cum bone remodeling that ultimately manifests in development of skeletal disorders like joint pains, bone pain (Aaseth et al., 2012; Dermience et al., 2015; Hirayama et al., 2011; Prasad, 2013; Zofková et al.,2013). Significantly reduced levels of above said trace metal **(Figure 3.1.4, Table 3.1.3)** noted in CKDu endemic study region's groundwater as compared to other two non-affected regions (study area 2 & 3) could possibly explain aggravation of skeletal disorders & joint pains witnessed among CKDu subjects residing in this region (Canacona taluka), probably stemming from decreased intake (through groundwater) of these elements in these subjects. These observations were consistent with the analysis of skeletal disorders in similar CKDu hit regions viz. Uddanam region (Andhra Pradesh) and Central America wherein the groundwater profile of this region displayed lowered trace elements and hence reduced intake by the affected subjects which was postulated to be involved in disturbance of bone mineral homeostasis and manifestation of skeletal injuries in such patients which ultimately triggered increased intake of NSAIDs,a well-known nephrotoxin that could have

contributed to renal damage worsening in these patients (Brooks, 2009; Correa-Rotter et al., 2014; Cozzolino et al., 2014; Khandare et al., 2015; Ganguli, 2016; Wesseling et al., 2013). Additionally ingestion of groundwater deficient in these essential trace metals (Zn, Cu, Mo, Fe, Mn, Ni, Co) enhances susceptibility to increased accumulation in the kidney and associated renal toxicity of heavy metals and silica whose evidence emerges from exposure studies (via groundwater intake) conducted in humans/animals for heavy metals and animals for silica (Carson et al., 2018; Nordberg et al., 2014; Jayatilake et al., 2013). The negative interaction of heavy metals or silica with trace metals stems from the competition of these elements for binding to common metal transporters involved in uptake and transport in the human body specifically in intestines and kidneys (like metallothioneins, metal transporter 1, divalent metal transporter 2, DMT2) due to similarities in chemical structure and valencies of these elements. This shared metal transporters under reduced trace metals(below optimal) and increased heavy metal or silica nephrotoxin conditions preferentially selects for nephrotoxin uptake resulting in competitive exclusion and disruption of intestinal or renal absorption of trace metals that consequently manifests in trace metal elimination from the body & accumulation of heavy metals & silica nephrotoxin in the kidney & associated induction of renal toxicity (Carson, 2018; Díaz-Gómez et al., 2017; Dashnyam et al.,2017; Gil & Hernández, 2015; Maret, 2017; Nordberg et al., 2014; Nordberg and Nordberg, 2016; Sergent et al., 2017; Yang and Shu, 2015). Thus, the deficiency in intake of these trace metals (possibly through groundwater intake) enhances susceptibility of nephrotoxin exposed population (CKDu subjects) to elevated uptake and accumulation of the nephrotoxic heavy metals and metalloid (silica) in the kidney which ultimately results in manifestation of severe renal injury specifically proximal tubular damage. Heavy metals nephrotoxin (like lead) are well reported in inducing oxidative renal tubular damage but silica's toxicity mechanism is unknown, hence pursued in Chapter 4.This heavy metal induced oxidative tubular damage could have been further aggravated by lack of anti-oxidant protection which is conferred by abovesaid trace metals under optimal concentrations as these metals serve as cofactors for anti-oxidative enzymes like SOD & glutathione peroxidase (Carson, 2018; Díaz-Gómez et al., 2017; Goyer, 2016 ;Jan et al.,2015;Khandare et al., 2015; Mozrzymas, 2018; Nordberg et al., 2014; Nordberg & Nordberg, 2016; Prashanth et al., 2015; Wu et al., 2016). Thus these findings provide supportive evidence to increased CKDu incidence (i.e. tubular nephropathy) in study region 1 due to below optimal concentration of trace metals in this region's groundwater as compared to study regions 2 & 3 **(Figure 3.1.4, Table 3.1.3)** that resulted in lowered trace metals intake which manifested in decreased prevention of nephrotoxin

accumulation and failure to provide anti-oxidant protection from associated renal tubular injury. Thus our results were consistent with similarly reduced trace metal profile of groundwater of CKDu hit regions like Sri Lanka &Andhra Pradesh that was proved to assist in increasing nephrotoxins bioavailability in human (i.e. lead, silica), associated toxin accumulation in the kidney and consequent aggravation of renal damage (Ganguli, 2016; Khandare et al., 2015; Jayasumana et al., 2015a; Jayatilake et al., 2013; Levine et al., 2016; Nanayakkara et al; 2014). Additionally, some trace metals (i.e. Fe, Mn, Al, Cu, Zn) have been reported in influencing bioavailability of emerging nephrotoxin viz.trace geogenic element-silica in groundwater for human exposure. These multivalent cations can bind & trap silica by inert metal silicate complex formation at an alkaline pH due to generation of silicate anion at this pH thereby decreasing silica availability in groundwater (Amjad et al., 2010; Zuhl et al., 2013).Thus in this study, reduced levels of these above said trace metals in CKDu region **(Figure 3.1.4 & Table 3.1.3)** as compared to other groups could have resulted in reduced silica trapping, thereby contributed in increased silica availability in CKDu endemic region's groundwater which resulted in enhanced silica exposure (described later).

3.1.3.3 Nephrotoxin (specifically heavy metal) content in the groundwater of the three study regions of Canacona taluka.

Heavy metals are elements possessing atomic weights between 63.546 and 200590 and a specific gravity of 4 that is 5 times greater than water, hence the name. They arise in water from 2 different sources which are (i) natural process[from natural minerals weathering, leaching from ore extraction & volcanic products] (ii) anthropogenic activities [from mining associated AMD induced metals leaching from aquifer's bedrock deposits; untreated effluent disposal into land & seepage into water table from industries (like tanning, paints, fossil fuel, steel electroplating, batteries, plastic, smelting); leaching of metal enriched agrochemical into water table & municipal/domestic solid waste clearance containing dental amalgam, Hg thermometers (Abiola, 2017;Gunatilake et al., 2015; Flora et al., 2012; Jaishankar et al., 2015 Kim et al., 2016; Mohod and Dhote, 2013;Sankhla et al., 2016;Tchounwou et al., 2012; Xu et al., 2018). Like trace metals, heavy metals are minimally present in groundwater but both these metals categories are characteristically different. Heavy metals unlike trace metals have no potential health benefits but are hazardous. Heavy metals unlike trace metals are persistent, thermochemically resistant, possess long half-lives, bioaccumulative, hence are not easily broken down in the environment nor metabolized. Due to their persistent and bioacumulative nature they can perish for several years accumulating in every level of the

food chain (including humans) ,which can inflict severe toxic health effects in humans on chronic exposure to excessive levels (Bánfalvi, 2011; Jaishankar et al., 2015).One such major health hazard inflicted is induction of nephrotoxicity on chronic and higher exposure levels. The well renowned heavy metals that have been reported over years in displaying significantly grave amount of nephrotoxic potency are lead, cadmium, arsenic and mercury and have been implicated in CKDu causation in various environmental monitoring studies conducted in affected countries like Sri Lanka, Central America, India (Bandara et al., 2008; Bandara et al.,2010 a &b; Bandara et al., 2011; Correa-Rotter, 2017;Dharma-Wardana, 2015; Franko et al., 2005; Ganguli, 2016; Gifford et al., 2017; Jayasumana et al., 2011; Jayasumana et al., 2013; Jayasumana et al., 2014; Jayasumana et al.,2016;Johnson et al.,2013;Johnson and Sánchez-Lozada,2013;Lopez-Marin et al.,2014;Orantes et al., 2014; Orr and Bridges,2017; Navas-Acien et al.,2009; Paranagama et al., 2018; Ramirez-Rubio et al., 2013; Rajapakse et al., 2016; Rastogi et al.,2008;Wanasinghe et al.,2018;Wanigasuriya,2011;Wanigasuriya et al., 2012;Wasana et al., 2016; Weaver et al., 2015; Wimalawansa, 2016;Wesseling et al., 2013).

Despite structural & chemical variations in the above said nephrotoxic heavy metals, they all share a common mechanism of nephrotoxicity i.e. mediated by induction of oxidative renal proximal tubular damage which is detailed individually in Chapter1. Nephrotoxicity is mediated by direct attack of heavy metals on mitochondria of nephron's proximal tubular cell (owing to role of these cells in toxin elimination and reconcentration) causing disruption of cellular respiration that results in increased ROS production surpassing the anti-oxidant defense manifesting in oxidative stress. The excessively generated ROS then stimulates inevitable induction of apoptosis and inflammation by respectively activating its key mediators viz. DNA damage and ROS sensitive inflammatory factor (NF-kβ). This heavy metal induced incessant apoptotic cell death and inflammation results in tubular atrophy and fibrosis development respectively causing complete disruption of tubular structural-functional integrity, manifesting in chronic tubulointerstitial nephritis (CTN), typical of CKDu (Bridges and Zalups, 2017; Fowler, 2015; Gifford et al., 2017; Jan et al., 2015; Lentini et al., 2017; Price, 2017; Robles-Osorio et al., 2015; Satarug, 2018; Weaver and Jaar, 2015).

Based on well-established nephrotoxic potencies of heavy metals (Pb, Cd, As, Hg) in inducing CTN, it is crucial to assess the contribution of these potential etiological agents in Canacona's CKDu causation in the current study via evaluation of their levels in their major exposure source viz. drinking groundwater (contaminated by anthropogenic activities).Hence, levels of these nephrotoxins were estimated in groundwater of 3 study regions of Canacona **(Table 3.1.4)** to assess for variations in exposure levels and associated CKDu incidence.

Table 3.1.4 :Nephrotoxic heavy metals profile of the groundwater in the three study regions during the three different seasons with reference to their respective WHO established permissible limits.

CKD status	CKDu Endemic				CKD Non-endemic				No CKD prevalence				WHO permissible limits ($\mu g\ L^{-1}$)
Sampling site and period/ Parameter ($\mu g\ L^{-1}$)	Study area/ group 1 (n=114)				Study area/ group 2 (n=28)				Study area/ group 3 (n=124)				
	PRM	MON	POM	MEAN [a]	PRM	MON	POM	MEAN [a]	PRM	MON	POM	MEAN [a]	
Hg	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0
As	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	10
Cd	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	3
Pb	8.79±0.04*	11.2±0.12*	9.96±0.07*	9.98±0.08*[b]	0.78±0.02	0.98±0.03	0.85±0.01	0.87±0.03	0.68±0.02	0.93±0.03	0.89±0.01	0.83±0.03	10
	(8.5-10.2)	(9.6-11.7)	(9.3-11.2)	(8.5-11.7)	(0.72-1.05)	(0.89-1.20)	(0.77-1.07)	(0.72-1.20)	(0.62-0.93)	(0.85-1.24)	(0.81-1.12)	(0.62-1.24)	

Abbreviations: BDL-below detectable levels; CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. Data are derived from two independent experiments with the levels of each nephrotoxin in an individual experiment measured in triplicates. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected.

a Values are represented as mean ± SE of the lead nephrotoxin estimated for all the samples(n) collected recurrently during three different sampling time points-Pre-Monsoon(PRM), Monsoon(MON) and Post-Monsoon(POM) in the three respective study areas . The range of minimum to maximum values attained for the lead nephrotoxin have been denoted in parenthesis individually for each sampling time-point (i.e. PRM, MON and POM) and cumulatively for the average of all the seasons combined. (i.e. mean of all seasons). All the values are in ($\mu g\ L^{-1}$).Differences at*$p<0.05$ were considered to be significant.

b Values represent the levels of lead which were on the borderline (approaching the upper WHO permissible limits) for drinking water indicating a serious cause for concern and were found to be significantly higher only in the CKDu endemic region (study area 1) which depicted a larger prevalence in the higher concentration range (with details of the prevalence described in Fig.3.1.5) as compared to the CKD non-endemic and control region.Thus highlighting the strong possibility of lead contribution in nephrotoxicity induction in this region.

In the present study, mean levels of 3 estimated nephrotoxic heavy metals viz. Cd, Hg and As were found to be well below detectable limits **(Table 3.1.4)** signifying absence of toxic exposure levels to these nephrotoxins in the taluka via groundwater consumption thereby suggesting no involvement of the same in etiological development of CKDu in Canacona. These results were consistent with findings of other CKDu environmental monitoring studies in Sri Lanka, Central America and Andhra Pradesh that ruled out contribution of these nephrotoxins via exposure through groundwater in CKDu causation endemic to those regions (Almaguer et al., 2014; Chandrajith et al., 2011; Jayasumana et al., 2014; Herath et al., 2018; Khandare et al., 2015,Ganguli, 2016; Levine et al., 2016).These estimated toxins showed no significant seasonal & spatial variations among 3 study areas except for Pb **(Table 3.1.4).**

Interestingly, lead levels in CKDu region's groundwater (study area1) (mean=9.98µg L^{-1}, min-max=8.5-11.7µg L^{-1}) **(Table 3.1.4)** were significantly ($p<0.05$) approaching WHO & BIS set upper permissible limit of 10 µg L^{-1}(on the borderline) for drinking water (Edition, 2011; WHO, 2011) as compared to remarkably lowered, non-toxic Pb levels noted in other 2 study areas **(Table 3.1.4).** 81.1% of the analysed groundwater samples of CKDu endemic region were significantly prevalent in the WHO set borderline range of 8-10 µg L^{-1} **(Figure 3.1.5A)** supporting occurrence of lead at borderline levels in groundwater consumed by CKDu endemic region. These raised Pb levels in CKDu endemic region's groundwater can be explained by confounding interactions between rich metal deposits (i.e. PbS) intruding the aquifer's granitic bedrock (at 2.5% by weight) (Fernandes and Widdowson, 2009), acidic pH **(Table 3.1.1)** and softness (containing low Ca, Mg levels, **Table 3.1.2**) of the groundwater. Acidic pH has been well reported in enhancing lead leaching into groundwater from its metal enriched deposits present in aquifer's bedrock on percolation of acidic groundwater over the bedrock. This was supported by observance of inverse and significant ($p<0.05$) correlations ($r=-0.856$, $p=0.037$) between decreasing pH and rising lead levels of this region's groundwater. This excessive lead leaching could have been further aggravated by CKDu region groundwater's softness (containing low Ca and Mg, **Table 3.1.2**) as these elements are reported to restrict lead dissolution and hence its bioavailability by forming inert complex metallic salts thereby trapping Pb (Chowdhury et al., 2016; Du et al., 2014; Halim et al.,2013; Förstner & Wittmann, 2012; Karim, 2011; Kavcar et al., 2009; Kumari et al.,2013;Machiraju et al., 2013; Sankhla et al., 2016). This was further backed by occurrence of strong, negative and significant ($p<0.05$) associations between Pb-Ca ($r=-0.899$, $p=0.025$) and Pb-Mg ($r=-0.785$, $p=0.014$), supporting these divalent metals capacity in decreasing lead availability.

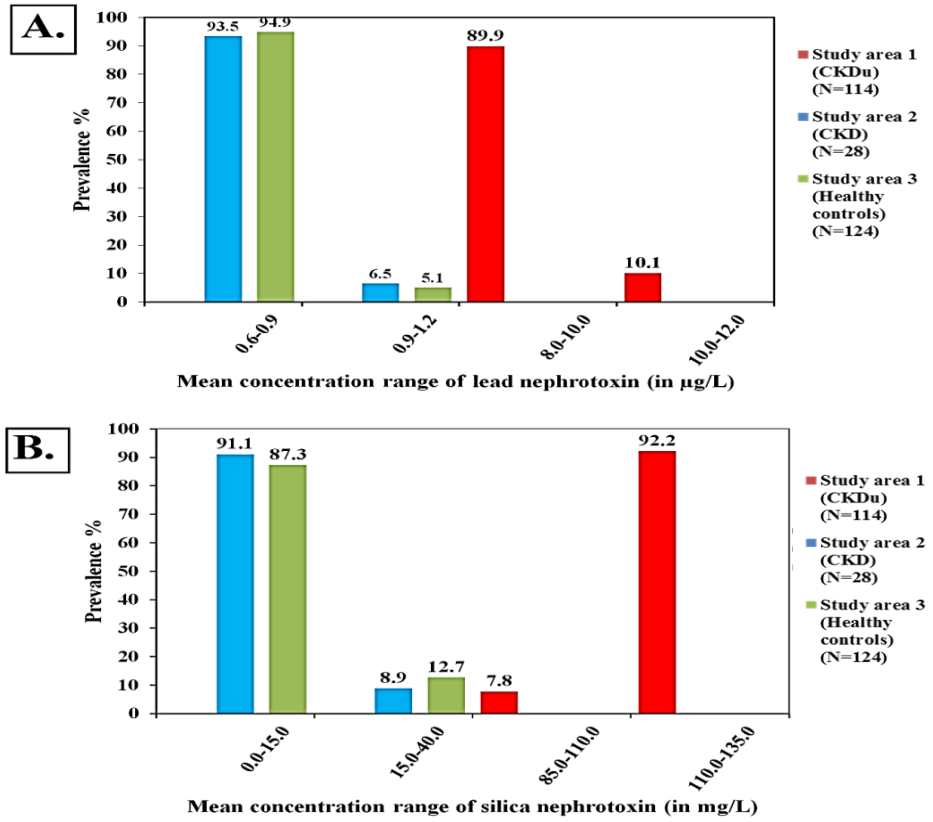


Figure 3.1.5. Prevalence of the noted nephrotoxins (viz. silica and lead) at various levels of their concentration range analysed in the groundwater regularly consumed by study population of Canacona

The occurrence of the mean concentrations of the lead(A) and silica(B) nephrotoxins (analysed and averaged over all the three sampling seasons combined) in various ranges of their levels in the groundwater of the three study regions have been represented by the prevalence %. The prevalence % denotes the proportion of groundwater samples from the total number analysed in particular study region bearing the nephrotoxins (lead or silica) at the stated concentration range. As indicated the levels of both silica and lead nephrotoxins in the groundwater of the CKDu endemic region (study region 1) were found to be prevalent in their respective higher concentration ranges as opposed to their levels in the groundwater of the other two study regions (viz. CKD-non endemic study region 2 and healthy control region 3) wherein they were highly prevalent in their corresponding lower concentration ranges. This prevalence of silica and lead in their respective higher concentration range in the CKDu endemic region's groundwater could be attributed to the acid mine drainage from the neighboring non-functional granite mine that could have caused the excessive leaching of silica and lead from their high reserves present in the constituting granitic bedrock of this region's aquifer. Whereas the prevalence of silica and lead in their respective significantly lower concentration ranges in the groundwater of study region 2 and 3 could be attributed to the neutral groundwater-bedrock interactions noted in these regions that could have resulted in the reduced leaching of silica and lead from the constituting aquifer's metabasitic bedrock (which are innately silica and lead deficient as analysed by Widdowson et al.). The prevalence of silica and lead in their respective higher concentration ranges in the CKDu affected regions suggests a higher degree of exposure of the affected subjects to these nephrotoxins via groundwater consumption that could have chronically manifested in severe renal tubular damaging effects that ultimately exaggerated into the development of a higher incidence of CKDu in the Canacona taluka.

Another plausible lead source could be ages old, inferior quality PVC based well casing lining the interior of bore wells in Canacona. These PVC casing are Pb enriched in form of lead stabilisers, since high quality pipes (devoid of Pb stabilisers) are unaffordable by this rural and economically poor population. Thus enhanced acidity **(Table 3.1.1)** and increased residence time of groundwater in bore wells of CKDu-endemic region could have caused increased lead leaching thereby substantially contributing to elevated lead levels noted in this region (Chowdhury et al., 2016; LEAD Action News, 2010; Parker et al., 1990). These levels were further escalated during monsoon as compared to other 2 seasons due to increased groundwater acidity as a result of intensified acid rock drainage during this season that causes significantly enhanced lead leaching from its potential sources i.e. granitic bedrock and bore

well casing in CKDu endemic region's groundwater (Chowdhury et al., 2016; Förstner and Wittmann, 2012; Machiraju et al., 2013; Sankhla et al., 2016).On the contrary, negligible lead levels were reported in CKD non-endemic region's (study area 2) [mean=0.87±0.03μgL^{-1}, range=0.72-1.2μgL^{-1}] & healthy control region's (study area 3)groundwater [mean=0.83± 0.03 μgL^{-1},range=0.62-1.24μgL^{-1}] **(Table 3.1.4)**,with 93.5% and 94.9% of samples in study region 2 and 3 respectively containing lead levels at their lowest concentration range of 0.6-0.9 μgL^{-1}**(Figure 3.1.5A).** This lowered Pb levels in groundwater of these two study regions (2 and 3) could be linked to groundwater's neutral pH (resulting in decreased bedrock-water interactions and Pb leaching from the well casing) and non-availability of Pb deposits in the metabasitic geological cover (Fernandes and Widdowson, 2009) constituting the aquifer bedrock of these regions (Chowdhury et al., 2016; Daae et al., 2015; Förstner and Wittmann, 2012; Henry, 2015; Singh et al., 2012). This was further supported by occurrence of inverse and insignificant ($p>0.05$) correlations of -0.098 ($p=0.213$) and -0.074 ($p=0.097$) between neutral pH and minimal lead levels in the groundwater of study regions 2 and 3 respectively.

Few epidemiological studies and dose-exposure analysis have shown that chronic exposure to borderline lead levels (i.e. 8-10 μgL^{-1}) via consumption of contaminated drinking water causes progressive induction of renal tubular dysfunction marked by decreased creatinine clearance, increased serum creatinine and urea, low plasma β2-microglobulin level, lowered GFR and hyperuricemia, all of which are major biomarkers of CTN. Levels of this disease biomarkers can be improved & reversed on adoption of chelation therapy (CDC, 2012; CDC, 2015; Fadrowski et al., 2013; Fowler et al., 1980; Flora et al., 2012; Lin et al., 2006; NIH, 2012; Payton et al., 1994; Rastogi, 2008; Weaver & Jaar, 2015).Moreover etiological assessment studies in CKDu hit regions of Sri Lanka & Central America have shown that low-level environmental Pb exposure (6-8μgL^{-1}) over time causes development of prominent lead inclusion bodies in proximal tubular cells which ultimately results in development of interstitial fibrosis & tubular dysfunction, characteristic features of CTN. This nephrotoxicity exhibited even at low level exposure over time is mainly attributed to intrinsic potency of proximal tubular cells to reconcentrate multivalent metals ions (like trace and heavy metals) and lead's bio-accumulative property and long-half life (30 yrs) in renal proximal tubule, which cumulatively causes lead accumulation and progressive aggravation of lead induced renal tubular damaging effects with time that consequently manifests in CTN (Chandrajith et al., 2011; Correa-Rotter, 2017; Levine et al., 2016; Jayatilake et al, 2013; McClean et al., 2012; Navas-Acien et al., 2009; Wesseling et al., 2013; Wijkström et al., 2018). Our results were consistent with findings of aforementioned previous epidemiological and environmental

monitoring analyses conducted in CKDu hit regions of Central America and Sri Lanka, wherein borderline lead levels noted in these studies were established to cause severe proximal tubular dysfunction (typical of CKDu) over chronic exposure thereby suggesting strong and significant involvement of lead nephrotoxin at such borderline exposure levels (via chronic groundwater consumption) in etiological development of CKDu in Canacona.

Additionally, few epidemiological studies have stated that these borderline lead levels could also induce indirect nephrotoxicity by interfering with bone mineral metabolism and calcium homeostasis causing skeletal disorders which explains increased skeletal disorder prevalence noted in 55% of CKDu population, thereby causing them to over-consume NSAID (well-reported nephrotoxin) for pain alleviation (Chapter 2), which could have further aggravated lead induced renal damage (Brooks, 2009; Correa-Rotter et al., 2014; Cozzolino et al., 2014; Ganguli, 2016; Jayatilake et al., 2013; Khandare et al., 2015;Wesseling et al., 2013).

Lead induced skeletal toxicity is mediated by two ways, each in bone and kidney. Lead on uptake and absorption sequesters to some extent in bone matrix as well due to its structural homology with the major matrix constituting metal ion viz. calcium thereby selectively displacing the latter metals ions from the matrix causing faulty calcification, decalcification, and bone remodeling manifesting in significant calcium loss, calcium homeostasis disruption and associated induction of bone-disorders. Whereas in the kidney, excessively accumulated Pb over time interferes with vitamin D metabolism associated with bone mineral homeostasis. Wherein Pb inhibits vitamin D activation (i.e. conversion of vitamin D to 1,25-dihydroxyvitamin D, its active form, which takes place primarily in renal tubule)ultimately resulting in Vitamin D deficiency. Vitamin D deficiency reduces intestinal calcium & phosphate absorption leading to parathyroid hormone(PTH)overstimulation causing increased turnover & calcium loss from bones to maintain optimum levels; ultimately impairing calcium homeostasis. This consequently leads to defective bone mineralization, bone density & apatite formation reduction manifesting as osteomalacia (Assi et al., 2016; Caito et al., 2017; de Souza et al., 2018; Flora et al., 2012; García-Esquinas et al., 2015; Gil et al., 2015; Dermience et al., 2015; Kalantar-Zadeh et al., 2010; Maret, 2017; Pemmer et al., 2013;Ruiz et al., 2016; Shroff et al., 2016;Tomaszewska et al., 2016). Thus osteomalacia caused by chronic borderline lead exposure (through groundwater consumption) could also be causative factors for severe body and joint pains reported in the CKDu endemic region (study region 1).

Overall, these findings highlights Pb's diverse nephrotoxicity mechanisms at our currently reported borderline exposure levels (9.98μgL^{-1}) **(Table 3.1.4)** wherein it can inflict direct nephrotoxicity by direct attack on targeted renal proximal tubule or indirectly by disrupting

calcium and mineral homseostasis in these affected subjects causing skeletal pains that inevitably resulted in elevated consumption of a potential nephrotoxin viz. NSAID. This lead induced direct and indirect nephrotoxicity cumulatively could have manifested in enhanced proximal tubular dysfunction that resulted in CTN which ultimately progressed into irreversible chronic renal failure which is typical of CKDu pathology (Brooks, 2009; Buser et al., 2016; Cozzolino et al., 2014; de Souza et al., 2018; Flora et al., 2012; Khandare et al., 2015; Kim et al., 2015; Laws, 2015; Ruiz et al., 2016; Tchounwou et al., 2012; Weaver and Jaar, 2015).Thus supporting strong contribution of Pb in CKDu causation in Canacona.

3.1.3.4 Trace geogenic element profile including the silica nephrotoxin levels in the groundwater consumed by the three study regions of the Canacona taluka

Geogenic elements as the name suggests include groups of elements derived from the earth's crust and are inevitably present at low levels in the groundwater as a result of natural groundwater-bedrock (of the aquifer) interactions. These naturally occurring geogenic elements possesses limited bioavailability under ideal environmental conditions due to its limited solubility in natural water (at a neutral pH) and is not essential for basic metabolic functions. However these elements on increased bioavailability (possibly due to low pH associated increased water interactions with bedrock (enriched in geogenic elements)has been reported in inducing health hazards (Centeno et al., 2016; Reimann and De Caritat, 2012; Reimann et al., 2007; Voutchkovaet al., 2015). The commonly occurring geogenic elements in groundwater include lithium, boron, strontium & silica. Owing to health damaging potency of these elements, it is mandatory to analyse presence of these elements in drinking water source of the exposed population to assess for exposure levels and possible health injuries that can be inflicted. Hence in this study, levels of these geogenic elements were determined in groundwater consumed by 3 study regions to check for variations in their levels & associated induction of nephrotoxicity (Khandare et al., 2015;Reimann and De Caritat, 2012; Sponholtz et al., 2016;Voutchkovaet al., 2015), results of which are presented in **Table3.1.5.** Due to lack of WHO permissible limits for Silica, Sr, Li, B, potable drinking source supplied by Canacona Municipal Corporation (from Galgibagh river) was chosen as reference.

As indicated in **Table 3.1.5,** all 3 trace geogenic elements viz. Li, B and Sr were present at comparable levels in all 3 study regions depicting no spatial variations among these regions. 100, 96.4 and 90.3% of water samples in study region 1 contained B, Li and Sr levels at their respective lowest concentration ranges of 0.1-0.2 mg/L, 0.002-0.004 mg/L and 18-36 mg/L depicting prevalence of these elements at low exposure levels in this CKDu endemic region.

This prevalence trend was resonated in study groups 2 and 3 depicting similar prevalence of these 3 geogenic elements in lower level range **(Table 3.1.5)**. The comparable levels of trace geogenic elements noted in all 3 regions could be attributed to presence of innately lower percentage of these elemental deposits/ores in aquifer's bedrock of all 3 study regions as noted by Fernandes and Widdowson (2009), thus resulting in low levels in the groundwater. Moreover, these three geogenic elements depicted seasonal variations at a comparable extent in all 3 study regions with maximum levels noted in post-monsoons due groundwater aquifer recharge that causes increased leaching of bedrock elements into groundwater as compared to other seasons. However this variation was not statistically significant ($p>0.05$) owing to inherently lower elemental constitution in aquifer's bedrock (Khan et al., 2010; Khandare et al., 2015; Jalgaonkar, 2008; Munoz et al., 2013; Nigro et al., 2018;Voutchkova et al., 2015). Moreover, levels of these 3 geogenic elements viz. Li, B, Sr in all study regions **(Table 3.1.5)** were found to be higher than levels noted in municipality treated potable water source used as a reference. These lowered levels in reference source could be attributed to its origin arising from surface waters (i.e. Galgibagh river) that inherently possesses decreased rock-water interactions and residence time that inevitably results in reduced leaching of earth's crust geogenic elements into groundwater. These levels could have been further reduced by water pretreatment(like reverse-osmosis, filtration, coagulation) by the municipality that inevitably and effectively will reduce these geogenic elements in the water source justifying lowered levels of these elements in reference source (Ding et al.,2015; Greenlee et al.,2009; Najm and Trussell,1999; Kamaraj and Vasudevan,2015; Öner et al., 2011). However, although these 3 geogenic elements viz. Li, B, Sr slightly exceeded levels present in reference source at a comparable extent in all 3 study regions, these concentrations are not concerning as their higher levels of exposure have been associated with toxic health effects. A good dose response relationship has been noted between increased exposure to Li, B, Sr and increased incidence of skeletal disorders. Exposure studies in rat models and few human epidemiological studies have stated that chronic exposure to Li, B, Sr in drinking water at respective exposure levels of 5, 25, 90 mg/L have been reported in causing defective bone formation and increased skeletal pains. This bone and skeletal toxicity is an outcome of structural and valencies resemblance (all being divalent) of these geogenic element with essential trace metal that majorly constitutes the bone's mineral matrix viz.Ca which causes Ca displacement from the matrix when these geogenic elements are present at increased levels in blood, causing significant Ca loss resulting in disrupted calcium homeostasis and defective bone-mineralization. This calcium homeostasis was reported to be further disrupted

by interference of these geogenic elements with vitamin D metabolism that is crucial for Ca absorption into bone structure, Ca homeostasis maintenance and bone-apatite formation. But the exact mechanism of vitamin D inhibition by these geogenic elements is unknown till date. Studies in rat models showed that administration of higher Sr and lower Ca dietary intake resulted in osteomalacia characterized by defective bone formation produced due to reduced bone apatite synthesis and mineral density (Cohen-Solal, 2002; Dahl et al.,2001; Dermience et al.,2015; Grynpas and Marie,1990; Kalantar-Zadeh et al.,2012; Mozrzymas, 2018).

However, in the current study, levels of these geogenic elements reported in CKDu endemic region's groundwater (study area1) **[Table 3.1.5]** were found to be significantly lower than the reported toxic levels known to cause bone and skeletal disorders, thus signifying no role of these elements exposure (through groundwater consumption) in induction of skeletal pains witnessed in half of the CKDu population. This observation was supported by presence of comparable levels of these trace geogenic elements in groundwater of CKD endemic region (study region 2) and healthy control region (study region 3) **[Table 3.1.5]** negating role of these elements viz. Li, B, Sr in skeletal disorders induction. These findings were consistent with the results of monitoring studies of these abovesaid geogenic elements in inducing skeletal toxicity and linked nephrotoxicity as a result of NSAID intake in CKDu hit regions of Sri Lanka & Central America, wherein lowered levels of these elements were noted in the major exposure route i.e. groundwater of these CKDu regions, thus had successfully negated their contribution in triggering skeletal discomfort noted in these regions (Correa-Rotter et al.,2014; Cozzolino et al.,2014; Ganguli, 2016; Khandare et al.,2015; Wesseling et al.,2013).

Interestingly, in the current study **(Table 3.1.5)**, mean levels of one of the analysed trace geogenic elements viz. silica were found to be significantly ($p<0.05$) higher CKDu endemic region's groundwater (study area1) (115.5 mg/L, range=95.6-132.9mg/L) as compared to other 2 areas (i.e. CKD non-endemic with mean levels=13.9 mg/L, range=10.2-22.3mg/L and healthy control with mean levels=13.5mg/L, range=11.2-25.4mg/L) and potable reference water source (mean=9.4 mg/L, range=9.2-9.8mg/L). Levels in CKDu region (study area1) were 8 fold higher than CKD non-endemic and control regions (study areas 2 and 3) and 12 fold higher than reference source suggesting it's possible involvement in CKDu development in this taluka. 92.2 and 7.2% of the CKDu region samples contained silica in respective higher concentration range of 110-135 mg/L and successively lower range of 85-110 mg/L indicating silica prevalence at higher levels in this region**.** Whereas silica levels in study area2 and 3 were similar with 91.1 and 87.3% of samples being prevalent in lowest range viz.0-15mg/L indicating no possible nephrotoxicity induction in these areas **(Figure 3.1.5B).**

Table 3.1.5 :Trace geogenic element profile including the emerging silica nephrotoxin in the groundwater in the three study regions during the three different seasons with reference to their respective permissible limits(from literature).

CKD status	CKDu Endemic					CKD Non-endemic					No CKD prevalence					Annnotation for the prevalence % of the concentration ranges (in mg L^{-1}) of the resepctive parameters	Reference-Galgibagh river (Potable source)
Sampling site and period/ Parameter (mg L^{-1})	Study area/ group 1 (n=114)					Study area/ group 2 (n=28)					Study area/ group 3 (n=124)						
	PRM	MON	POM	MEAN[a]	Prevalence %[a]	PRM	MON	POM	MEAN[a]	Prevalence %[a]	PRM	MON	POM	MEAN[a]	Prevalence %[a]		
Silica	98.6.±1.01*	129.5±0.94*	118.31±1.02*	115.5±1.11*[b]	-	11.34±0.3	14.1±0.2	16.36±0.3	13.9±0.3	-	12.2±0.4	13.29±0.51	15.16±0.52	13.52±0.51	-		9.4±0.04
	(95.6-125.3)	(114.6-139.7)	(109.6-132.9)	(95.6-132.9)		(10.2-16.5)	(11.9-19.9)	(14.7-22.3)	(10.2-22.3)		(11.2-18.7)	(13.4-21.3)	(14.7-25.4)	(11.2-25.4)			(9.2-9.8)
B	0.18±0.002	0.15±0.004	0.19±0.005	0.17±0.003	A=100%	0.23±0.003	0.22±0.002	0.25±0.004	0.23±0.003	B=100%	0.25±0.007	0.21±0.002	0.27±0.003	0.24±0.003	B=100%	A=0.1-0.2; B=0.2-0.3	0.08±0.001
	(0.11-0.20)	(0.10-0.17)	(0.13-0.20)	(0.10-0.20)		(0.21-0.25)	(0.20-0.25)	(0.23-0.27)	(0.20-0.27)		(0.23-0.28)	(0.20-0.24)	(0.25-0.30)	(0.20-0.30)			(0.05-0.10)
Li	0.0025±0.0007	0.0035±0.0006	0.004±0.0007	0.0036±0.0007	A=96.4%; B=3.6%	0.004±0.0002	0.0022±0.0008	0.003±0.0004	0.003±0.0006	A=98.7%; B=1.3%	0.0031±0.0012	0.0028±0.0011	0.003±0.0012	0.002±0.0012	A=100%	A=0.002-0.004; B=0.004-0.006	0.0034±0.001
	(0.0020-0.0041)	(0.0028-0.0043)	(0.0035-0.0045)	(0.0020-0.0045)		(0.036-0.044)	(0.0020-0.0042)	(0.0025-0.0041)	(0.0025-0.0044)		(0.0021-0.0037)	(0.0022-0.0032)	(0.0025-0.035)	(0.0022-0.0037)			(0.0029-0.0037)
Sr	32.83±1.1	28.56±0.72	38.90±0.83	33.43±1.01	B=90.3%; C=9.7%	16.2±0.6	13.5±0.6	18.6±0.4	16.1±0.6	A=85.5%; B=14.5%	14.56±0.4	11.31±0.5	15.82±0.6	13.89±0.5	A=100%	A=0-18; B=18-36; C=36-54	11.4±0.05
	(25.6-39.4)	(22.7-36.9)	(32.3-43.1)	(22.7-43.1)		(12.6-19.8)	(9.4-18.9)	(14.8-21.1)	(9.4-21.1)		(12.6-17.2)	(10.5-14.6)	(13.9-18.0)	(10.5-18.0)			(10.9-11.9)

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. Data are derived from two independent experiments with the levels of each geogenic element in an individual experiment measured in triplicates. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected.

a Values are represented as mean ± SE of each individual geogenic element estimated for all the samples(n) collected recurrently during three different sampling time points-Pre-Monsoon(PRM), Monsoon(MON) and Post-Monsoon(POM) in the three respective study areas . The range of minimum to maximum values attained for a given geogenic element have been denoted in parenthesis individually for each sampling time-point (i.e. PRM, MON and POM) and cumulatively for the average of all the seasons combined. (i.e. mean of all seasons). All the values are in (mg L^{-1}). Differences at*$p<0.05$ were considered to be significant. Since no permissible limits have been established by the WHO for these geogenic elements including the silica nephrotoxin till date, the levels determined in a potable water source (i.e. municipality treated river). The prevalence of the mean concentration of each geogenic element(averaged over all the sampling seasons combined) in various ranges of their levels in the groundwater of the three study regions have been represented by prevalence % and annotated by uppercase letters(i.e. A to C) for convenience. The annotations for each estimated parameter varies and have been independently described. The prevalence % denotes the proportion of samples from the total number in particular study region bearing the parameter at the stated concentration range .For example, the prevalence % annotation of 'A' in the case of boron levels denotes the percentage of the total number of samples analysed in a particular study region possessing concentrations at the range of 0.1-0.2 mg/L.

b Values indicates the silica levels in the groundwater of the CKDu endemic region that were observed to be largely prevalent in the highest concentration range as compared to the other two study regions(2 and 3) which depicted a larger prevalence in the remarkably lower concentration range (with details of the prevalence described in Fig.3.1.5). Thus suggesting the possible nephrotoxic role of silica in significant renal damage development witnessed in this CKDu endemic region (study region 1).

The elevated silica levels in CKDu endemic region's groundwater (study area1) **(Table 3.1.5)** could be attributed to close proximity of endemic villages of this region to the abandoned granite mining area located in the taluka's Chaudi village (Fernandes and Widdowson, 2009; Nadaf, 2009a) & also to excessive silica leaching from aquifer's granitic bedrock in these areas by acidic groundwater as a result of acid mine drainage from the abandoned mine (Fantong et al.,2009; Jacobs et al.,2014; Machiraju et al.,2013; Khan et al.,2015; Kumar and Kumar, 2013; Kumari et al.,2013). It is well known that granite being a felsic rock is a rich silica source (containing >75% by weight) & in current study the granitic bedrock was found to contain 81% silica by weight as analysed by Fernandes and Widdowson (2009); during geological survey of the taluka. This silica laden granitic bedrock of the CKDu endemic region aquifer can enrich groundwater with silica through normal rock-water interactions (Carroll, 2012; Larson, 1984; Subramani et al., 2010; Srinivasamoorthy et al., 2008). However, this silica enrichment in the groundwater was found to be significantly intensified at an acidic pH as observed in CKDu region since silica solubility is greatly enhanced at an acidic pH (<6.5) and reduces at a neutral or alkaline pH (Icopini et al, 2015; Halim et al.,2013; Kumar and Kumar, 2013; Pradeep et al.,2016; Zuhl et al.,2013).These findings were further supported by presence of strong, negative and significant ($p<0.05$) correlations noted between silica levels and pH of the groundwater of study area1($r=-0.927, p=0.019$).This trend of increased silica availability noted at a reduced pH in CKDu region provided supporting evidence to low silica levels obtained in CKD non-endemic (study area2) and control regions (study area3) **(Table 3.1.5)** attributed to neutral groundwater and metabasitic geological constitution of the aquifer's bedrock (which is inherently silica poor-containing <40% silica), hence do not enrich groundwater with high silica (Chowdhury et al.,2016; Daae et al.,2015; Fernandes and Widdowson,2009; Förstner and Wittmann, 2012; Henry,2015; Nadaf, 2009a; Noack et al.,2014; Singh et al.,2012; Srivastava et al.,2013).These observations were backed by insignificant ($p>0.05$) and negative correlations of -0.067 ($p=0.114$) and -0.059 ($p=0.056$) noted between groundwater's neutral pH and minimal silica levels in respective areas 2 & 3.

Another contributing factor to elevated silica levels in CKDu endemic region was limited availability of multivalent cations like Ca, Mg in this region's groundwater **(Table 3.1.2)**. It is well documented that silica forms insoluble and inert metal silicate complexes with multivalent cations present at high concentrations and at high pH (alkaline pH) due to formation of reactive silicate anion at this pH (Amjad et al.,2010; Diop and Tijani,2008; Förstner and Wittmann,2012; Kumar and Kumar,2013;Machiraju, et al., 2013; Ng et al.,1993; Pradeep et al.,2016; Zuhl et al.,2013). In line with this evidence we can safely presume that

since the study area 1's cation concentrations and pH of groundwater were lowered, inert silicate complexes formation was inhibited, enhancing silica availability (levels) in CKDu endemic region's groundwater.These findings were supported by significantly($p<0.05$) strong & inverse correlations of -0.901 ($p=0.021$) & -0.815($p=0.041$) noted between silica and Ca and silica & Mg respectively in this region's groundwater highlighting strong tendency of these cations in restricting silica availability. This silica limiting potency of these multivalent cations justifies lowered silica content noted in groundwater of non-endemic(study area2) and control regions (study area 3) due to comparatively higher Ca and Mg levels and neutral pH observed in this region **(Table 3.1.1 and 3.1.2).** These observations were further backed by occurrence of significantly ($p<0.05$) comparable and negative correlations in study group2/3 of -0.20($p=0.023$)/-0.167($p=0.015$) and -0.178($p=0.037$)/0.154($p=0.041$) noted between silica and Ca and silica and Mg respectively signifying silica inhibiting ability of these cations (Amjad et al.,2010; Daae et al.,2015; Diop and Tijani,2008; Förstner and Wittmann,2012; Henry,2015; Noack et al.,2014; Singh et al.,2012; Srivastava et al.,2013; Zuhl et al.,2013).

Epidemiological and animal studies have pointed out a strong association between prolonged exposure to excess silica levels and induction of chronic tubular dysfunction. Preliminary evidence of silica renal toxicity in humans was provided by cross sectional studies of silicotic and non silicotic workers which showed chronic tubulo-interstitial nephritis (CTN) manifestation in silicotic workers. This was evident from significantly higher levels of urea, creatinine, uric acid in serum, normoalbuminuria (normal plasma albumin levels) and elevated urinary β2-microglobulin (B2M) levels in silicotic workers. These high tubular damage biomarkers levels persisted even after silica exposure ceased, suggesting that chronic silica exposure causes enhanced silica deposition (due to its bioaccumulative tendency) and related induction of irreversible nephrotoxicity (Mcdonald et al.,2005; Mohner et al.,2017; Ng et al., 1993; Rapiti et al.,1999; Rosenman,2000; Steenland,2005; Vupputuri et al., 2012).

Recently few epidemiological studies have highlighted relationships between high silica exposure levels through another route viz. consumption of contaminated drinking water and induction of tubular-toxicity in various CKDu endemic regions. Cross sectional studies of CKDu cases in Balkan region of Europe and Uddanam region of Andhra Pradesh (India) revealed urinary levels of major tubular injury markers viz. b2M, NAG heightened in positive & strong association with elevation of blood silica levels noted in CKDu affected subjects. This high mean blood silica levels in CKDu subjects [i.e. Balkan region=93.3 mg/L in cases v/s 9.2 mg/L in controls; Uddanam region=89.6 mg/L in cases v/s 7.9 mg/L in controls] positively associated with increased silica contamination in well-water consumed by these

subjects in the CKDu prevalent region [i.e. Balkan region=97.9 mg/L in cases v/s 17.2 mg/L in controls; Uddanam region= 92.2 mg/L in cases v/s 13.3 mg/L in controls], that suggested alimentary contamination to be the major silica exposure route in these CKDu subjects. This silica contamination differences in well-water of CKDu affected and unaffected regions proportionately translated into remarkable differences in blood silica levels that consequently induced severe renal proximal tubular toxicity (evident from elevated tubular proteinuria i.e. raised level of tubular injury protein markers-b2M & NAG in urine), which is typical of CTN presentation that was directly linked with pathological manifestation of CKDu in those regions. Thus depicting role of this nephrotoxic geogenic element viz. silica in CKDu induction (Goldsmith & Goldsmith,1993; Khandare et al.,2015; Markovic & Lebedev,1965; Markovic,1968; Markovic,1971; Radovanovic et al.,1991; Stiborova et al.,2016)

All these epidemiological studies have highlighted significant dose and time response relationship in silica induced nephrotoxicity wherein chronic exposure to high silica doses induced grave proximal tubular injuries (evident from enhanced tubular proteinuria) that possibly could have resulted in structural and functional integrity disruption of proximal tubules which ultimately manifested in renal failure, the hallmarks of CKDu pathology. This dose-response relationship in silica induced nephrotoxicity was supported by animal studies as well. In a study carried out in guinea pigs fed with drinking water containing 50mg/L silica for 6 months, two prominent signs of CTN (renal pathological manifestation of CKDu) were noted:-interstitial tissue inflammatory scarring and dystrophic-atrophic lesions in tubular parenchyma which ultimately resulted in kidney shrinkage and contraction due to it becoming fibrotic and atrophic that subsequently manifested in complete renal function disruption which advanced into chronic renal failure (Markovic and Arambasic,1971). Similar effects were noted in beagle dogs (Newberne and Wilson,1970),other guinea pigs (Dobbie & Smith; 1982) & mice models (Kawanabe et al.,1992) administered with silica doses of 80-150mg/L; 60-100mg/L;55mg/L in drinking water for 10months,8 months and 1 year respectively. Additionally, in these models, significant enhancement in tubular proteinuria (i.e. excessive urinary excretion of tubular injury specific LMV proteins-b2M, NAG) was also noted (before biopsy) with chronic exposure to high silica. Contrarily, reduced histopathological alterations & decreased urinary tubular proteins excretion were noted on treatment of independent animal groups (for each category i.e. guinea pigs, dogs, mice) with low silica doses (10 mg/L) for the same time-period suggesting no significant induction of tubular toxicity at lower dosing. Supporting evidence for these silica induced renal histopathological alteration emerged from a few human renal pathological analysis conducted in kidney biopsy studies of

CKDu cases in Yugoslavia and autopsy studies of patients in Bulgaria who died of renal failure due to advanced CKDu. In these studies as well, histopathological alterations revealed primary tubular lesions with diffused dystrophic cum atrophic processes in parenchyma; proliferative inflammatory lesions in interstitial tissue (surrounding proximal tubules) and silica deposition in renal cortico-tubular portion. Thus these histopathological studies in animal and human indicated that chronic exposure to high silica levels induced renal pathological alterations typical of CTN comprising of tubular atrophy and interstitial inflammatory lesions coupled with fibrosis which is reported to be the CKDu pathological manifestation, thus suggesting silica's nephrotoxic potential in CKDu causation (Bolton et al.,1981; Markovic and Lebedev, 1965b; Markovic, 1972 and 1974; Policard and Collet, 1954; Policard et al.,1960 a and b; Policard et al.,1961; Radovanovic et al.,1991).

Thus, since mean silica levels (115.5 mg/L, range=95.6-132.9 mg/L, **Table 3.1.5**) obtained in CKDu endemic region's groundwater were found to significantly exceed nephrotoxic levels (of around 90 mg/L) established from previously reported silica toxicity animal studies (Dobbie and Smith,1982; Markovic and Arambasic, 1971; Newberne and Wilson,1970) and a few chronic exposure based epidemiological analyses (>35yrs via groundwater intake) (Khandare et al.,2015; Markovic, B., 1968; Markovic, 1971; Radovanovic et al.,1991; Stiborova et al.,2016), we can conclusively suggest the strong & significant contribution of higher silica exposure through chronic groundwater consumption in development of high CKDu incidence in Canacona. This was further supported by safe and non-toxic levels notedin groundwater of CKD-non endemic (mean=13.9 mg/L, range=10.2-22.3 mg/L, **Table 3.1.5**) and healthy control regions (mean=13.5 mg/L, range=11.2-25.4 mg/L, **Table 3.1.5**), indicating silica as a major nephrotoxin responsible for CKDu manifestation in the taluka.

3.1.3.5 Correlations between and within groups of trace metals, nephrotoxins and trace geogenic elements present in groundwater consumed by 3 study regions of Canacona

Various sets of relationship assessments were carried out between different groups of elements analyzed in groundwater of the 3 study regions of Canacona by calculations of Pearson's correlation coefficients(r) with their respective p-values to identify common sources of origin of these elements in groundwater which can allow us to take necessary remedial/preventive measures to get rid of contaminating source thereby decreasing exposure to various nephrotoxins and associated induction of renal damage (Jayatilake et al.,2013). Firstly, relationship between average concentrations of measured trace metals in ground water of 3 three study regions were assessed and the results are presented in **Table 3.1.6.**

Table 3.1.6: Correlation assessments between the mean levels of various trace metals (averaged over three sampling seasons) analysed in the groundwater regularly consumed by the study population of Canacona taluka for identification of the common source(s) of origin(if any) for occurrence of these trace metals in this exposure matrix

Trace metal (in the groundwater)	Study group	Correlation coefficients [r]										
		Trace metal (in the groundwater)										
		Al	Fe	Mn	Zn	Cu	Ba	Co	Ni	Mo	V	Ag
Al	1	1	-	-	-	-	-	-	-	-	-	-
	2	1	-	-	-	-	-	-	-	-	-	-
	3	1	-	-	-	-	-	-	-	-	-	-
Fe	1	**0.215**	1	-	-	-	-	-	-	-	-	-
	2	**0.856**	1	-	-	-	-	-	-	-	-	-
	3	**0.893**	1	-	-	-	-	-	-	-	-	-
Mn	1	**0.189**	**0.227**	1	-	-	-	-	-	-	-	-
	2	**0.756**	**0.879**	1	-	-	-	-	-	-	-	-
	3	**0.804**	**0.906**	1	-	-	-	-	-	-	-	-
Zn	1	**0.101**	**0.154**	**0.135**	1	-	-	-	-	-	-	-
	2	**0.559**	**0.714**	**0.637**	1	-	-	-	-	-	-	-
	3	**0.601**	**0.819**	**0.739**	1	-	-	-	-	-	-	-
Cu	1	**0.099**	**0.598**	**0.312**	**0.209**	1	-	-	-	-	-	-
	2	**0.571**	**0.705**	**0.516**	**0.614**	1	-	-	-	-	-	-
	3	**0.619**	**0.812**	**0.678**	**0.596**	1	-	-	-	-	-	-
Ba	1	**0.115**	**0.103**	**0.087**	**0.129**	**0.09**	1	-	-	-	-	-
	2	**0.879**	**0.714**	**0.689**	**0.812**	**0.775**	1	-	-	-	-	-
	3	**0.785**	**0.707**	**0.612**	**0.845**	**0.699**	1	-	-	-	-	-
Co	1	-0.078	-0.098	-0.101	-0.087	-0.059	-0.067	1	-	-	-	-
	2	-0.156	-0.229	-0.141	-0.139	-0.256	-0.187	1	-	-	-	-
	3	-0.209	-0.113	-0.196	-0.21	-0.299	-0.247	1	-	-	-	-
Ni	1	-0.045	-0.033	-0.024	-0.099	-0.021	-0.037	**0.113**	1	-	-	-
	2	-0.123	-0.203	-0.117	-0.212	-0.109	-0.211	**0.908**	1	-	-	-
	3	-0.368	-0.165	-0.214	-0.314	-0.198	-0.189	**0.857**	1	-	-	-
Mo	1	-0.012	-0.045	-0.032	-0.028	-0.054	-0.018	**0.121**	**0.132**	1	-	-
	2	-0.114	-0.265	-0.114	-0.147	-0.203	-0.101	**0.756**	**0.915**	1	-	-
	3	-0.203	-0.187	-0.199	-0.132	-0.298	-0.157	**0.741**	**0.857**	1	-	-
V	1	-0.024	-0.018	-0.058	-0.069	-0.035	-0.047	**0.04**	**0.079**	**0.127**	1	-
	2	-0.156	-0.298	-0.196	-0.247	-0.101	-0.198	**0.689**	**0.557**	**0.896**	1	-
	3	-0.263	-0.274	-0.203	-0.301	-0.189	-0.145	**0.719**	**0.618**	**0.812**	1	-
Ag	1	-0.011	-0.026	-0.036	-0.041	-0.014	-0.035	**0.032**	**0.047**	**0.113**	**0.12**	1
	2	-0.213	-0.114	-0.215	-0.189	-0.103	-0.231	**0.589**	**0.612**	**0.914**	**0.871**	1
	3	-0.154	-0.158	-0.207	-0.236	-0.189	-0.174	**0.664**	**0.703**	**0.875**	**0.779**	1

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology. 'n' in each study region depicts the total number of groundwater samples that were collected recurrently over three sampling seasons for analysis of the trace metals. The total number of groundwater samples collected during each sampling season from the study regions 1,2 and 3 were 114, 28 and 124 respectively. Values are represented as Pearson's correlation coefficients (r) with their respective p-values also calculated for identification of statistically significant associations (if any)(viz. a mutual contaminating source) present between any given two trace metals. Differences at $p<0.05$ were considered to be statistically significant correlations, which are highlighted in bold. a 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, positive and statistically significant ($p<0.05$) associations were noted between various dual combinations in two individual broad groups of trace metals viz. group1 (comprising of Fe, Mn, Zn, Cu and Al) and group 2(constituting of Co, Ni, Mo, V and Ag) in the groundwater of all of the three study regions. This signified the existence of a common contaminating source for each group of trace metals. Moreover, as expected inverse and insignificant ($p>0.05$) correlations were noted between these two aforementioned broad groups of elements when the relationship between one element from group 1 was assessed with an individual element from group 2, suggesting occurrence of different contaminating sources for both these groups of elements. For units and details of each individual trace metal concentration, kindly refer Table 3.1.3 of this section of the chapter.

As indicated in **Table 3.1.6**, higher and significantly ($p<0.05$) positive correlations(r) between various dual combinations of a group of elements comprising of Fe, Mn, Zn, Cu, Ba, Al were noted in groundwater of study regions 2 and 3 at comparable extents. These higher associations (evident from their higher correlation coefficient-'r'values) indicated a common origin source of these elements. It is well-reported that these elements are known to originate in groundwater from natural sources like weathering of minerals ores and limited influence of common anthropogenic sources like discharge of untreated effluents from industries like iron ore extraction, steel processing, etc. Therefore in line with the existing literature, the

aforementioned sources could be responsible for correlated presence of these trace metals in groundwater of these two study regions (Förstner and Wittmann,2012; Giri and Singh, 2015; Ghazali et al.,2018; Karim, 2011; Wongsasuluk et al.,2014).Moreover,geological constitution of these elements in aquifer's bedrock of these 2 regions is significantly higher than that of CKDu region owing to metabasitic makeup of the bedrock in the former regions which are well reported to be laden with reserves of Fe, Mn, Zn, Cu, Ba, Al (as analysed by Fernandes & Widdowson, 2009) & thus can inherently enrich groundwater with these elements through natural bedrock-water interactions (Chowdhury et al.,2016; Daae et al., 2015; Förstner & Wittmann, 2012; Henry,2015; Noack et al.,2014; Srivastava et al.,2013; Singh et al., 2012).

Additionally, comparatively weaker and significant ($p<0.05$) associations between the same combinations of abovesaid group of elements in CKDu endemic region's groundwater (study area1) were noted signifying a common contaminating source of these elements like the other 2 study regions **(Table 3.1.6).** The weaker associations in this region could be attributed to the presence of reduced reserves of these elements in this region's granitic bedrock of aquifer (Fernandes & Widdowson,2009) leading to lower groundwater enrichment with these elements & thus reduced relation (Chandrajith et al.,2011).This was backed by low industrial impact on CKDu region groundwater as compared to other study areas with latter being located close to industrial source (Fantong et al.,2009; Galhardi and Bonotto, 2016; Halim et al.,2013; Khan et al.,2015; Machiraju et al.,2013; Pradeep et al,2016; Subramani et al.,2010).

Additionally significantly strong ($p<0.05$) correlations were noted between various dual combinations of group of elements comprising of Co, Ni, Mo, V, Ag in groundwater of study regions 2 & 3 **(Table 3.1.6),** suggesting existence of a common contaminating source of all these elements in both regions groundwater. Its well-stated that these elements contamination in this region's groundwater could have arose from untreated industrial effluent discharge from electroplating, smelting, tanning, piping etc.(Giri and Singh,2015; Karim, 2011;Kavcar et al.,2009). Similarly,positive but weaker correlations between these same group of elements were noted in CKDu affected region's groundwater suggesting a common origin source of these elements in this region as well but lower correlation could be due to reduced industrial effluents discharge in this region, owing to contaminating industrial source being located far from this region (Fantong et al.,2009; Galhardi and Bonotto, 2016; Halim et al.,2013; Kumar and Kumar, 2013; Machiraju et al., 2013; Pradeep et al.,2016; Srinivasamoorthy et al.,2008).

Inverse & insignificant ($p>0.05$) correlations were noted between these 2 abovesaid broad groups of elements viz.group1(comprising of Fe,Mn,Zn,Cu,Al) & group 2(Co,Ni,Mo,V,Ag) in groundwater of all 3 study regions, possibly attributed to different contaminating sources

of both of these groups of elements (Chandrajith et al.,2011; Fantong et al.,2009; Galhardi & Bonotto,2016;Giri and Singh,2015;Halim et al.,2013;Jayatilake et al.,2013;Kavcar et al.,2009 ;Karim,2011;Machiraju et al.,2013;Pradeep et al.,2016).Despite strong correlations witnessed between various groups of trace metals in groundwater of 3 study regions, levels of the same were found to be within their respective WHO permissible levels (Edition,2011;WHO,2011) signifying absence of strong industrial impact in these regions that averted groundwater quality compromise interms of trace metal level(Chandrajith et al.,2011;Jayatilake et al.,2013 Secondly, relationship between mean of various analyzed nephrotoxins in groundwater of 3 study regions were assessed and results presented in **Table 3.1.7** Cd, As, Hg were excluded from correlation analysis owing to below detectable levels of the same noted in groundwater of the 3study regions signifying absence of groundwater contamination with these elements.

Table 3.1.7: Correlation assessments between mean levels of various nephrotoxins (averaged over three sampling seasons) analysed in groundwater regularly consumed by the study population of Canacona taluka,for identification of a common contaminating source of these nephrotoxins in this exposure matrix.

Nephrotoxin (in the groundwater)	Study region (total number of samples collected recurrently over three sampling seasons)	Correlation coefficients [r] *(p values)*	
		Nephrotoxin (in the groundwater)	
		Silica	Lead
Silica	1	1	
	(n=114)	-	
	2	1	
	(n=28)	-	
	3	1	
	(n=124)	-	
Lead	1	0.933	1
	(n=114)	*0.016*	-
	2	-0.041	1
	(n=28)	*0.212*	-
	3	-0.062	1
	(n=124)	*0.156*	-

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology.'n' in each study region depicts the total number of groundwater samples that were collected recurrently over three sampling seasons for analysis of the nephrotoxins. Values are depicted as Pearson's correlation coefficients (r) with their respective p-values also calculated and italicized for identification of statistically significant associations (if any)(viz. a common contaminating source) present between the given two nephrotoxins viz. silica and lead. Correlation coefficients were not computed for the other three anlaysed nephotoxins viz. Cd, Hg and As owing to the below detectable levels of the same detected in the groundwater of all of the three study regions. Differences at $p<0.05$ were considered to be statistically significant correlations, which are highlighted in bold. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, positive and statistically significant($p<0.05$) associations were noted between the two nephrotoxins viz. silica and lead in the groundwater of the CKDu endemic region suggestive of the occurrence of a common contaminating source of these nephrotoxins in the groundwater. The source could have most likely been the enhanced leaching from the granitic aquifer's bedrock constituting this region which was previously reported by Widdowson et al to be intrinsically enriched in these nephotoxins. The increased leaching was possibly attributed to the increased acidity of the groundwater as a result of acid mine drainage from the abandoned mine located in the vicinity that caused the elevated concentrations of these nephrotoxins in the groundwater as their availability is well-known to rise at an acidic pH and hence resulted in increased associations between these two nephrotoxins. For units and details of each individual nephrotoxin concentration, kindly refer Tables 3.1.4 and 3.1.5 of this section of the chapter.

As indicated in **Table 3.1.7**, significantly ($p<0.05$) strong and positive correlations of 0.933 ($p=0.016$) were noted between mean lead and silica levels in CKDu hit region's groundwater (study area1) indicative of common origin source of these 2 nephrotoxins (Chandrajith et al., 2011; Jayatilake et al.,2013; Khandare et al.,2015; Levine et al.,2016; Markovic & Lebedev, 1965; Radovanovic et al.,1991). The common origin source of these nephrotoxins could be

attributed to this region's granitic bedrock of the aquifer that has been proved to contain rich silica (81% by weight) and considerable lead deposits (2.5% by weight) (Fernandes and Widdowson, 2009); that may have excessively released these nephrotoxins on interaction with this region's acidic groundwater as these toxin availability is noted to rise at an acidic pH (Chowdhury et al.,2016; Du et al.,2014; Fantong et al.,2009;Halim et al.,2013;Khan et al., 2015; Karim,2011; Kavcar et al.,2009; Kumar & Kumar,2013; Kumari et al.,2013; Machiraju et al.,2013; Sankhla et al.,2016; Zuhl et al.,2013). Thus excessive leaching from CKDu region aquifer's bedrock by acidic groundwater was strongly suggested as common source of groundwater contamination with these 2 nephrotoxins viz. lead and silica in this region.

Contrarily, inverse associations of -0.041($p=0.212$), and -0.062 ($p=0.156$) were noted between the Pb and silica levels in groundwater of study regions2 and 3 respectively at a comparable extent **(Table 3.1.7).** This signified a differential source of origin of these 2 nephrotoxins in the groundwater(at minimal but WHO safe concentrations)of these two regions. From literature, Pb could have probably arose from release of untreated effluents from smelting, paint and battery industries and silica could have inevitably arisen from deficient silica reserves that are present in these region's metabasitic bedrock [as analysed by Fernandes and Widdowson (2009)] and neutral groundwater that caused reduced leaching of silica nephrotoxin (Chowdhury et al., 2016; Daae et al., 2015; Förstner and Wittmann, 2012; Henry, 2015; Noack et al., 2014; Srivastava et al., 2013; Singh et al., 2012).

Thirdly,association between means of various geogenic elements including silica nephrotoxin in groundwater of 3 study regions were analysed & results depicted in **Table 3.1.8**. As indicated in **Table 3.1.8**,significant and positive associations were noted between 3 of the analysed geogenic elements(i.e.Li, B, Sr) at comparable extents in all 3 study region's groundwater indicating a common source of origin these elements which is inevitably the earth's crust or the aquifer's bedrock(hence the name geogenic).However, significantly ($p<0.05$) weaker associations were noted between silica and each of the geogenic elements (i.e.Li, B, Sr) in CKDu affected region **(Table 3.1.8)** indicating differences in content of each of geogenic element in the aquifer's bedrock. As described by Fernandes and Widdowson (2009), silica content significantly surpassed the negligible reserves of other 3 geogenic elements (at an average of 0.8% by weight) present in the aquifer's granitic bedrock explaining weaker relationships noted between silica and other geogenic elements owing to the differential enrichment of the groundwater with these elements (Pradeep et al,2016; Khan et al, 2015;Khandare et al.,2015; Kumar & Kumar,2013; Machiraju et al.,2013; Markovic & Lebedev,1965; Markovic,1968; Radovanovic et al.,1991; Subramani et al.,2010; Voutchkova

et al.,2015). Contararily, higher correlations were noted between silica nephrotoxin and other 3 geogenic elements in groundwater of other two study areas (2 & 3) as compared to region 1 **(Table 3.1.8)**. This could be attributed to a similar distribution of the concentration content of all 4 geogenic elements as reported in these region's metabasitic bedrock (by Fernandes and Widdowson,2009) resulting in comparatively higher associations between these elements in these 2 study regions groundwater (Chowdhury et al,2016; Daae et al.,2015; Förstner and Wittmann,2012; Henry,2015; Noack et al.,2014; Srivastava et al.,2013; Singh et al.,2012).

Table 3.1.8: Correlation assessments between the mean levels of various trace geogenic element including the silica nephrotoxin (averaged over three sampling seasons) analysed in the groundwater regularly consumed by the study population of Canacona taluka, for identification of a common contaminating source of these geogenic elements in this exposure matrix.

Trace geogenic element (in the groundwater)	Study group (total number of subjects)	Correlation coefficients [r] *(p values)*			
		Trace geogenic element (in the groundwater)			
		Silica	Li	B	Sr
Silica	1	**1**	-	-	-
	(n=114)	-	-	-	-
	2	**1**	-	-	-
	(n=28)	-	-	-	-
	3	**1**	-	-	-
	(n=124)	-	-	-	-
Li	1	**0.032**	**1**	-	-
	(n=114)	*0.023*	-	-	-
	2	**0.18**	**1**	-	-
	(n=28)	*0.047*	-	-	-
	3	**0.103**	**1**	-	-
	(n=124)	*0.033*	-	-	-
B	1	**0.079**	**0.067**	**1**	-
	(n=114)	*0.012*	*0.029*	-	-
	2	**0.185**	**0.113**	**1**	-
	(n=28)	*0.028*	*0.045*	-	-
	3	**0.212**	**0.147**	**1**	-
	(n=124)	*0.017*	*0.036*	-	-
Sr	1	**0.199**	**0.029**	**0.35**	**1**
	(n=114)	*0.036*	*0.021*	*0.039*	-
	2	**0.668**	**0.115**	**0.169**	**1**
	(n=28)	*0.018*	*0.032*	*0.025*	-
	3	**0.589**	**0.125**	**0.114**	**1**
	(n=124)	*0.027*	*0.011*	*0.047*	-

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; 'n' in each study region depicts the total number of groundwater samples that were collected recurrently over three sampling seasons for analysis of the trace geogenic elements including the silica nephrotoxin. Values are depicted as Pearson's correlation coefficients (r) with their respective p-values also calculated and italicized for identification of statistically significant associations (if any) (viz. a common contaminating source) present between any given two geogenic elements. Differences at $p<0.05$ were considered to be statistically significant correlations, which are indicated in bold. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As depicted in the results, positive and statistically significant ($p<0.05$) associations were noted between all the geogenic elements in the groundwater of all of the three study regions. This signified the existence of a common contaminating source for these geogenic elements as expected, since they are intrinsically known to arise from the earth's crust specifically the aquifer's bedrock as result of natural bedrock-groundwater interactions, hence conferred the name geogenic. However the strength of association between the silica nephrotoxin in combination with the other three geogenic elements (i.e. Li, B and Sr) varied between the three study regions as evident from the variations in the correlation coefficient values. Higher associations (indicated from the higher 'r' values) were noted in the groundwater of the study regions 2 and 3 due to similar extents of enrichment of the constituting metabasitic bedrock with these aforementioned elemental reserves (as analysed by Widdowson et al) and the neutral groundwater-bedrock interactions that resulted in comparable concentrations of these elements in the groundwater, hence manifested in higher associations between the same. Contrarily, weaker associations (indicated by lower 'r' values) were noted between these stated geogenic elements in the groundwater of the CKDu endemic region. This could be possibly attributed to the enhanced enrichment of the groundwater with silica as compared to the other geogenic elements as a result of excessive leaching of the silica from the high reserves that are innately present in the constituting granitic aquifer's bedrock by the acidic groundwater of this region.Hence this resulted in weaker associations due to the dissimilar concentrations of silica and the other geogenic elements present in the groundwater of this region. For units and details of each individual geogenic element concentration, kindly refer Table 3.1.5.

Lastly, correlation between means of various trace metals and nephrotoxins (viz. silica, Pb) analysed in groundwater of all 3 study regions were assessed as trace metals are widely reported to influence nephrotoxins bioavailability especially geogenic nephrotoxin-silica & to some extent-lead heavy-metal (Jayatilake et al.,2013), whose results are listed in **Table3.1.9**

Table 3.1.9: Correlation assessments between the mean levels of various nephrotoxins and trace metals (analysed and averaged over three sampling seasons) in the groundwater regularly consumed by the study population of Canacona taluka, for identification of an association(if any) between these nephrotoxins and trace metals.

Trace metal (in the groundwater)	Correlation coefficients [r] *(p-value)*					
	Nephrotoxin (in the groundwater) in the respective study regions					
	Silica			Lead		
	1	2	3	1	2	3
Al	**-0.814**	**-0.615**	**-0.754**	**-0.745**	**-0.633**	**-0.658**
	0.012	*0.032*	*0.047*	*0.018*	*0.033*	*0.014*
Fe	**-0.779**	**-0.518**	**-0.625**	**-0.674**	**-0.458**	**-0.514**
	0.028	*0.036*	*0.041*	*0.011*	*0.028*	*0.045*
Mn	**-0.723**	**-0.578**	**-0.511**	**-0.689**	**-0.526**	**-0.451**
	0.011	*0.037*	*0.012*	*0.016*	*0.025*	*0.034*
Zn	**-0.547**	**-0.336**	**-0.451**	**-0.443**	**-0.206**	**-0.385**
	0.033	*0.042*	*0.027*	*0.034*	*0.021*	*0.047*
Cu	**-0.685**	**-0.423**	**-0.498**	**-0.605**	**-0.559**	**-0.596**
	0.011	*0.029*	*0.043*	*0.018*	*0.041*	*0.015*
Ba	**-0.803**	**-0.682**	**-0.778**	**-0.699**	**-0.587**	**-0.656**
	0.025	*0.032*	*0.012*	*0.028*	*0.037*	*0.019*
Co	**-0.178**	**-0.087**	**-0.115**	**-0.147**	**-0.055**	**-0.089**
	0.017	*0.026*	*0.041*	*0.039*	*0.014*	*0.033*
Ni	**-0.254**	**-0.118**	**-0.098**	**-0.169**	**-0.098**	**-0.067**
	0.033	*0.048*	*0.015*	*0.036*	*0.027*	*0.044*
Mo	**-0.198**	**-0.147**	**-0.121**	**-0.156**	**-0.112**	**-0.103**
	0.019	*0.036*	*0.025*	*0.041*	*0.019*	*0.039*
V	**-0.112**	**-0.077**	**-0.096**	**-0.101**	**-0.056**	**-0.079**
	0.014	*0.023*	*0.045*	*0.021*	*0.042*	*0.018*
Ag	**-0.107**	**-0.058**	**-0.079**	**-0.084**	**-0.043**	**-0.065**
	0.026	*0.017*	*0.039*	*0.047*	*0.011*	*0.029*

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; 'n' in each study region depicts the total number of groundwater samples that were collected recurrently over three sampling seasons for analysis of the nephrotoxins (viz. silica and lead) and the trace metals Values are depicted as Pearson's correlation coefficients (r) with their respective p-values also calculated and italicized for identification of statistically significant associations (if any) (such as a common contaminating source) present between the stated nephrotoxin(viz. silica or lead) and the trace metals. Differences at $p<0.05$ were considered to be statistically significant correlations, which are highlighted in bold. ~~a~~ 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, negative and statistically significant($p<0.05$) associations were noted in the groundwater of all of the three study regions between each of the two nephrotoxins (viz. silica or lead) and the stated trace metals of the two individual groups of the metals viz. group1 (comprising of Fe, Mn, Zn, Cu and Al) and group 2(constituting of Co, Ni, Mo, V and Ag).This signified the existence of different contaminating sources for both the groups of trace metals and the aforementioned nephrotoxins. However the strength of association (evident from the correlation coefficient values) varied between combinations of each of the two nephrotoxins(viz. silica and lead) with the trace metals from group 1 versus that from group 2 at a comparable extent in the groundwater of all the three study regions. Higher associations (indicated by higher 'r' values) were noted between the stated nephrotoxins (i.e. silica or lead) and the trace metals from group 1 signifying the presence of a differential elemental constitution of the nephrotoxins and the group 1 trace metals in the same contaminating source i.e. the aquifer's bedrock in each of the three study regions. Contrarily, weaker (indicated by lower 'r' values) but negative associations were observed between the aforementioned nephrotoxins (i.e. silica or lead) and the trace metals from group 2 in the groundwater of all of the three study regions. This could be possibly attributed to the differential origin of the contaminating source of the nephrotoxins and the group 2 trace metals with the former probably emerging from a natural source(i.e. aquifer's bedrock) and the latter arising from an anthropogenic source(i.e. discharge of untreated effluents in the groundwater from industries like electroplating that contain these trace metals to some extents. For units and details of each individual nephrotoxin and trace metal concentration, kindly refer Tables 3.1.3 to 3.1.5 of this section of the chapter.

As indicated in **Table 3.1.9**,significantly($p<0.05$) strong, and inverse correlations of silica and Pb with various permutations of a group of trace metals viz.Fe, Mn, Zn,Ba,Al were noted in CKDu region's groundwater (Chandrajith et al.,2011; Jayatilake et al.,2013; Khandare et al., 2015; Levine et al.,2016; Markovic and Lebedev,1965;Radovanovic et al.,1991).This was

attributed to the differences in nephrotoxins and trace metals composition in the aquifer's granitic bedrock as previously analysed by Fernandes and Widdowson (2009), with nephrotoxins (silica, Pb) and trace metals being largely enriched and deficient in the bedrock respectively which is typical of a granitic geological makeup of the bedrock(Chowdhury et al, 2016; Du et al.,2014; Förstner and Wittmann,2012; Ghazali et al.,2018; Halim et al.,2013; Khan et al.,2015;Karim,2011;Kavcar et al.,2009;Kumar and Kumar,2013;Kumari et al.,2013; Machiraju et al.,2013;Sankhla et al.,2016;Wongsasuluk et al.,2014;Zuhl et al.,2013).A similar level of negative correlations between these nephrotoxins & trace metals were also noted in groundwater of study regions 2 & 3**(Table 3.1.9)**.This was also attributed to variations in toxin and trace-metal makeup of aquifer's metabasitic bedrock in these regions which contained significantly higher trace metals reserves as compared to nephrotoxins viz. silica, Pb This resulted in decreased levels of nephrotoxins (silica, Pb) and comparatively increased levels of mentioned trace metals in these region's groundwater owing to natural rock-water interactions which resulted in inverse relationship between these toxins and trace metals. (Chowdhury et al.,2016; Daae et al.,2015; Förstner and Wittmann,2012; Henry,2015; Srivastava et al.,2013; Singh et al.,2012). Moreover, significantly($p<0.05$) negative associations were noted between silica and Pb nephrotoxins in various combination with another group of trace metals comprising of Co,Ni,Mo,V,Ag in CKDu region's groundwater **(Table 3.1.9)** (Chandrajith et al.,2011; Jayatilake et al.,2013; Khandare et al.,2015; Levine et al, 2016; Markovic & Lebedev,1965; Radovanovic et al.,1991).This indicated the differential source of origin of nephrotoxins and trace metals with nephrotoxins (Pb, silica) emerging from a natural source i.e.aquifer's bedrock and stated trace metals probably arising from an anthropogenic source viz.discharge of untreated effluents from electroplating, smelting industries (Chowdhury et al.,2016; Du et al.,2014; Fantong et al.,2009; Giri & Singh,2015; Halim et al.,2013; Khan et al.,2015; Karim,2011; Kavcar et al.,2009; Kumar & Kumar,2013; Kumari et al.,2013; Machiraju et al.,2013; Sankhla et al.,2016; Zuhl et al.,2013).This same trend at a lesser magnitude (indicated by decreased 'r' values) was replicated in groundwater of other two study regions (2, 3) as well **(Table 3.1.9**).This also indicated a different origin source of nephrotoxins and mentioned trace metals, with nephrotoxins arising naturally from the bedrock and trace metals possibly from an anthropogenic industrial source (Chowdhury et al.,2016; Daae et al.,2015; Förstner and Wittmann,2012; Giri and Singh,2015;Henry,2015; Noack et al.,2014; Kavcar et al.,2009; Karim, 2011; Srivastava et al.,2013;Singh et al., 2012). The extent of association in these regions was lesser as compared to that noted in CKDu region, attributed to lesser and safe nephrotoxin levels noted in these regions groundwater.

Thereby, these correlation findings were in strong agreement with results of similar studies conducted in other CKDu regions of Sri Lanka, Balkan (Europe) and Uddanam region (Andhra Pradesh). Herein lowered trace metals levels in groundwater of the CKDu endemic region enhanced bioavailability of potential nephrotoxins viz. Pb and silica to humans, resulting in enhanced toxin accumulation in the kidneys and consequent aggravation of the induced renal tubular damage, preventing renal protection from these toxins which ultimately manifested in chronic renal failure associated with CKDu in this taluka **(**Chandrajith et al, 2011; Goldsmith and Goldsmith,1993; Khandare et al.,2015; Jayatilake et al., 2013; Levine et al.,2016; McClean et al.,2012; Nanayakkara et al;2014; Navas-Acien et al., 2009; Markovic and Lebedev,1965; Radovanovic et al.,1991; Wesseling et al.,2013; Wijkström et al.,2018).

3.1.3.6 Nephrotoxicity risk assessments

3.1.3.6.1 Estimated daily intake of the potential nephrotoxins through groundwater consumption in the three study regions of the Canacona taluka

Presence of nephrotoxins in its major exposure sources (viz. water and food) does not guarantee that the population exposed to those nephrotoxin sources will develop nephrotoxicity unless amounts/levels of exposure to the nephrotoxins are calculated and proved to surpass toxic levels. Thus for reliable assessments of nephrotoxicity induction in humans, a standard index by WHO is formulated called estimated daily intake(EDI) of nephrotoxins that helps to measure the amount of exposure to a particular nephrotoxin by calculation of the toxin intake from ingestion of its potential exposure sources (i.e. water or food). Since the current study is focused on groundwater exposure source of nephrotoxins, the nephrotoxin intake from this source will be described in this section. EDI is an accurate indicator of the rate of nephrotoxin transfer from its environmental exposure source (water or food) into human body that consequently determines the extent of induction of renal tubular damage in an exposed population. EDI of nephrotoxin is dependent on 3 major factors: nephrotoxin concentration in the exposure source (i.e. groundwater); daily ingestion rate of source and body-weight (Bw) of human (as Bw impacts tolerance to nephrotoxin) (Bandara et al., 2008 and 2011; Gadde et al., 2017; Jayasumana et al.,2013; Jayasumana et al.,2015; Jayatilake et al.,2013; Nanayakkara et al., 2014; Wanigasuriya, 2011;Wijkström et al.,2018).

In this study, EDI was calculated according to WHO set guidelines detailed in Materials and methods section. For EDI calculations, Bw was set at 60kg and daily ingestion rate of water as reported by Goa state's water legislative authority i.e. Department of water resources (2016) was 3.5L/day (Lopez-Marin et al.,2014; Ma et al.,2016; Wongsasuluk et al.,2014).

For validation of EDI,WHO has recommended a permissible intake limit of nephrotoxins called tolerable daily intake (TDI) which represents maximum amount of nephrotoxin that can be ingested from an individual source (water or food) over a lifetime without inducing significant nephrotoxic effects risk,thus used to denote safe nephrotoxin exposure levels. TDI values are specific for exposure source, thus varies between exposure sources (i.e. TDI of a nephrotoxin from exposure via water is different from that of food source due to differential rates of absorption of these nephrotoxins into blood circulation from these exposure sources (that is after ingestion into human body).Thus EDI of the nephrotoxin is generally compared with respective TDI from a particular exposure source (i.e. water in this case) to assess whether the calculated intake of nephrotoxins in the study population is hazardous enough in developing the anticipated renal tubular damage i.e. Chronic tubulointerstitial nephritis (the hallmark of CKDu) on prolonged exposure. Thus helping in establishing toxic levels of nephrotoxin exposure in the population which allows adoption of remedial measures to decrease nephrotoxins exposure and avert associated development of CKDu (Bandara et al.,2010a,b; Bandara et al.,2011;Giri and Singh,2015;Rango et al., 2015;Wu et al.,2009).

Although higher levels of two nephrotoxins viz. Pb & silica were reported in CKDu endemic region's groundwater as compared to lowered and safe levels noted in control region, the former's contribution in CKDu development can be further validated by estimation of these nephrotoxin intake levels (amount of exposure) from this exposure source viz. groundwater (Bandara et al.,2008; Bandara et al.,2010a; Rango et al.,2015; Paranagama et al.,2018), hence was determined in this study. Mean EDI values along with minimum to maximum range of their levels for these 2 potential causal nephrotoxins viz. Pb & silica from its major exposure source viz. contaminated groundwater consumption were determined in all 3 study regions and compared with their respective WHO set TDI reference limits, depicted in **Table 3.1.10.** The EDI for 3 of the nephrotoxins i.e. Cd, Hg, As were excluded from these calculations owing to below detectable levels of the same detected in groundwater of all 3 study regions signifying no intake and subsequent induction of renal dysfunction by these nephrotoxins.

As indicated in **Table 3.1.10**, mean EDI values of Pb (0.58±0.03µg/day kgbw, min-max=0.49-0.68µg/day kgbw) from groundwater were found to be significantly ($p<0.05$) higher in CKDu hit region as compared to CKD non-endemic (0.050±0.03 µg/day kgbw, min-max=0.042-0.063 µg/day kgbw) & healthy control region (0.048±0.03 µg/day kgbw, min-max=0.036-0.072 µg/ day kgbw). Moreover, 90% of CKDu region's samples displayed prevalence of EDI values in higher borderline range (i.e.0.5-0.6 µg/day kgbw).

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Table 3.1.10: Mean and range of estimated daily intake (EDI) of various nephrotoxins from the consumption of groundwater by the study population of Canacona taluka and their comparison with the respective WHO recommended tolerable daily intake (TDI) allowances.

CKD status	CKDu Endemic					CKD Non-endemic					No CKD prevalence					Annnotation for the prevalence % of the ranges of the intake levels (in their respective units) of the resepctive nephrotoxin	Permissible tolerable daily intake (TDI) of the npohrotoxin
Sampling site and period/ EDI of the nephrotoxin	Study area/ group 1 (n=114)					Study area/ group 2 (n=28)					Study area/ group 3 (n=124)						
	PRM	MON	POM	MEAN[a]	Prevalence %	PRM	MON	POM	MEAN[a]	Prevalence %	PRM	MON	POM	MEAN[a]	Prevalence %		
Lead	0.51±0.005*	0.65±0.009*	0.58±0.007*	0.58±0.007*[b]	C=89.9%; D=10.1%	0.045±0.002	0.057±0.003	0.049±0.001	0.050±0.002	A=93.5%; B=6.5%	0.039±0.001	0.054±0.002	0.051±0.001	0.048±0.002	A=94.9%; B=5.1%	A=0.03-005; B=0.05-0.07; C=0.45-0.60; D=0.60-0.75	0.6 µg/day kg bw
	(0.49-0.59)	(0.56-0.68)	(0.54-0.65)	(0.49-0.68)		(0.042-0.061)	(0.051-0.070)	(0.044-0.063)	(0.042-0.063)		(0.036-0.054)	(0.049-0.072)	(0.047-0.065)	(0.036-0.072)			
Silica	5.75±0.05*	7.55±0.09*	7.0±0.06*	6.74±0.07*[b]	C=7.8%; D=92.2%	0.66±0.012	0.82±0.009	0.95±0.008	0.81±0.011	A=91.1%; B=8.9%	0.71±0.014	0.77±0.009	0.88±0.012	0.78±0.013	A=87.3%; B=12.7%	A=0.0-1.0; B=1.0-2.0; C=4.5-6.5; D=6.5-8.5	3 mg/ day kg bw
	(5.57-7.30)	(6.67-8.14)	(6.39-7.75)	(5.57-8.14)		(0.59-0.90)	(0.69-1.10)	(0.85-1.24)	(0.59-1.24)		(0.65-1.01)	(0.78-1.16)	(0.85-1.40)	(0.65-1.40)			

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected. Values are represented as mean±SE with the range of minimum to maximum daily intake levels (shown in parenthesis) of the two nephrotoxins viz. silica and lead also depicted, that was measured from groundwater consumption in the three study groups or regions. The EDI for the other three nephrotoxic heavy metals i.e. Cd, Hg and As were not computed owing to the below detectable levels of the same detected in the groundwater of all of the three study regions. The EDI levels for the nephrotoxic heavy metal viz. lead (Pb) are indicated in µg/ day kg bw and for the nephrotoxic geogenic element viz. silica in mg/ day kg bw. The average concentrations of each individual nephrotoxin(refer Tables 3.1.4 to 3.1.5) in the groundwater estimated over three different sampling time points i.e. pre-monsoon, monsoon and post-monsoon were used for EDI calculations of the nephrotoxin. The EDI was calculated by considering the Department of water resources (Goa) established average rates of daily water consumption (in L/day) i.e. 3.5L/day for an average person bearing a body weight of 60kg (applicable for the study population of Canacona taluka as well). The EDI of the nephrotoxins were then compared to their respective WHO established tolerable daily intake levels (TDI) for assessment of the danger of developing renal dysfunction at the prevalent levels of exposure in the three study regions. Owing to the recently emerging nephrotoxic potency of silica, no accurate TDI for silica has been formulated by the WHO. Hence we utilized the maximum permissible and non-nephrotoxic intake levels established in various animal silica-toxicity studies and few epidemiological analyses as a reference threshold, for assessing the nephrotoxic danger imposed by silica at the prevalent exposure levels. As indicated, the intake levels of lead were noted to be significantly($p<0.05$) higher in the CKDu endemic region and approaching the borderline of the WHO recommended maximum tolerable allowance as opposed to the CKD-non endemic and healthy control regions wherein safe and non-toxic intake levels were noted in these regions. Thus suggesting a strong contribution of lead in the development of CKDu in the endemic region (study region 1) of the taluka. Moreover, higher intake levels of silica were also noted in the CKDu endemic region that were observed to be significantly ($p<0.05$) surpassing the previously established nephrotoxic threshold levels, highlighting the strong involvement of silica exposure in the development of CKDu as well. This finding was supported by the observance of safe and non-nephrotoxic intake levels of silica (significantly well below the toxic threshold) being noted in the other two study regions (CKD non-endemic and healthy control) of the taluka. .

Whereas, around 93.5 and 94.9% of respective study area 2 and 3's samples were occurring at the lowest EDI range (i.e. 0.03-0.05 µg/day kgbw) and well below WHO permissible or safe lead intake levels (viz. 0.6 µg/day kgbw) with a small proportion of 8.3 and 8% of its intake contributing to tolerable daily intake (TDI) respectively. Thereby, indicating no danger of nephrotoxicity in these 2 study regions. The lowered intake values observed in study region 2 and 3 were attributed to lower and safe Pb concentrations noted in groundwater of these 2 regions. Although, daily lead intake (EDI values) from groundwater in study region 1 were significantly higher than intakes in other 2 study areas, the former intake were approaching WHO threshold i.e. were on the borderline levels, with its intake contributing to 96% of TDI levels, which denoted possible risk of developing nephrotoxicity **(Table 3.1.4).** Hence these EDI values were compared with previously existing literature for analysis of the role of such intake levels in causing nephrotoxicity. Our results were consistent with borderline intake levels of Pb from groundwater consumption noted in studies of similar CKDu affected regions of Central America and Sri Lanka wherein Pb intake at such borderline levels (0.4-0.5 µg/day kgbw) over a prolonged exposure for more than 30 yrs were proved to be involved in CKDu causation in those regions (Brooks, 2009; Chandrajith et al., 2011; Correa-Rotter, 2017; Laws, 2015; Levine et al., 2016; Jayatilake et al, 2013;McClean et al.,2012; Navas-Acien et al.,2009;Wesseling et al.,2013;Wijkström et al.,2018) .On renal biopsy examination of those subjects it was found that this Pb induced nephrotoxicity at borderline exposure levels was mediated by formation of prominent Pb inclusion bodies in proximal tubular cells that directly attacked mitochondrial respiration causing enhanced oxidative stress which consequently induced incessant apoptosis & inflammation that resulted in tubular atrophy and fibrosis, manifesting in CTN (typical of CKDu pathology).This nephrotoxic potency exhibited by Pb at such borderline intakes was attributed to its longer-half life and bioaccumulative ability that caused significant Pb deposition with time which induced renal tubular damaging effects that progressively accumulated and ultimately aggravated into renal failure (Chandrajith et al.,2011; Flora et al., 2012; Levine et al.,2016; Jayatilake et al,2013; McClean et al.,2012; Navas-Acien et al.,2009; Wesseling et al.,2013; Weaver and Jaar, 2015; Wijkström et al., 2018). Moreover our results were in agreement with borderline lead intake levels (0.55 µg/day kgbw) noted in few other epidemiological dose response analysis wherein such intake levels on chronic exposure (i.e. via contaminated groundwater intake for >25 yrs) were implicated in development of CTN (CKDu pathological manifestation) (CDC,2015; Fadrowski et al.,2013;Fowler et al.,1980;Flora et al., 2012; Lin et al.,2006; NIH, 2012; Payton et al., 1994; Rastogi, 2008; Weaver & Jaar, 2015).

In coherence with the previous observations, our results strongly highlighted the significant contribution of Pb intake even at such borderline exposure levels (via chronic groundwater intake) in the etiological development of CKDu in Canacona's endemic region. This finding was further backed by low and safe Pb intakes noted in other 2study regions, confirming Pb as the causal nephrotoxin responsible for CKDu manifestation in Canacona.

Furthermore daily intake levels of an emerging nephrotoxin viz. silica were also analysed in this study to validate the contribution of silica in CKDu causation in Canacona.

As indicated in **Table 3.1.10**, average EDIs of silica from groundwater in CKDu affected region (6.74±0.01 mg/day kgbw, min-max=5.57-8.14 mg/day kgbw) were found to be significantly ($p<0.05$) as compared to CKD (0.81±0.02 mg/day kgbw, min-max= 0.59-1.24mg/day kgbw) & healthy control region (0.78±0.01 mg/day kgbw, min-max=0.645-1.40mg/day kgbw). Since no safe threshold or permissible TDI values for silica have been established by WHO till date due to its recently emerging nephrotoxic potency, we have adopted the average threshold intake levels (i.e.3 mg/day kgbw) from various silica toxicity animal studies and few epidemiological studies for renal toxicity risk assessments, wherein silica intake levels beyond this threshold were reported to inflict severe proximal tubular nephrotoxicity. As evident from **Table 3.1.10**, EDI values in 91.1 & 87.3% of respective study region 2 and 3 samples were prevalent in the lowest range (i.e.0.1-1 mg/day kgbw) and were well below the previously established nephrotoxic threshold with a small proportion of 26 and 25% of its intake contributing to the tolerable safe threshold (i.e .non-nephrotoxicity inducing permissible level).Therefore, suggesting no danger of silica induced renal damage in these 2 study regions. The lowered intake values noted in study region 2 and 3 were accredited to lower and safe silica concentration observed in these 2 regions groundwater **(Table 3.1.5).** Whereas, silica intake levels (from groundwater ingestion) in CKDu hit region (6.7 mg/day kgbw) were found to significantly ($p<0.05$) surpass previously established safe threshold (of 3 mg/day kg bw) by 2.2 folds with 92.2% of their intake levels being prevalent in the highest range (i.e.6.5-8.5 mg/day kg bw) **(Table 3.1.10).**Thus signifying a significantly elevated risk of developing proximal tubular nephrotoxicity. Our results were in agreement and significantly surpassed silica intake levels(i.e.3 mg/day kg bw)noted from groundwater consumption in similar CKDu affected populations of Andhra Pradesh and Balkan (Europe),located in close proximity to granite mines, wherein chronic intake or exposure (>35 yrs) of silica at such levels were confirmed to be responsible for CKDu development in these regions (Bolton et al.,1981;Goldsmith and Goldsmith,1993; Khandare et al.,2015; Markovic and Lebedev.,1965; Markovic, 1968, 1971, 1972 & 1974; Ng et al.,1993;

Radovanovic et al.,1991; Stiborova et al., 2016). Our findings were found to be consistent with and surpassed the renal damage inducing intake levels noted in various silica toxicity animal studies, wherein average intake of 4.2, 3.9 and 3.5 mg/day kgbw in beagle-dogs, mice and guinea pigs from consumption of silica dosed drinking water over a long-term exposure (i.e. for a period of 6-10 months) resulted in severe induction of tubular dysfunction in these models which manifested in renal failure (Dobbie and Smith, 1982; Kawanabe et al.,1992; Markovic and Arambasic,1971; Newberne and Wilson,1970).The increased incidence of tubular dysfunction (CKDu) on silica exposure in the abovementioned human epidemiological and animal studies were confirmed from biochemical analysis in both models (human, animal), renal biopsy examinations (in humans) and autopsy evaluation (in animals). In biochemical examinations, enhanced tubular proteinuria (i.e. elevated excretion of tubular damage specific LMV proteins in urine viz. NAG, b2M) with significant increase in serum creatinine, urea and uric acid were noted in affected humans and animals of these studies which was supported by existence of strong correlations between enhanced incidence of tubular proteinuria and silica intake levels from groundwater over chronic exposure. This was further backed by the presence of normal levels of urinary excretion of glomerular damage specific proteins (viz. albumin) in these studies validating role of silica in induction of tubular injuries & not glomerular dysfunction in these exposed models. These tubular injuries were confirmed by biopsy (in humans) or autopsy (in animals) analysis wherein renal histopathological alterations of tubular atrophy, mononuclear cell infiltration & interstitial fibrosis were noted which are typical of CTN (CKDu pathological manifestation). Moreover, renal histological analysis also depicted high number of silica deposits in proximal tubular cells of renal cortex, thus indicated occurrence of silica bioaccumulation on chronic exposure & associated buildup of renal tubular injuries that manifested in renal failure. Thus overall signifying role of high levels of silica exposure via groundwater intake in CTN development in these studies (human epidemiological & animal toxicity).

Therefore, in line with these aforementioned reported findings of silica toxicity animal studies and few human epidemiological analyses, our results reported higher silica intake level which significantly surpassed previously established nephrotoxic threshold (of 3 mg/day kgbw), thus highlighting the role of silica at such higher intake or exposure levels (through chronic silica contaminated groundwater consumption) in the etiological development of CKDu in Canacona's endemic region. These findings were further backed by significantly low non-toxic silica intake levels noted in other study regions (i.e. general CKD & healthy control regions), confirming silica & Pb contribution in in Canacona CKDu induction

3.1.3.6.2 Target hazard quotient of the potential nephrotoxins analysed in the groundwater of the three study regions of the Canacona taluka

Nephrotoxins that display bioaccumulation tendencies, biological and environmental persistence and chronic toxicity over long-term exposure needs to be assessed for their lifetime risk of developing renal damage in order to take necessary preventive or remedial measures to reduce exposure to such nephrotoxins. Thus for measurement of nephrotoxicity risk, WHO has formulated a tool called target hazard quotient(THQ) (Gifford et al.,2017; Gunatilake et al.,2015; Lentini et al.,2017; Ma et al.,2016b; Sabath and Robles-Osorio, 2012; Tchounwou et al.,2012; Wanigasuriya et al.,2012; Wimalawansa,2014; Wu et al., 2009).

THQ is another statistical tool that is used for predicting the risk of developing nephrotoxicity during the individual's lifespan when continuously (or daily) exposed to the levels of nephrotoxin (intake dose) estimated to be prevalent in a given population via the ingestion route (i.e. by consumption of major nephrotoxin exposure sources-water or food).Thus THQ is depicted as a fractional contribution of the estimated daily intake levels of the nephrotoxin to a permissible reference intake set by WHO called oral reference dose (RfD) with THQ calculation detailed in Materials and methods. RfD is the estimate of maximum permissible daily human exposure to the nephrotoxin (or daily toxin intake) through oral consumption of toxin's exposure source (i.e. groundwater) that is likely to occur without inflicting any appreciable renal toxicity risk during the lifetime. RfD helps to assess the extent by the which EDI of nephrotoxin from a particular exposure source (i.e. water) differs from it, thus assists in prediction of the risk of renal damage at prevalent toxin intake levels over a lifetime. As per WHO, risk of nephrotoxicity through water ingestion route exhibits a safe threshold (of 1) below which no adverse effect is expected. Hence, THQ<1 indicates the population is assumed to be exposed to safe nephrotoxin levels with no risk of developing renal damage on oral intake of exposure source over lifetime, wherein reversal of condition occurs when THQ>1 (Bandara et al.,2008 & 2011; Gadde et al.,2017; Giri & Singh, 2015; He et al.,2018; Karim, 2011; Nanayakkara et al.,2014; Wanasinghe et al.,2018; Wanigasuriya et al.,2011; Wasana et al.,2016; Wesseling et al.,2013; Wongsasuluk et al.,2014; Xu et al.,2018).

Although higher concentrations of 2 major nephrotoxin viz. Pb & silica were reported in CKDu region's groundwater as compared to lowered & non-toxic levels noted in the control region, Pb & silica's significant involvement in CKDu development can be accurately confirmed by calculations of these toxins's hazard quotient on exposure via groundwater intake (Bandara et al.,2008; Bandara et al.,2010a; Bandara et al.,2011; Paranagama et al.,2018, Wasana et al.,2016; Wanasinghe et al.,2018); hence estimated in this study. Herein,

mean THQ values along with minimum and maximum values for the 2 established causal nephrotoxins viz. Pb and silica from its major exposure source viz. contaminated groundwater intake were estimated in all 3 study regions and compared with their respective WHO established oral reference dose, which is demonstrated in **Table 3.1.11**.

THQ values for 3 of the nephrotoxins i.e. Cd, Hg, As were excluded from these estimations owing to below detectable levels of the same detected in all 3 study region's groundwater, hence negating possibility of considerable intake of these nephrotoxins (through groundwater) thus signifying no exposure and subsequent induction of renal dysfunction by these toxins. As indicated in **Table 3.1.11**, mean THQ values of Pb nephrotoxin on exposure through groundwater intake were found to be significantly ($p<0.05$) elevated in CKDu region (0.96±0.03, min-max=0.81-1.13) as compared to CKD non-endemic (0.083±0.03, min-max= 0.07-0.114) and healthy control region (0.080±0.03, min-max=0.06-0.11). 93.5 and 94.9 % of THQ values of Pb estimated in study region 2 and 3 were noted to be prevalent at the lowest range of their values (i.e. 0.06-0.09) respectively. These THQ values were well below WHO set safe and non-toxic threshold of 1 with small contributions of around 8.3 and 8% respectively to the maximum permissible hazard index (HI) of developing nephrotoxicity which was also set by WHO to be of a value of 1 **(Table 3.1.11)**. HI is the maximum permissible risk quotient that is likely to occur with no development of nephrotoxicity whatsoever from combined effect of exposure to all possible nephrotoxins (i.e. Pb & silica in this case) through a particular exposure source (i.e. groundwater in this case) (Bandara et al., 2008; Chotpantarat et al.,2014; He et al.,2018; Jayatilake et al.,2013; Karim,2011; Levine et al., 2016; Wongsasuluk et al.,2014). Remarkably lowered THQ values of Pb in regions 2 & 3 were attributed to lowered Pb intake from groundwater owing to low & safe Pb levels in the same **(Table 3.1.4)**; which indicated population is exposed to safe Pb levels at which no renal toxicity will be induced (Bandara et al.,2010a; Nanayakkara et al.,2014; Rango et al.,2015).

Parallely, 89.9% of Pb THQ values in CKDu-affected region were found to be prevalent in the higher borderline range (i.e.0.8-1**) (Table 3.1.11**). Although THQ values of Pb were significantly ($p<0.05$) higher than other 2 regions, they were noted to approach the borderline range of maximum permissible risk of 1 as established by WHO, significantly contributing to a major extent of 96% to the maximum hazardous risk (HI) from the cumulative effect of all possible nephrotoxins (i.e. silica and Pb) through groundwater intake which highlighted an enhanced potential risk of developing renal damage **(Table 3.1.11)**. Hence obtained THQ values were matched with those noted in related studies assessing contribution of Pb in inducing renal damage at borderline exposure levels.

Table 3.1.11: Mean and range of target hazard quotients (THQ) of various nephrotoxins from the consumption of groundwater by the study population of Canacona taluka and their comparison with the respective WHO recommended safe and non-toxic threshold.

CKD status	CKDu Endemic					CKD Non-endemic					No CKD prevalence					Annnotation for the prevalence % of the ranges of the THQ values of the resepctive nephrotoxin	WHO established safe and permissible threshold of THQ	Hazard index(HI)= mean THQ (lead) + mean THQ (silica)
Sampling site and period/ THQ of the nephrotoxin	Study area/ group 1 (n=114)					Study area/ group 2 (n=28)					Study area/ group 3 (n=124)							
	PRM	MON	POM	MEAN[a]	Prevalence %	PRM	MON	POM	MEAN[a]	Prevalence %	PRM	MON	POM	MEAN[a]	Prevalence %			
Lead	0.85±0.004*	1.08±0.009*	0.96±0.005*	0.96±0.008*[b]	C=89.9%; D=10.1%	0.075±0.002	0.095±0.003	0.081±0.001	0.083±0.002	A=93.5%; B=6.5%	0.065±0.001	0.09±0.003	0.080±0.001	0.080±0.002	A=94.9%; B=5.1%	A=0.06-0.09; B=0.09-0.12; C=0.8-1; D=1-2	1	HI(study group 1) =3.21; HI(study group 2) =0.353; HI(study group 3) =0.34
	(0.81-0.98)	(0.93-1.13)	(0.9-1.07)	(0.81-1.13)		(0.070-0.101)	(0.085-0.114)	(0.073-0.105)	(0.070-0.114)		(0.060-0.090)	(0.081-0.11)	(0.078-0.104)	(0.060-0.110)				
Silica	1.92±0.01*	2.51±0.04*	2.33±0.03*	2.25±0.03*[b]	C=7.8%; D=92.2%	0.22±0.003	0.27±0.004	0.31±0.005	0.27±0.004	A=91.1%; B=8.9%	0.23±0.004	0.25±0.005	0.29±0.009	0.26±0.006	A=87.3%; B=12.7%	A=0.1-0.3; B=0.3-0.6; C=1-2; D=2-3		
	(1.85-2.43)	(2.23-2.71)	(2.13-2.58)	(1.85-2.71)		(0.20-0.25)	(0.23-0.31)	(0.27-0.35)	(0.20-0.35)		(0.21-0.28)	(0.26-0.33)	(0.28-0.39)	(0.21-0.39)				

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; HI-hazard index; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; WHO-World health organization. 'n' in each study region depicts the number of subjects in that study region or group from which the groundwater samples were collected. Values are represented as mean±SE with the range of minimum to maximum THQ values (shown in parenthesis) of the two nephrotoxins viz. silica and lead also depicted, that was measured from groundwater consumption in the three study groups or regions. The THQ values being a ratio possess no units and details and details. The average EDI of an individual nephrotoxin (refer Table 3.1.10) through groundwater consumption were used for THQ estimations of the nephrotoxin by calculating the fractional contribution of the measured nephrotoxin's EDI to their respective WHO formulated(for lead) and literature established(for silica) oral reference doses as described earlier. The WHO and previously determined set oral reference doses for Pb and silica are 0.6 µg/day kg bw and 3 mg /day kg bw respectively. As indicated the THQ values of Pb and silica in the CKDu endemic region were found to be significantly ($p<0.05$) borderline and exceeding their respective reference limits. Thus suggesting higher risk of developing severe renal damage (specifically tubular dysfunction) over a lifetime of an individual at the current levels of enhanced exposure to both of the nephrotoxins prevalent in this region through the gravely contaminated groundwater consumption. This was supported by the observance of safe and non-nephrotoxic risk values of silica (significantly well below the toxic threshold) being noted in the other two study regions (CKD non-endemic and healthy control) of the taluka. Furthermore, the cumulative risk of developing renal toxicity (i.e. Hazard index, HI) by the combined effects of both of these aforementioned nephrotoxins on exposure at such levels was computed by the summation of the THQ's of each individual nephrotoxin[i.e. HI= THQ(lead)+ THQ(silica)].As indicated, the hazard index in the CKDu endemic region was also found to significantly exceed the permissible threshold of 1 unlike the other two study regions(2 and 3) that were well below the non-toxic limit. Thus suggesting that the individual and combined nephrotoxins (i.e. silica and lead) exposure via groundwater consumption conferred a remarkably severe lifetime risk of developing CKDu disease in the Canacona taluka, at the prevalent exposure levels noted in this endemic region. .

Our findings were in agreement with the borderline hazardous risk values of Pb from groundwater ingestion noted in studies of similar CKDu affected regions of Sri Lanka and Central America wherein chronic exposure or intake (for over 30yrs) to borderline Pb levels (0.4µg/day kgbw) through groundwater significantly enhanced the susceptible population's risk in developing nephrotoxicity in a lifetime. This observation was deduced from absence of glomerular injury indicated by normal urinary albumin levels with simultaneous enhanced induction of tubular dysfunction markers viz. increased serum creatinine, uric acid and urea, increased urinary b2M and NAG levels in exposed subjects that proportionately escalated with increasing exposure to borderline levels of Pb intake, suggesting a strong role of Pb in inducing tubular damage (i.e. CTN, typical of CKDu) on chronic exposure at such borderline levels as well. These finding were further confirmed by presence of significant Pb deposits in renal proximal tubule & presence of CTN specific renal histopathological changes of tubular atrophy cum fibrosis and interstitial scarring which supported Pb's bioaccumulative ability (Flora et al.,2012; Weaver and Jaar, 2015),which were possibly responsible for accumulated renal tubular damage and exaggeration into renal failure on chronic exposure (Brooks, 2009; Chandrajith et al.,2011; Correa-Rotter,2017; Laws, 2015; Levine et al.,2016; Jayatilake et al,2013; McClean et al.,2012; Navas-Acien et al.,2009; Wesseling et al.,2013; Wijkström et al.,2018).

In coherence with the previous findings, our results strongly highlighted that exposure to borderline Pb levels (occurring in the CKDu endemic region) through the oral ingestion route (i.e. groundwater intake), significantly enhanced the risk of susceptible population present in CKDu regions in developing severe nephrotoxicity (renal tubular damage-CKDu) on chronic exposure. This was backed by safe and non-toxic THQ values noted in other 2study regions which indicated that the populations residing in these regions are at no significant risk of developing nephrotoxicity throughout the lifetime at such non-toxic levels of exposure to Pb in groundwater of these regions. Therefore, confirming strong involvement of Pb in the etiological development of CKDu in the endemic region of the Canacona.

Furthermore, THQ risk values of an emerging nephrotoxin viz. silica were also analysed in the current study to further confirm involvement of silica in CKDu causation in Canacona.

As indicated in **Table 3.1.11**, average THQ risk of silica in inducing nephrotoxicity from contaminated groundwater intake were significantly ($p<0.05$) higher in CKDu affected region (2.25±0.01, min-max=1.85-2.71) as compared to CKD non-endemic (0.27±0.02, min-max=0.2-0.35) and healthy control region (0.26±0.01, min-max=0.21-0.39). Since no safe threshold of THQ for silica has been set by WHO till date due to its recently emerging

nephrotoxic potency, we have adopted the safe threshold reference value of 1 for a nephrotoxin in general in this study as well. This method was adopted from similar usage of the safe threshold of 1 in aforementioned human epidemiological studies and animal toxicity analyses for silica nephrotoxicity risk assessments, wherein THQ value of silica exceeding 1 was considered to denote a strong risk of inflicting grave proximal tubular damage. As witnessed in current study, 91.1 and 87.3% of THQ values in study regions 2 and 3 respectively were found to be prevalent in the lower range (0.1-0.3) **(Table 3.1.11)** & below the WHO set safe threshold of 1.Moreover,this THQ values contributed to a small extent of 26 and 25% to the maximum permissible cumulative nephrotoxicity risk (Hazard index) of 1 that can be inflicted from combined exposure to all nephrotoxins (i.e. Pb and silica) from a particular exposure source (i.e. groundwater) **(Table 3.1.11).**This reduced THQ risk values were ascribed to lower silica intake (exposure levels) from groundwater due to safe Pb levels in the water **(Table3.1.5).** Thus denoting no risk of developing renal damage in these 2areas.

Parallely, mean THQ risk values of silica in CKDu affected region significantly exceeded the adopted safe non-nephrotoxic threshold of 1 by 2.2 folds with 92.2% of THQ values being prevalent in severely nephrotoxic higher range of 2-3 **(Table 3.1.11)**. Thus denoting a significantly high risk of developing proximal tubular damage in prone population on chronic exposure to silica levels prevalent in this region. Our findings were found to be consistent and significantly exceeded the nephrotoxicity inducing risk values of silica (i.e.1.2) from groundwater intake in similar CKDu affected regions of Andhra Pradesh & Balkan (Europe), wherein these THQ values highlighted the susceptible population's high risk of developing tubulotoxicity in a lifetime on chronic exposure/intake (> 35yrs) of 3mg/day kgbw levels via groundwater. These findings were deduced from increased prevalence of renal tubular dysfunction markers in urine (viz. b2M, NAG) and serum (viz. creatinine, urea, uric acid) which proportionally rose with chronic exposure (>35yrs) to higher silica intake levels (3mg/ day kgbw), which highlighted silica's bioaccumulative potency that caused amplification of induced renal tubular damaging effects that manifested in CKDu development which exaggerated into renal failure (Goldsmith & Goldsmith,1993; Khandare et al,2015; Markovic, 1968 & 1971; Markovic & Lebedev,1965; Radovanovic et al.,1991; Stiborova et al., 2016).

Additionally our findings of silica's THQ values were consistent and remarkably exceeded THQ values (i.e.1.4, 1.3, 1.1) noted in silica toxicity risk analysis in animals like guinea pigs, dogs, mice respectively. Herein these silica's THQ risk values noted in beagle dogs, mice, guinea pigs suggested a strong risk of developing nephrotoxicity in a susceptible group on chronic exposure (>6-8 months) to respective daily intake levels of 4.2,3.9,3.5mg/day kgbw

via drinking water ingestion. This was concluded from the progressive increase in the extent or magnitude of induced CTN specific renal histopathological alterations (of tubular atrophy and fibrosis) with increasing period of exposure (i.e. from 1 to 7months) to high silica intakes (>3mg/day kgbw). Moreover, strong correlations were noted between increasing levels of tubular proteinuria (i.e. increased urinary excretion of tubular injury specific proteins-b2M, NAG) & rising period of silica exposure (i.e. 1 to7 months) to high silica intakes (>3mg/day kgbw), suggesting high risk of tubular-toxicity (noted in CKDu) on chronic & high silica exposure (Dobbie & Smith, 1982; Kawanabe et al.,1992; Newberne & Wilson, 1970).

Therefore, in agreement with previous findings, our results strongly highlighted that chronic exposure to remarkably higher silica levels (prevalent in the CKDu endemic region) through the oral route (i.e. intake of contaminated groundwater) significantly escalated the vulnerable population's risk in CKDu endemic regions in developing grave proximal tubular damage (i.e. CKDu) in a lifetime. This was supported by safe and non-toxic THQ values noted in other 2 study regions which depicted that these regions are at no substantial risk of developing CKDu throughout their lifespan at such non-toxic silica exposure levels in these region's groundwater. Therefore further justifying strong involvement of silica along with Pb in the etiological development of CKDu in the endemic region of Canacona taluka.

Overall, on evaluating the cumulative risk of developing nephrotoxicity (i.e. hazard index, the sum of THQ values of each nephrotoxin) from the combined exposure to all possible nephrotoxins (i.e. silica & Pb in this case) through groundwater intake, it was observed that the HI values in CKDu endemic region (i.e. 3.21) **(Table 3.1.11)** significantly ($p<0.05$) exceeded the WHO set safe threshold of 1.These HI values were also significantly higher than safe and non-toxic cumulative risk values noted in study area 2 (HI=0.35) and study area 3 (HI=0.34) **(Table 3.1.11).** This suggested a remarkably elevated and severe risk of developing proximal tubular damage (i.e. CKDu) among the susceptible inhabitants of this CKDu affected region over a lifetime, on chronic and combined exposure to both nephrotoxins viz. silica and Pb at the significantly elevated levels that were prevalent in this region's groundwater (these nephrotoxin major exposure source).This signified additive effects of these nephrotoxins (Pb, silica) that caused severe amplification of renal tubular damaging effects with time that consequently progressed into induction of chronic renal failure which ultimately manifested in CKDu development disease in this endemic region of Canacona (Bandara et al.,2008; Dharma-Wardana, 2018; Jayasumana et al.,2015b; Jayasumana et al.,2016; Jayatilake et al.,2013; Levine et al.,2016; Wesseling et al.,2013).

3.1.3.7 The role of various intake levels of the nephrotoxins- silica and lead (via groundwater consumption) in the manifestation of tubular or glomerular specific nephropathy in the study population of Canacona taluka

It is well reported that EDI of nephrotoxins on surpassing their respective WHO tolerable limits on chronic exposure induces tubular damage viz. Chronic tubulointerstitial nephritis (CTN), typical of CKDu pathology. Thus effect of daily intake (via groundwater intake) of the two established causal nephrotoxins viz. silica and Pb on induction of tubular (CKDu) and glomerular (typical of diabetes or hypertension triggered CKD) based nephropathies in study population of Canacona was analyzed. This was achieved by assessing the relationship between estimated daily intake (EDI) levels of these nephrotoxins and alterations in tubular or glomerular dysfunction specific biomarker profile via calculation of significant Pearson correlation coefficients(r) (Bandara et al.,2008; Bandara et al.,2010a; Bandara et al.,2011; Giri and Singh,2015; Herath et al.,2018; Jayasumana et al.,2015b; Jayatilake et al.,2013;Ma et al.,2016b; Nanayakkara et al.,2014; Weaver et al.,2015; Wongsasuluk et al.,2014).Tubular damage specific markers include uBCR(urinary b2M to creatinine ratio) and uNCR (urinary NAG to creatinine ratio) which depicts levels of low-molecular weight proteins (b2M) and tubular specific enzyme (NAG) respectively, that significantly escalate in urine on tubular injury due to nephrotoxin induced disruption in intrinsic tubular reabsorption of these proteins into plasma (Laws et al.,2016; Ramírez-Rubio et al.,2015; Wanigasuriya et al.,2017). Glomerular injury specific markers include uPCR (urinary protein/creatinine ratio), uACR (urinary albumin/creatinine ratio), uAPR (urinary albumin/protein ratio) and uAlb/b2M ratio (urinary albumin/b2M ratio) which indicates proteins levels majorly albumin, that increases in the urine owing to diabetes or hypertension induced glomerular damage, which on good-health is averted due to these protein's high molecular weight that prevents filtration into urine (Fisher et al.,2013; Solini et al.,2014; Levey et al.,2015; Warady et al.,2015).Therefore, correlation assessments between EDI (intake levels) of the nephrotoxins (via groundwater) and tubular or glomerular injury specific biomarkers will help in accurate evaluation and validation of contribution of these potential causal nephrotoxins viz. silica and Pb at the prevalent exposure/intake levels (via groundwater intake) in CKDu induction (i.e. tubular pathology) in Canacona and negate their involvement in glomerular injury induction, the results of which are presented in **Table 3.1.12.**

As indicated in **Table 3.1.12**, significantly ($p<0.05$) strong and positive correlations were noted between increasing intake of silica and Pb nephrotoxins (via groundwater intake) and rising levels of tubular dysfunction markers(evident from increased levels of uBCR and

uNCR) in CKDu affected population (study group 1).The correlation values of EDI of Pb/silica with uBCR and uNCR were found to be 0.936(p=0.027)/ 0.947(p=0.015) and 0.903 (p=0.019)/ 0.912(p=0.036) respectively. Thus highlighting strong contributions of silica & Pb nephrotoxins at the significantly higher levels of intake (exposure) which are prevalent in the CKDu endemic region in development of tubular pathology, typical of CKDu in Canacona. This was further supported by presence of comparatively weaker, negligible and insignificant (p>0.05)correlations noted between these nephrotoxin intake levels and tubular injury markers in study group 2 and 3 populations at a comparable extent **(Table3.1.12)**. Thus signifying no role of these nephrotoxins viz. silica & Pb in induction of tubular damage in these study group2 and 3 subjects (Cabral et al.,2015; Ganguli,2016; Jayatilake et al.,2013; Khandare et al.,2015; Levine et al.,2016; Markovic,1968; Markovic and Lebedev, 1965; McClean et al.,2012; Radovanovic et al.,1991; Tatapudi et al.,2018; Wesseling et al., 2013).

Table 3.1.12: Correlation assessments between the estimated daily intakes of each individual nephrotoxin and the average urinary based nephropathy specific biomarkers of the Canacona taluka's study population for analysis of the contribution (if any) of the nephrotoxin exposure (through contaminated groundwater consumption) in the induction of tubular or glomerular pathology in the taluka.

Mean EDI of each nephrotoxin (from groundwater consumption)	Study group (total number of subjects)	Correlation coefficients [r] *(p values)*					
		Urinary based nephropathy specific biomarkers					
		uPCR	**uACR**	**uBCR**	**uNCR**	**uAPR**	**uAlb/b2M ratio**
Silica	**1**	-0.036	-0.011	**0.947**	**0.912**	-0.041	-0.021
	(N=114)	*0.102*	*0.059*	*0.015*	*0.036*	*0.077*	*0.213*
	2	-0.045	-0.029	0.044	0.067	-0.051	-0.043
	(N=28)	*-0.204*	*-0.098*	*0.078*	*0.099*	*-0.065*	*-0.084*
	3	-0.022	-0.008	0.054	0.041	-0.022	-0.015
	(N=124)	*-0.077*	*-0.103*	*0.102*	*0.058*	*-0.098*	*-0.054*
Lead	**1**	-0.044	-0.021	**0.936**	**0.903**	-0.057	-0.033
	(N=114)	*-0.21*	*-0.132*	*0.027*	*0.019*	*-0.069*	*-0.079*
	2	-0.033	-0.018	0.023	0.039	-0.043	-0.029
	(N=28)	*-0.089*	*-0.054*	*0.095*	*0.103*	*-0.146*	*-0.098*
	3	-0.011	-0.005	0.041	0.035	-0.027	-0.02
	(N=124)	*-0.087*	*-0.098*	*0.077*	*0.069*	*-0.13*	*-0.074*

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; EDI-estimated daily intake; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. 'N' in each study region depicts the number of subjects in that study region or group from whom the groundwater samples were collected recurrently over three sampling seasons and the same subjects were analysed for nephropathy specific biomarkers in the urine. Values are represented as Pearson's correlation coefficients (r) with their respective p-values 'italicized' for identification of statistically significant associations (if any) between any given two parameters [i.e. daily intake of the nephrotoxins and the variations (increase or decrease) in the tubular or glomerular nephropathy specific biomarkers]. Differences at $p<0.05$ were considered to be statistically significant correlations, which are highlighted in bold. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, strong (indicated by higher 'r values'), positive and statistically significant($p<0.05$) associations were noted between the intake or exposure levels (through groundwater consumption) of both of the nephrotoxins (viz .lead and silica) and the tubular nephropathy specific biomarkers (i.e. uBCR and uNCR) in the CKDu affected study group 1.This highlighted the strong involvement of these aforementioned nephrotoxins in triggering the tubular dysfunction typical of CKDu manifestation in the Canacona taluka at such high and prolonged levels of exposure through long-term contaminated groundwater consumption. Furthermore, negligible, inverse and insignificant ($p>0.05$) correlations were noted between the intake levels of the aforementioned nephrotoxins and the glomerular dysfunction biomarkers (i.e. uPCR, uACR, uAPR and uAlb/b2M ratio) at an analogous extent in all of the three study groups, indicating no contribution of these nephrotoxin exposure in triggering the glomerular pathology observed in the study group 2 as well. For units and details of the estimated daily intake levels of the nephrotoxins and the renal dysfunction biomarkers, kindly refer Table 3.1.10 of the current section of this chapter and Table 2.8 of chapter 2 respectively.

In totality, these findings were further supported by presence of insignificantly ($p>0.05$) inverse associations noted between exposure doses (intake levels) of silica and Pb nephrotoxins and levels of glomerular injury biomarkers (i.e. uPCR, uACR, uAPR, uAlb/b2M ratio) at a comparable extent in all 3 study groups **(Table 3.1.12)**. Thus justifying intrinsic potency of these nephrotoxins in solely inducing proximal tubular dysfunction at high levels of prolonged exposure with no involvement whatsoever in triggering glomerular function disruption. This inverse relationship provided supporting evidence for confirming diabetes and hypertension to be main causative agents of glomerular CKD witnessed among study group 2 subjects (Rajapakse et al.,2016; Ratnayake et al.,2017; Smith et al.,2011).

Overall, our correlation findings were in agreement with individual values obtained in independent etiological assessments of the role of silica & Pb in CKDu causation. Herein these studies autonomously and strongly supported the role of prolonged exposure (intake) to silica and Pb nephrotoxins via groundwater intake in significant induction of tubular pathology associated with CKDu development in similarly affected regions of Andhra Pradesh (supporting silica's role in CKDu) [Ganguli,2016; Khandare et al.,2015; Tatapudi et al., 2018]; Balkan region of Europe (supporting silica's role in CKDu) [Markovic, 1968; Markovic and Lebedev, 1965; Radovanovic et al.,1991]; Sri Lanka (supporting Pb's role in CKDu) [Levine et al.,2016; Jayatilake et al.,2013] & Central America (supporting Pb's role in CKDu) [Brooks, 2009; Cabral et al.,2015; McClean et al.,2012; Laws,2015; Laws et al.,2015; Wesseling et al.,2013]. Thus, in coherence with previously reported findings, our results provide confirmatory evidence to the strong contribution of silica & Pb nephrotoxins in etiological development of CKDu in Canacona, being only manifested on chronic & higher levels of exposure/intake (via contaminated groundwater ingestion)of these nephrotoxins.

3.1.4 Conclusion

As per our knowledge, the current study is the 1st report to highlight that high predominance of CKDu (i.e. chronic tubulo-interstitial nephritis) witnessed in some villages of Canacona could be an outcome of individual or synergistic effects of chronic exposure to high-levels of nephrotoxic silica and borderline lead levels via long-term consumption of untreated contaminated groundwater. This study highlighted groundwater to be the major exposure route to these potential causal nephrotoxins viz. silica and lead whose increased levels in groundwater could have stemmed from AMD from an abandoned granite mine located in the vicinity of Canacona's CKDu endemic region, which resulted in increased groundwater

acidity and related enhanced leaching of these nephrotoxins from the aquifer's granitic bedrock that innately contains higher silica and Pb deposits (Fernandes & Widdowson,2009) as compared to healthy region. This was supported by significantly($p<0.05$) higher negative correlations noted between pH & nephrotoxin levels in groundwater supporting nephrotoxin (silica, Pb) bioavailability enhancing effect with decreasing pH. This enhanced nephrotoxins bioavailability(especially silica) could also be attributed to trace metals (i.e. Fe, Mn, Zn, Cu) and major cations (viz. Ca, Mg) deficiency of CKDu region's groundwater as these metals ions are reported to trap silica and limit its availability and nephrotoxicity by inert metal silicate complexes formation at high metal level and basic pH. This was backed by significantly inverse correlations noted between trace metals, major metal, Pb & silica toxin.

This study also highlighted Pb's potency to inflict nephrotoxicity at borderline (low-exposure levels) in CKDu endemic region's groundwater possibly attributed to its bioaccumulative tendency and long half-life that could have caused progressive accumulation and aggravation of Pb induced nephrotoxic effects with time which ultimately could have exaggerated into CKDu development. This work also highlighted possibility of lead to two exhibit modes of nephrotoxic action-direct and indirect. Direct nephrotoxicity was possibly mediated by direct oxidative damage to nephron's proximal tubule (1˚nephrotoxin target)which resulted in excess apoptotic cell death and inflammation that ultimately manifested in tubular atrophy and fibrosis typical of CTN (hallmarks of CKDu). Indirect nephrotoxicity was possibly potentiated by Pb interfering with bone-mineral-homeostasis that may have resulted in skeletal-disorder, which triggered chronic NSAID's intake in this CKDu region (noted from demographic study) that could have aggravated renal-damage as NSAID's nephrotoxic nature is reported.

The potential etiological contribution of the two nephrotoxins (silica and Pb) highly prevalent in Canacona's CKDu region's groundwater was confirmed by assessment of daily intake (EDI) or exposure levels of these nephrotoxins and lifetime risk of developing nephrotoxicity at such toxin exposure levels (viz. THQ). Results highlighted that daily intake levels of lead and silica nephrotoxins were found to significantly approach the borderline of WHO set maximum tolerable intake (TDI) limit (i.e. 0.6 µg/day kg bw) and remarkably surpassed previously established TDI limit (i.e. 3 mg/day kg bw) respectively in CKDu affected region. This suggested the strong involvement of Pb and silica nephrotoxins in induction of nephrotoxicity (viz. tubular damage) on prolonged exposure to such high nephrotoxins levels through direct intake of untreated groundwater. This was further supported by toxicity risk assessment values (i.e. THQ) of Pb and silica nephrotoxins correspondingly being on the

borderline and exceeding WHO formulated non-hazardous threshold of 1 respectively in this CKDu region. Thus suggesting a significantly enhanced risk of developing nephrotoxicity (i.e. CKDu pathology) over a lifetime in susceptible populations of Canacona's CKDu endemic region on prolonged exposure to high nephrotoxins levels that are prevalent in this region's groundwater (through direct consumption).This nephrotoxicity risk was significantly elevated on combined exposure to both nephrotoxins as evident from cumulative THQ values of nephrotoxins viz. hazard index significantly surpassing WHO established non-toxic threshold of 1, highlighting the possibility of additive renal tubular damaging effects of both nephrotoxins in CKDu hit population, that could have significantly exaggerated into manifestation of irreversible chronic renal failure. This strong contribution of Pb and silica toxins in increased CKDu manifestation in Canacona was supported by presence of significantly ($p<0.05$) strong & positive associations (via correlation analysis) noted between higher nephrotoxin exposure levels and increased induction of tubular injury biomarkers noted in CKDu subjects with no correlation noted with glomerular injury markers.

Thus this study highlighted the significant role of lead and silica nephrotoxins at such high exposure levels through chronic untreated groundwater consumption in the etiological development of the tubular pathological manifestation associated with CKDu in Canacona.

Section 3.2

Environmental monitoring of nephrotoxins in the 'food' exposure matrix and assessment of their contribution in CKDu manifestation

3.2.1 Introduction

Our previous chapter has established CTN to be pathological manifestation of CKDu in Canacona that is caused by environmental nephrotoxins, hence role of nephrotoxins in CKDu causation was explored. Human exposure to nephrotoxins is mediated via two major routes viz. food and untreated groundwater (well-water) (Lusco et al., 2017).This section focuses on detailed analysis of nephrotoxins in commonly consumed foods (food matrix) of Canacona.

Food is a major exposure route for nephrotoxins arising naturally or by anthropogenic invasion of the balanced ecosystem. Naturally originating nephrotoxins include toxins produced as by-products in living organisms under favorable growth conditions and include ochratoxin (OTA) and aristolochic acid (AA). OTA and AA are produced by fungal sp. i.e. *Aspergillus ochraceus* and plant weed sp. i.e. *Aristolochia indica* (birthwort) respectively under corresponding inappropriate storage (low temperature, high moisture) and harvest conditions (intermingling of weed seeds and cereal grains). Both toxins are endemic to cereal & pulses fields and possess high thermochemical stability causing them to be unaffected by food processing, thus contaminating food grains.Their nephrotoxicity mechanism is mediated by oxidative damage, detailed in Chap.1 (Weaver et al.,2015;Rajapakse et al., 2016).

Anthropogenically arising nephrotoxins include toxins contaminating exposure matrices-food or water on disturbances of the same by human (anthropogenic activities) and includes heavy metals like lead, cadmium, arsenic, and mercury. Heavy metal pollution in aquatic and terrestrial environments (that ideally contain trace metal levels) can arise from human activities like industrial discharge, metal-laden fertilizers usage which leads to adverse effects on fish & plants (vegetable, cereal crops) that serve as 1˚components of staple diet in developing countries-India (Gunatilake et al.,2015;Tchounwou et al.,2012;Xu et al.,2018).

Heavy-metals are non-biodegradable, toxic, possess long half-life and bioaccumulative. In aquatic ecosystem, fish directly accumulate heavy metals in muscles via oral route by chronic consumption of metal contaminated water for survival. Whereas in terrestrial ecosystems, plants are a way of heavy metal transfer from contaminated soils to humans. In metal contaminated soils, plants uptake & accumulate heavy metals (due to long half-life) in different organs (edible parts) viz. seeds, leaves,vegetables.This metal accumulation in edible parts of fish & plants result in human exposure to nephrotoxins via direct ingestion through food chain leading to metal accumulation & induction of nephrotoxicity in humans. All heavy metals share a common renal-toxicity mechanism i.e. mediated by oxidative injury explained in Chap.1 (Lentini et al.,2017; Wu et al., 2016; Xu et al.,2018; Orr and Bridges,2017).

In developing countries like India, fish, vegetables, pulses and staple cereals (rice, wheat) are important protein sources contributing to 51% of animal protein, 35%, 42% & 60% of plant protein respectively (Tilman and Clark, 2014; D'Odorico et al.,2018;Von Braun, 2007). Moreover, Canacona being a rural populace located in India's coastal belt primarily obtains animal protein from regular consumption of cheap fish types like mackerels, sardines etc. They also derive plant protein from developing countries's staple pulses and cereal crops like chickpea, rice, wheat, green-gram, ragi respectively being a rural community (Statistical Handbook of Goa, 2016). Further protein intake in this rural taluka is achieved via consumption of small-scale local cum organically cultivated vegetables-red/green amaranthus, spinach, pumpkin, brinjal. Consumption of lean meat like white meat (poultry) and red meat (cattle, goats) are highly limited owing to inability to afford these expensive protein due to low economic status. In Canacona, fish, vegetables, pulses & cereals accounted for 72% of animal protein, 42%, 35% & 49% of plant protein respectively with each category contributing 50.3g/capita/day, 58.6g/capita/day, 35.6g/capita/day & 81.5g/capita/day relatedly. As fish, vegetables, pulses and cereals are primary indicators of nephrotoxin accumulation in the food chain and established as principal constituents of rural Canacona's staple diet, fish and [vegetables,pulses and cereals] can be deemed as suitable bio-indicators of contamination in aquatic and terrestrial ecosystem respectively with nephrotoxins like ochratoxin, aristolochic acid and heavy metals (Haldankar , 2016; Velip, 2016).

Hence, in the current study, the levels of nephrotoxins like ochratoxin and aristolochic acid were examined in cereals (rice, wheat, green gram and ragi) and pulses (chickpea) collected from Canacona as they are restricted to these matrices. Heavy metals viz. Pb, Cd, Hg, As were analysed in daily consumed fish (mackerel, sardines), vegetables (red and green amaranthus, spinach, pumpkin, brinjal) and abovesaid cereals and pulses in Canacona owing to diverse prevalence distribution of heavy metals in aquatic and terrestrial ecosystems and are not merely restricted to cereals and pulses food matrices like OTA and AA. To the best of our knowledge, the currently described study is the first report that conducted a detailed analysis of principal food originating natural (OTA and AA) and anthropogenic (heavy metals) nephotoxins in a variety of commonly consumed food (right from plant to animal sources) in CKDu affected and non-affected regions of Canacona using validated and accredited test methods, in order to assess the role (if any) of these nephrotoxins on chronic exposure in causation and development of CKDu in this rural taluka.

3.2.2 Materials and methods

3.2.2.1 Study design/study area

The similar design of the study groups as incorporated in the demographic and biochemical analysis of the CKDu cases in the Canacona taluka (Chapter 2) was adopted in the current study as well. Herein, a detailed-list of Canacona sub-district's CKD-affected patients was obtained from two main hospitals in Goa-Apollo Victor hospital and Canacona Health Centre. On analysis it was found that 142 from a combined total of 180 CKD affected patients were hailing from the Canacona taluka. From this 80 % of the CKD affected patients (n=114) possessed no defined etiology hence the CKD among these subjects was recategorized as Chronic kidney disease of unknown etiology (CKDu).These CKDu affected subjects were residents of two villages namely Ponsulem and Chaudi, that were located in the vicinity of a non-operational granite mine. Hence for convenience the residing subjects in these two CKDu endemic villages were cumulatively grouped under one broad category i.e. study-group 1 and their area of residence was clubbed as study-area 1.The remaining 28 patients from the grand total of 142 patients possessed known etiology of CKD related to traditional causals of diabetes and hypertension. These CKD affected subjects were hailing from scattered villages of the taluka namely Cola, Poinguinim and Anvali and were collectively grouped under the non-endemic study-group 2 and their area of residence categorized as study-area 2. For selection of true-controls, a total of 124 volunteers with a 1: 1 sex ratio from two healthy villages of the taluka namely Molorem and Endrem (showing no prevalence of CKD whatsoever) were randomly chosen to match the age and sex distribution of the CKDu cases. These healthy volunteers from both the non-CKD prevalent villages were clubbed together under study-group 3 with their area of residence grouped as study-area 3. The details of the three study-groups are presented in Table 2.1 of Chapter 2 (demographic and biochemical study of the CKDu cases) of this thesis.

3.2.2.2 Demographic survey of the types of food and their rate of consumption in the study population

The food legislative authority of Government of Goa i.e. Directorate of Agriculture and Directorate of Fisheries provides lists of consumption rates of major food categories (i.e. cereals, pulses, vegetables and fish) in Goa for appropriate analysis of intake of various health hazardous toxins. Despite information on food consumption rates, detailed description of food types/species under each broad category commonly consumed in the taluka are unavailable.

In order to appropriately analyse the levels of nephrotoxin exposure via the food route in an affected study population, it is mandatory to know the types or the species of food commonly consumed under each category by the targeted study groups. Analysis of the nephrotoxins in the wide spectrum of food matrices available for human consumption will be practically impossible and unfeasible. Hence, to avoid unnecessary wastage of resources and input of effort in the irrelevant sample analysis, a detailed demographic survey of the commonly consumed type or species of food samples under the three broad food categories (i.e. cereals or pulses, vegetables and fish) in the three study groups or regions of Canacona taluka was conducted (Munoz et al., 2017).

The subjects of the three study groups or regions were interviewed at the selected hospitals using a structured questionnaire to acquire information on various parameters like the number and type of cereals regularly consumed, the number and type of pulses frequently consumed, the number and type of vegetables commonly consumed ,the number of fish varieties consumed and the approximate daily rate of consumption of each food type/species (measured in g/day); the results of which are presented in **Table 3.2.1**. The demographically derived average consumption rates (in g/day) of each food category (i.e. cereals and pulses, vegetables and fish) was calculated by taking the mean of the consumption rates of each food type or species under the respective individual categories. This demographically obtained average consumption rates for each food category were then compared with their respective average consumption rates listed by the state's Food legislative authorities i.e. Directorate of Agriculture and Directorate of Fisheries (2016, 2017) to validate the obtained data and were found to be more or less homologous **(**Directorate of Agriculture, 2015; Directorate of Agriculture, 2016; Directorate of Fisheries report, 2016; Fishery Resource Potentiality Of Goa, 2015; Indiastat, 2017; MoSPI, 2014). Since the Food legislative authorities listed data was obtained from a vast population sampling and survey, the results (i.e. average consumption rate for each food category) of the same were utilized for further analysis of the nephrotoxin intake through various food categories (Mendil et al., 2015; Levine et al., 2016; Siriwardhana et al., 2014). The food types/species under each broad food category (i.e. cereals and pulses; vegetables and fish) were chosen for analysis in the three study groups based on the demographically obtained average rate of consumption (in g/day) of these food types in the respective food categories. Based on this, the first five highly consumed food types or species (deduced from their higher consumption rates per day) under each broad category (i.e. cereals and pulses, vegetables and fish) were selectively chosen for analysis of various food specific nephrotoxins.

Table 3.2.1: Demographic survey of the types of food regularly consumed in the three study cohorts of the Canacona taluka and their average rate of consumption daily (in g/day)

Food category	Sub-type of each food category	Botanical name	Average rate of consumption per day (in g/day)						Directorate of Agriculture and Directorate of Fisheries estabished daily consumption of each food category (in g/day)
			Study group 1		Study group 2		Study group 3		
			Mean	Range (Min-max)	Mean	Range (Min-max)	Mean	Range (Min-max)	
Cereals and pulses	Rice	*Oryza sativa*	262.1	(251.6-280.9)	284.6	(272.6-293.6)	274.5	(264.5-287.3)	132g/day
	Wheat	*Triticum aestivum*	206.3	(201.3-210.9)	207.6	(205.6-213.9)	204.6	(200.3-215.6)	
	Green gram (Moong dal)	*Vigna radiata*	163.7	(160.3-171.2)	171.4	(165.5-181.3)	169.1	(163.9-171.5)	
	Ragi	*Eleusine coracana*	143.6	(139.5-151.7)	147.9	(135.6-152.1)	141.3	(135.6-147.2)	
	Chickpea	*Cicer arietinum*	120.5	(119.3-125.6)	121.3	(118.3-125.6)	119.4	(115.7-123.9)	
	Bajra	*Pennisetum glaucum*	98.3	(89.6-100.3)	103.1	(100.3-107.9)	109.9	(99.8-115.6)	
	Black gram (Urad dal)	*Vigna mungo*	78.9	(74.6-85.1)	70.6	(65.6-84.3)	95.6	(94.3-108.7)	
	Jowar	*Sorghum bicolor*	66.7	(62.6-70.3)	55.1	(52.1-38.9)	72.3	(69.5-81.3)	
	Average (of all cereals and pulses)	-	***142.5***	***(62.6-280.9)***	***145.2***	***(52.1-293.6)***	***148.3***	***(69.5-287.3)***	
Vegetables	Red amaranthus	*Amaranthus cruentus*	315.5	(303.8-321.5)	325.6	(318.6-331.6)	317.9	(305.9-321.7)	205 g/day
	Green amaranthus	*Amaranthus viridis*	241.3	(235.6-251.7)	272.3	(269.5-281.4)	214.5	(208.5-218.9)	
	Spinach	*Sipinacia oleracea*	239.7	(232.1-245.6)	259.4	(249.6-260.3)	278.9	(265.9-284.5)	
	Pumpkin	*Cucurbita pepo*	227.9	(220.4-230.1)	217.4	(215.3-221.6)	209.3	(205.9-217.3)	
	Brinjal	*Solanum melongena*	213.6	(205.6-221.3)	230.9	(221.4-241.3)	227.4	(218.4-230.6)	
	Lady finger	*Abelmoschus esculentus*	180.9	(175.6-185.6)	187.6	(178.6-190.3)	184.5	(181.3-192.3)	
	Raddish	*Raphanus raphanistrum*	145.6	(140.5-156.1)	147.3	(141.7-151.6)	156.9	(147.5-160.3)	
	Bottle gourd	*Lagenaria siceraria*	120.3	(115.9-131.3)	115.6	(109.6-121.3)	120.3	(114.5-126.9)	
	Average (of all vegetables)	-	***210.6***	***(115.9-321.5)***	***219.5***	***(109.6-331.6)***	***213.7***	***(114.5-321.7)***	
Fish	Mackerels	*Rastrelliger kanagurta*	69.3	(65.4-73.5)	74.6	(69.5-76.9)	71.9	(69.3-74.5)	65 g/day
	Sardines	*Sardinella longiceps*	58.1	(54.6-67.8)	66.4	(62.3-68.4)	62.7	(60.3-64.7)	
	Average (of fish)	-	***63.7***	***(54.6-73.5)***	***70.5***	***(62.3-76.9)***	***67.3***	***(60.3-74.5)***	

Values are represented as mean±SE with the range of minimum to maximum rate of consumption of each food sub-type of the three respective food categories (i.e. cereals and pulses or vegetables or fish) in the three study groups or regions. The mean rate of consumption (g/day) of each food category (i.e. cereals and pulses or vegetables or fish) was estimated by taking the average of the consumption rates of their respective sub-types. No statistically significant ($p>0.05$) differences were observed in the rates of consumption of each food category between the three study cohorts or regions signifying similar eating habits and additionally were found to be comparable with the values reported by the food screening legislative authority of the Government of Goa viz. The Directorate of Agriculture (2015, 2016) and the Directorate of Fisheries (2016, 2017).

Moreover the types of food species consumed among the three study groups were similar and their consumption rates were more or less comparable indicating the presence of common food habits in the three study groups (Chandrajith et al., 2011; Jayatilake et al., 2013). Therefore the type or species of food samples chosen for nephrotoxin analysis in all the three study groups or regions of the current study were arrived at rice, wheat, green gram (moong

dal), ragi and chickpea belonging to the cereals and pulses category; red amaranthus, green amaranthus, spinach, pumpkin and brinjal representing the vegetable category and mackerels and sardines signifying the fish categories **(Table 3.2.1).** The food samples were selectively chosen taking into consideration the consumption rate and ensuring appropriate representation of each of the broad food categories for accurate analysis of nephrotoxins (Chandrajith et al., 2011; Jayatilake et al., 2013; Siriwardhana et al., 2014).

3.2.2.3 Sample collection and preservation/storage

For the appropriate analysis of nephrotoxins in the food matrix, all the types of food samples were collected from each of the subjects belonging to the three different study regions (groups) to assess for variations (if any) in the levels of nephrotoxins in a similar type of food consumed in either of these study areas (Jayatilake et al., 2013). Based on the demographic survey, the types of food samples to be anlaysed based on their regular and highly prevalent consumption in the taluka were narrowed down to cereals like rice, wheat, green gram (moong dal) and ragi; pulses like chickpea, vegetables like red amaranthus, green amaranthus, spinach, pumpkin and brinjal and fish varieties like mackerels and sardines. In short, from study groups or regions 1, 2 and 3, the number of collected rice, wheat, green gram (moong dal), ragi, chickpea, red amaranthus, green amaranthus, spinach, pumpkin, brinjal, mackerels and sardines samples amounted to a total number of 114, 28 and 124 of each type in the respective study groups, representing geographical diversity in the sample collection as these groups belong to three different topographical areas of Canacona taluka.

300g of all the types of a food samples (cereals, vegetables and fish samples) were collected in ziplock polypropylene plastic packets from each of the subjects of the three study groups [i.e. study group 1 (CKDu endemic cases), 2 (CKD non-endemic cases) and 3 (healthy controls)] to assess the difference in the levels of nephrotoxin in a particular type of food matrix consumed in all of these three regions for the purpose of establishing the contribution of any of these nephrotoxins in the development of CKDu in the Canacona taluka. The food sampling was replicated during three different seasonal periods which are the pre-monsoon period (PRM) (summer month-May 2016), monsoon period (MON) (July 2016) and post-monsoon periods (POM) (winter month-November 2015) in order to assess for seasonal variations (if any) in the levels of the analyzed nephrotoxins that could affect the induction of CKDu. Following sample collection, they were transported to the lab for further processing for storage until analysis. The surveyed food samples collected from each of the three study areas were grouped into three broad categories namely fish, vegetables and cereals. These

broad categories were further divided into subcategories with the cereals category consisting of rice, wheat, green gram (moong dal) and ragi, the pulses group comprising of chickpea, the vegetable category being composed of red amaranthus, green amaranthus, pumpkin, brinjal and fish category being constituted by mackerels and sardines. The preservation and storage of each of these four broad food categories varied marginally, the details of which are listed below (Jayatilake et al., 2013; Levine et al., 2016).

Cereals and pulses:- Following transportation of cereal and pulses samples to the lab, the dust and impurities were eliminated and the husk was discarded as necessary. After which the cereal and pulses samples were oven dried at 70°C to constant weight and ground or homogenized into a fine powder. This fine powder was sieved by a 20-mesh sieve and the filtered powder was mixed evenly and stored in clean air tight polypropylene ziplock plastic packets at room temperature until analysis (Chandrajith et al., 2011; Khlifa et al., 2012).

Vegetables:- The transported samples were subjected to basic pre-treatment for storage of the samples until analysis. For this the vegetables were thoroughly cleaned with tap water followed by five rinses in deionized water to remove any dirt or particulate matter present on its surface. Then the edible part was separated using a steam cleaned stainless steel knife and chopped into pieces & allowed to oven dry at 60°C to eliminate high moisture content that is intrinsically present in fresh vegetables. This was followed by thorough grinding in a homogenizer, even mixing & storage of the samples in airtight ziplock polypropylene packets at −20 °C until analysis (Gunatilake et al.,2018; Jayatilake et al., 2013).

Fish samples:- Fish are well-known to concentrate heavy metals in muscles which is mainly consumed by humans therefore, we selected muscles as the primary site of metal uptake and accumulation in the present study. Immediately after transportation to the laboratory, samples were washed with fresh water to remove the mud or other fouling substances. Then the muscle tissue of each sample was removed and chopped into pieces with the aid of a steam cleaned stainless steel knife. The muscle tissues were then washed with deionized water and air dried to remove the extra water and subsequently, homogenized in a blender and 200 g of test portions were stored at −20 °C. Metal contents were expressed as mg kg^{-1} dry weight basis of fresh fish (Bandara et al., 2008; Faragher and DeHaan, 2018; Jayatilake et al., 2013).

3.2.2.4 Analytical methods

3.2.2.4.1 Reagents/chemicals

AAS standard solutions of Pb, Cd, As & Hg with each bearing concentration of 1000 ug/L were procured from Sigma-Aldrich, India. Ochratoxin & aristolochic acid standard solution

(100 ng/ml) were purchased from Merck-Millipore, India. All containers and glassware were cleaned by soaking into 20 percent nitric acid for at least 24 h and rinsed three times with deionized water prior to use. Deionized water (18 MΩ/cm resistivity) prepared from Merck Millipore system (MilliQ IQ/7003 ultrapure and pure water system, MA, USA) was used for preparation of all working solutions and utilized for all spectrophotometric analysis. All the reagents used were of analytical grade and all solutions.

3.2.2.4.2 Glassware and instruments

All the glassware and plastic ware used for analysis were carefully cleaned and rinsed with HNO_3 or HCl to avoid heavy metal, ochratoxin, and aristolochic acid contamination. This was achieved by first washing the apparatus thoroughly with water and detergent followed by rinsing with tap water and deionized water. The cleaning was eventually terminated by the final soaking in dilute acid (i.e. 500 ml conc.HNO_3 + 4500 mL deionized water) for 2 hours followed by rinsing with the same dilute acid and 5 extensive washings in deionized water and ultimately drying in a class-100 laminar flow hood (Thomas Scientific, NJ, USA) before use (Al-Saleh et al., 2017). Programmable furnace (Lindberg/Blue, Thermoscientific, Waltham, MA, USA) was used for sample digestion with thermostat maintaining temperatures of 450±25ºC. Ashing was supported by use of hot plate with heating control upto 200ºC. The AAS spectra for Pb and Cd were recorded on a Shimadzu AA7000 atomic absorption spectrometer (AAS) (Shimadzu, Tokyo, Japan). Moreover the AAS spectra for Hg and As were determined using a Shimadzu AA-7000 atomic absorption spectrophotometer equipped with hydride vapor generator (HVG-1 system, Shimadzu, Tokyo, Japan). Absorbance spectra for ochratoxin and aristolohic acid was measured on a ELISA plate reader (MultiSkanFC, Thermo-Fisher, MA, USA).

3.2.2.4.3 Sample digestion and extraction of different nephrotoxins from various food matrices in solution collected from the study population

Most food samples require a procedure of extraction of the nephrotoxins into the sample solution before analysis by atomic absorption spectrometry (AAS) or ELISA as these nephrotoxins inherently accumulate (owing to longer biological half-lives) and localize within the structure of the living-organism owing to the uptake of the nephrotoxins by these target organisms from the surrounding contaminated environments into the system for fulfillment of survival tendencies (Weaver et al., 2015). Hence it is mandatory to employ the correct extraction protocol based on the sample type to avoid unnecessary losses of the

analyte by volatilization to avoid false erroneous results (Smichowski, P. and Londonio, 2018; Ma et al., 2016a, Yu et al., 2016; Zhang et al., 2015).

3.2.2.4.3.1 Extraction of heavy metals (Pb, Cd, As and Hg) into the sample solution

In the current study, the heavy metals in all the three types of food samples (i.e. fish, cereals and vegetables) were extracted by the dry-ashing method as reported by Akinyele and Shokunbi (2015) with a few modifications. For this, the sample was initially homogenized into a fine paste. This was followed by accurately weighing 10g of the homogenized test paste to the nearest 0.01 g in a crucible and drying at 100°C, in a drying oven. Subsequently, the crucible was placed in a furnace at an initial temperature not higher than 100°C with the temperature steadily increased at a maximum rate of 50°C/h to 450°C for a period of 8h.Following ashing the crucible was allowed to cool and the ash was wetted with 1-3ml of 1:1 HNO_3 which was again evaporated to dryness on a hot plate. Subsequently the crucible containing the test ash sample was placed back in the furnace at temperature of not more than 200°C with the temperature gradually increased at a rate of (50–100°C/h) to 450°C for 2h. This process was repeated until the test sample was completely ashed i.e. ash should be white/grey or slightly colored. Following digestion and cooling, the test sample's ash residue was dissolved in 10 ml of 0.1M HNO_3 to the nearest 0.1 ml and filtered through a 0.45 μm acid-resistant filter paper (Whatmann no.44) into a 25 ml volumetric flask. The residual contents in the crucible were further washed two more times with 0.1M HNO_3 and the washings were also transferred into the volumetric flask. The final volume in the flask was adjusted to the mark with same diluent. The reagent blanks (devoid of test portions) were maintained for each set of samples collected from the three study areas and treated in the same way as the test samples. Three blank were included for each of the three batches of samples collected from the three study areas.

3.2.2.4.3.2 Extraction of ochratoxin into the sample solution

Since ochratoxin is a nephrotoxic mycotoxin contaminant endemic to only cereal food grains (like rice and wheat) and pulses (like chickpea) samples due to improper storage conditions of such samples, the extraction of ochratoxin is hence restricted to cereal and pulses samples only. Ochratoxin was extracted from the sample by the method adopted by Huertas-Perez et al. (2017) with a few modifications. For this, a 20g aliquot of each of the finely ground and filtered cereal samples (sieved through a 20 mm mesh-screen) collected from the entire study population were extracted with 100 ml of 70% methanol for ochratoxin determination on a

rotary shaker (MaxQ 2000, ThermoFisherScientific, Waltham, MA, USA) for 90 min. This was followed by filtration of the digest solution through Whatmann no. 1 filter paper and the filtrate containing the extracted ochratoxin from the cereal food grain and pulses sample were further subjected to analysis by ELISA.

3.2.2.4.3.3 Extraction of aristolochic acid into the sample solution

Aristolochic acid being a phytotoxin produced by the commonly occurring plant weed(i.e. birthwort) of the rice, wheat and pulses i.e. chickpea fields, its extraction in solution was majorly restricted to cereal food grain samples like rice and wheat, sharing homology with ochratoxin extraction (Huertas-Perez et al., 2017). In the current study, an extraction method similar to that for ochratoxin extraction as described by Li et al (2018) was adopted with a few modifications. For this, a 25g aliquot of each of the finely powdered and sieved cereal samples (filtered through a 20 mm mesh-screen) collected from the entire study population was extracted with 100 ml of (acetonitrile/water (60:40, v/v) for aristolochic acid determination using a homogenizer (DB5000A, ThermoFisherScientific, Waltham, MA, USA) for 10 min. Subsequently the digest solution was filtered through a Whatmann no. 1 filter paper and the filtrate containing the extracted aristolochic acid from the cereal food grain or pulses samples were further subjected to analysis by ELISA.

3.2.2.4.4 Estimation of various nephrotoxins in varied types of food matrices collected from the study population

Various analytical methods like atomic absorption spectrometry (AAS), Inductively coupled Plasma Mass spectrometry (ICP-MS), HPLC (high performance liquid chromatography), ICP-AES, ELISA have been widely utilized for the determination of the currently studied food based nephrotoxins in a number of toxicity studies till date, with each method possessing their respective share of advantages and disadvantages (Araya et al., 2018;Bueno et al., 2015; Daşbaşı et al., 2015; Mendil et al., 2015; Ferreira et al., 2015; Ma et al., 2016a; Shanakhat et al., 2018; Smichowski and Londonio,2018; Yu et al., 2016; Zhang et al., 2015). After critical evaluation of these analytical methods from detailed review of literature and appropriate weighing of the pros and cons for each of these methods, we narrowed down the analysis of heavy metals in various food samples by AAS (Akinyele and Shokunbi, 2015; Daşbaşı et al., 2015; Ferreira et al., 2015; Fernández et al., 2017; Ma et al., 2016a; Muñoz et al., 2017) and ochratoxin and aristolochic acid by ELISA (Shanakhat et al., 2018; Zang et al., 2015). These methods were shortlisted for analysis owing to the ease of sample extraction for

analysis, rapid and energy saving detection process displays significantly low limit of detections and confers high sensitivity and specificity for determinations as compared to other reported methods. The details of each analytical method employed for determination of various nephrotoxins (viz. heavy metals, ochratoxin and aristolochic acid) in different food matrices are described below.

3.2.2.4.4.1 Determination of Heavy metals (i.e. lead, cadmium arsenic and mercury)by atomic absorption spectrometry (AAS)

Atomic absorption spectrometry AAS is now probably the most widely used technique for determination of metals in biological materials. In practice, flame AAS (FAAS) is generally applied for heavy metal analysis as compared to graphite furnace AAS (GFAAS) as the former is less time consuming and less sensitive to interference(e.g., background absorption) (Smichowski and Londonio, 2018).

A Shimadzu AA7000 flame atomic absorption spectrometer (AAS) with Zeeman background correction system equipped with an air acetylene burner and an auto sampler (ASC-7000) was used for the determination of Pb and Cd. Moreover, Hg and As were determined using cold vapor AAS (CV-AAS) and hydride generation AAS (HG-AAS) techniques using a Shimadzu AA7000 flame atomic absorption spectrometer equipped with a cold vapor generator and hydride vapor generator (i.e. CVG-1 and HVG1-system, Shimadzu, Tokyo, Japan) (Chandrajith et al., 2011; Bandara et al.,2008). The measurement of Hg by CV-AAS was based on the reduction and volatilization of mercury from the sample digest solution to its elemental state following a chemical transformation by sodium borohydride without generating hydrides. Whereas on the other hand, the estimation of As by HG -AAS relied on the liberation of arsenic (III) hydride following a chemical reaction between a strong reductant (sodium tetrahydroborate) and arsenic compounds in the digest solution. Since most of the commercially available Hg and As standards are usually prepared from their respective inorganic salts (i.e. Hg (I) salts and either arsenic (III) or (IV) salts), it was mandatory for the Hg and As-standard and sample digest solutions containing unknown concentration of these metals to be correspondingly reduced to the elemental form of Hg or the hydride form of As for accurate estimation of the same (Ma et al., 2016a; Muñoz et al., 2017).

The analytical procedure employed for the quantification of the heavy metals was a slight modification of the method described by the following groups viz. Akinyele and Shokunbi (2015) for Pb, Cd and Munoz et al. (2017) and Fernández et al. (2015) for Hg and As. In this method, the purity of argon and acetylene gases used for flame generation was 99.999% and

99.99% respectively. Six working standard solutions bearing concentrations of 0.010, 0.050, 0.1, 0.2, 0.5 and 1 mg/L for each of the heavy metals i.e. Pb, Cd, As and Hg to cover the range of the concentration of the elements to be determined were daily prepared in 25ml volumetric flasks by appropriate dilution of their respective 10 mg/L stock standard solutions using 1%(w/w) suprapure grade nitric acid (Merck, Mumbai, India) to prepare the calibration curve for calculation of the unknown concentrations. Hollow cathode lamps were used for Pb (217 nm and slit 0.5 nm), Cd (228.8 nm and slit 0.5 nm), As (193.7 nm slit 0.5 nm) and Hg (253.7 nm and slit 0.5 nm) and they were operated according to the conditions recommended by the manufacturer as given in the Instruction manual accompanying the instrument for estimation of the unknown concentrations of the respective heavy metals in the test samples. For verification, the absorbance of the reagent blank (prepared in the same manner as the digested sample to determine the contamination of the reagents used during sample extraction), working standards of each of the analysed heavy metals and the sample solutions were recorded at their respective metal specific wavelength, after which the corresponding heavy metals content were calculated from their standard calibration curves by interpolation. Atomic signals were measured for Pb, Cd and Cr in peak area mood and for As and Hg in integration mode. The accuracy of the method was evaluated by analysing a certified reference material viz. NIST CRM specific for the type of food matrix by the same procedure used for the test sample analysis for every 20 samples. If the mean recoveries of the analysed metals remarkably deviated from the certified values, the instrument would have to be recalibrated. However in the current study the mean recoveries of the analysed metals were between 97.3 % to 99.1%, indicating a good agreement between certified and measured value thereby negating the requirements for frequent recalibration (Akinyele and Shokunbi, 2015; Fernández et al., 2017; Muñoz et al., 2017).

3.2.2.4.4.2 Determination of ochratoxin by ELISA

The measurement of ochratoxin levels in the sample digest or extract was done by solid-phase direct competitive enzyme immunoassay technique using Ochratoxin A ELISA assay kit (Helica Biosystems Inc., Fullerton, CA, USA) according to the manufacturer's instructions (Robinson et al., 2017; Zhang et al., 2015).

In brief, ochratoxin levels in test samples (food grain samples) were estimated from the standard curve of 6 provided ochratoxin standards of increasing concentrations i.e. 0.2,0.4,1,2, 4, 8 µg/L. For this, initially 100µL of ochratoxin standard or test sample (food grain) was added to their corresponding antibody (Ab) coated microtitre wells provided and

incubated at room temperature (RT) for 30 min. After which the contents of the microtitre plate were decanted, washed with deionized water (D.W) thrice, and tapped to remove residual water. Then 100µL of ochratoxin-HRP (horse raddish peroxidase) conjugate was added to each of Ochratoxin-Ab coated well treated with the respective ochratoxin standard and test samples containing the unknown ochratoxin levels which was further subjected to incubation at RT for 30 min. Following incubation, the well contents were decanted, washed with D.W thrice and tapped to remove the residual water. Subsequently 100µL of substrate reagent was added to each well and incubated for 10 min in the dark, following which 100µL of stop solution was added to terminate the reaction and the absorbance of the products formed were measured at 410 nm. The absorbance generated will be inversely proportional to the concentration of ochratoxin present in the standard or test sample due to competition of the ochratoxin from the bound HRP conjugate for the antigenic sites on the primary Ab coated in the wells that are intrinsically occupied by the ochratoxin present in the standard or test samples. A dose-response curve of absorbance versus concentration using the provided working standards of ochratoxin in the range from 0.2-8.0 µg/L was constructed and the unknown concentration in the test samples were measured by interpolation from the standard curve after consideration of the sample dilution factor (of 4) and the final concentration in the dry weight of the food sample was according to the formula mentioned below. Each type of food sample was analysed in triplicates and the average results were reported for better reproducibility.

3.2.2.4.4.3 Determination aristolochic acid by ELISA

The levels of aristolochic acid (AA) in the sample digest or extract was estimated by the solid-phase sandwich enzyme immunoassay technique using Aristolochic acid ELISA assay kit (Helica Biosystems Inc., Fullerton, CA, USA)in accordance with the manufacturer's guidelines (Li et al., 2015; Li et al., 2018).

To summarize, levels of aristolochic acid in the test samples (food grain samples) were determined from the standard curve of 6 furnished standards of increasing concentrations i.e. 0.05, 0.1, 0.2, 0.5, 1 and 2 µg/L. For this, primarily 100µL of AA standard or test sample (food grain) was added to their corresponding primary capture antibody (Ab) coated microtitre wells provided and incubated at room temperature (RT) for 30 min. Following which the contents of the microtitre plate were decanted, washed with deionized water (D.W) thrice, and tapped to remove residual water. Then 100µL of HRP (horse raddish peroxidase) enzyme conjugated secondary Ab for detection of bound aristolochic acid (from the standard

or the test samples) in each of the primary anti-aristolochic acid antibody (AA-1˚Ab) coated wells was added, which was further subjected to incubation at RT for 30 min. After incubation, the well contents were decanted, washed with D.W thrice and tapped to remove the residual water. Subsequently 100µL of substrate reagent was added to each well and incubated for 10 min in the dark, following which 100µL of stop solution was added to terminate the reaction and the absorbance of the formed products were measured at 410 nm. The absorbance generated will be directly proportional to the concentration of aristolochic acid present in the standard or test sample owing to the direct binding of the secondary detector Ab bound enzyme conjugate to ancillary antigenic sites of aristolochic acid (present in the standard or test sample) trapped by the primary capture antibody. A dose-response curve of absorbance versus concentration using the provided working standards of AA in the range from 0.05-2 µg/L was constructed and the unknown concentration in the test samples were determined by interpolation from the standard curve after consideration of the sample dilution factor (of 4) and the final concentration in the dry weight of the food sample was according to the formula mentioned below. Each type of food matrix was evaluated in triplicates and the mean values were reported for better reproducibility.

3.2.2.4.5 Calculations

3.2.2.4.5.1 Concentration of nephrotoxin in mg/kg or µg/kg

The concentration of nephrotoxin present in mg/kg dry weight (for heavy metals) or µg/kg dry weight (for ochratoxin and aristolochic acid) in each of the analysed food samples were calculated from the concentration derived in mg/L or µg/L from their respective standard calibration curves. The concentration in mg/kg or µg/kg as applicable was calculated according to the formula:

$$C = \frac{(a-b) \times V}{m}$$

where C = concentration in the test food sample [mg/kg (for heavy-metals) or µg/kg (for ochratoxin and aristolochic acid)]; a = concentration in the test solutions [mg/L (for heavy-metals) or µg/L (for ochratoxin and aristolochic acid)]; b = mean concentration in the blank solutions (mg/L or µg/L); V = volume of the test solution (mL); m = weight of the test portion (g).

For calculation of the concentration of heavy metals (in mg/kg dry weight) in the test sample, the weight and volume of test sample was considered as 10g and 25ml respectively (as described in the sample extraction procedure (Akinyele and Shokunbi, 2015). Whereas for

calculation of the concentration of ochratoxin and aristolochic acid (in µg/kg dry weight) in the test sample, the weight and volume of test sample was considered as 2g and 10 ml respectively (as described in the sample extraction procedure) (Li et al., 2018; Zhang et al., 2015). If test food sample solution was diluted owing to nephrotoxin concentration exceeding measurable range, then dilution factor was additionally taken into account in calculation of nephrotoxin concentration in food samples for appropriate analysis. Each sample was measured for nephrotoxin in triplicates and average results were reported (Akinyele & Shokunbi, 2015; Fernández et al.,2017; Li et al., 2018; Muñoz et al.,2017; Zhang et al.,2015)

3.2.2.4.5.2 EDI (estimated daily intake)

Estimated daily intake is an accurate indicator of the nephrotoxin transfer from food plants (i.e. cereals, pulses and vegetables) and fish in the terrestrial and aquatic ecosystem respectively to humans which has been used to predict the risk of developing renal toxicity in related studies (Chen et al., 2018; Islam et al., 2015; Jayatilake et al., 2013). The daily intake of the nephrotoxins depends on both the nephrotoxin concentration in the food sample and the daily rate of food consumption. In addition, the body weight of the human can influence the tolerance to various nephrotoxic contaminants. The EDI is a concept introduced to take into account these factors. The EDI (in µg/ day kg bw for heavy metals and in ng/ day kg bw for ochratoxin and aristolochic acid) was calculated according to *equation no.1* (stated in section number - 3.1.2.5.1)

In this case for EDI calculation by *equation no. 1*, 'C' represents the concentration of nephrotoxin (µg/kg for heavy metals and ng/ kg for ochratoxin and aristolochic acid) in the contaminated food sample; 'Cons' stands for the daily average consumption (in g/day) of a particular food type in the study region; and 'Bw' represents the body weight (in kg) of an individual.

The average daily intake (in g/day) of each food category (i.e. cereals and pulses or vegetables and pulses) in the three study regions as established from the demographic survey of the food samples are listed in Table 3.2.1. These demographically estimated daily consumption rates were found to be homologous to the values established by the Food legislative authorities of Goa, the Directorate of Agriculture, and Directorate of Fisheries. Hence in order to maintain uniformity in estimation, the Directorate of Agriculture and Directorate of Fisheries established average daily consumption rates of each food category were utilized for EDI calculations which were as follows:- 132g, 205g and 65 g for cereals and pulses, vegetables and fish respectively (Directorate of Agriculture, 2015; Directorate of

Agriculture, 2016; Directorate of Fisheries report ,2016; Fishery Resource Potentiality Of Goa , 2015; Indiastat, 2017; MoSPI , 2014). Moreover the average body weight the subjects belonging to all the three study regions was set to 60 kg (which is the general body weight considered for a studied individual) in the current study for consistency in calculations (Abdullah et al., 2017; Ahmed et al., 2015; Bortey-Sam et al., 2015; Chandrajith et al., 2011; Mitchell et al., 2017; Oueslati et al., 2018; Shaheen et al., 2016).

3.2.2.4.5.3 Risk characterization via calculation of target hazard quotient (THQ)

THQ is used to express the risk of developing a health hazard (renal toxicity in this case) on dietary exposure to a toxin (nephrotoxin in this case) and is expressed as a ratio of estimated daily intake (EDI) to oral reference dose (RfD, in µg/ day kg bw for heavy metals and in ng/ day kg bw for ochratoxin and aristolochic acid) (USEPA, 2000).

To assist in evaluation of the role of contaminating nephrotoxins in food in inducing certain health hazards (renal damage), JECFA has formulated a specific oral reference dose for each of these toxins. The oral reference dose is an estimation of maximum permissible risk on human population through daily exposure to nephrotoxin via food consumption taking into consideration a sensitive group during the lifetime. The RfD is specific and different for exposure (consumption) via food and water route (Chen et al.,2018; Islam et al.,2015; Jayatilake et al.,2013).

The THQ was calculated according to the formula described in *equation no.2* (stated in section number - 3.1.2.5.2)

In this case for THQ calculation by *equation no. 2*, 'EDI' stands for estimated daily intake and 'RfD' stands for oral reference dose with both possessing units of µg/ day kg bw for heavy metals or ng/day kg bw for ochratoxin and aristolochic acid.

The RfDs for Pb, Cd, As and Hg through food consumption are 4, 1, 2.5, and 3 µg/ day kg bw respectively and for ochratoxin and aristolochic acid is 17 and 4 ng/ day kg bw respectively. If the THQ value is below 1, the exposed population is expected not to develop any adverse health effect (i.e. nephrotoxicity in this case). The THQ equation was formulated on the basis that the ingestion dose is equal to the absorbed toxin dose, wherein cooking has no influence on the toxin/contaminants levels (USEPA, 1989) and the average body weight of an adult for EDI calculation was considered as 60 kg (Ahmed et al., 2015; Bortey-Sam et al., 2015; Chandrajith et al., 2011; Shaheen et al., 2015).

It has been well reported that exposure to multiple toxins or pollutants may result in additive and/or interactive effects. Thus, in this study, cumulative health risk was evaluated by

addition of the individual THQ values of each nephrotoxin for a particular exposure pathway (i.e. food in this case) and expressing it as the total THQ (TTHQ) (also known as the hazard index, HI)

The formula for HI/ TTHQ is = THQ (toxin 1) + THQ (toxin 2) +. . . . + THQ (toxin n)

It is assumed that the extent of the effect is directly proportional the summation of multiple nephrotoxin exposure and that analogous attack mechanism linearly disturb the target organ (which is kidney in this case). Here also the HI just like THQ is compared to a benchmark that is 1.The larger the value of HI, higher is the concern level. The study population is considered to be safe if the HI is lesser than 1. The HI value greater than 1, generally indicates the probability of adverse human health effects arising from the action of multiple toxins and denotes the need for adopting further investigation or remediative measures (Abdullah et al., 2017; Barone et al., 2015; Giri and Singh, 2017; Javed and Usmani, 2016; Mitchell et al., 2017; Oueslati et al., 2018; Nuapia et al., 2018; Qureshi et al., 2016).

3.2.2.4.6 Statistical analysis

All the results were expressed on a dry weight basis unless specified. The analysis of each nephrotoxin in each food sample was duplicated for better data reproducibility. All the statistical analyses were performed with SPSS (Version 20.0) for Windows.

The data for each continuous variable were expressed as a mean±SE with a range of minimum and maximum values provided of two independent experiments. The calculation of the values were conducted independently for categories stratified temporally (i.e. mean values with the minimum to maximum range measured for a variable at three different time points i.e. PRM, MON, POM in each group) and spatially (i.e. mean values with the minimum to maximum range of a variable compared between the three study regions). The statistical differences in the continuous variables between the three study groups (spatial) and between three different time points in each study group(temporal) were independently and separately compared using one-way analysis of variance test and the significance of the differences within these two separate comparison groups (i.e. temporally and spatially) were identified post-hoc using Dunnet's test. The relationship or association between various measured nephrotoxins in different food samples were analysed via calculation of Pearson's correlation coefficients (r) with their respective p-values to identify the significantly ($p<0.05$) common source of origin (if any) of these nephrotoxins, enabling the adoption of necessary remediation measures to eradicate the source of exposure which could assist in the reduction of the CKDu incidence in the taluka. Moreover the role of the dietary contribution of these

nephrotoxins in the development of the tubular or glomerular specific nephropathy in the taluka was assessed by calculation of the Pearson's correlation coefficients (r) with their respective p-values between the estimated daily intake of these nephrotoxins and the urinary selectivity indices for tubular or glomerular nephropathy. Differences at *p-values<0.05 were deliberated to be statistically significant in all the sets of assessments (i.e. analysis of the continuous variables between the three study groups and correlation assessments) (Chandrajith et al., 2011; Jayatilake et al., 2013; Wanigasuriya et al., 2011).

3.2.2.3 Results and Discussion

3.2.2.3.1 Concentrations of various nephrotoxins in different food samples consumed in the three study groups

Nephrotoxins were estimated in commonly consumed food samples in the 3 study groups to analyse for dietary contribution (if any) of nephrotoxins in CKDu causation in Canacona.

3.2.2.3.1.1 Concentration of heavy metals (i.e. Pb, Cd, As, Hg) in food consumed by the study population.

According to Directorate of Agriculture and Directorate of Fisheries(Government of Goa), Canacona taluka being rural, poor and coastal community heavily rely on cheap animal sources viz. fish (like mackerels and sardines), basic cereals and pulses (like rice, wheat, moong dal, ragi, chickpea) & locally grown vegetables (like spinach, red and green, pumpkin, brinjal) for daily nourishment (Directorate of Agriculture,2015; Directorate of Agriculture, 2016; Directorate of Fisheries report,2016; Fishery Resource Potentiality Of Goa, 2015; Indiastat, 2017; MoSPI, 2014). Hence, heavy metal levels in these foods were analysed.

The results of Pb, Cd, As and Hg contents (on dry weight basis) estimated in various food samples collected during 3 different seasons [i.e. pre-monsoon (summer), monsoon, post-monsoon (winter)] to check for seasonal variations in heavy metals levels in 3 study areas are shown in **Table 3.2.2; 3.2.3; 3.2.4 and 3.2.5** respectively. As shown in these tables Pb, Cd, As and Hg levels in plant and animal based foods were comparatively slightly higher in pre-monsoon (PRM), followed by post-monsoon (POM) with lower values noted in monsoon (MON), with statistically insignificant ($p>0.05$) differences noted. However, heavy-metals levels in all food samples were below legislative limits set by Joint FAO/WHO Expert Committee on Food Additives (JECFA). Lower values noted in MON could be attributed to incessant precipitation causing heavy-metal run-off from soil in addition to further diluting heavy metals concentration in soil and water bodies resulting in reduced heavy metal uptake

and accumulation by food crops and marine fish. The relatively negligibly higher values noted in POM could be accounted to receding water levels in soil and natural water bodies owing to precipitation cessation that averts terrestrial run-off into coastal environments and further heavy-metal dilution in aquatic and terrestrial environment thereby enriching pollution. The comparatively ($p>0.05$) higher heavy-metal values noted in PRM (summer) could be explained by increased evaporation from water bodies and soil moisture due to higher temperatures that could have concentrated heavy metals in natural waters and soil resulting in slightly higher uptake by inhabiting fish and food crops respectively. Additionally, minimally higher values in fish could be accounted to higher respiratory rates due to low dissolved oxygen that induces excessive consumption of metal contaminated water for survival. Moreover, enhanced dehydration of food plants by transpiration during summers could have contributed to reconcentration of heavy-metals in edible tissues (Achary et al.,2017; Gall et al.,2015; Chen et al., 2018; Feng et al.,2011; Jayatiilake et al.,2013; Noli and Tsamos,2016; Saha et al.,2016). However, despite similar trend of insignificant ($p<0.05$) and negligible seasonal variations noted in heavy metals measured in food in three study groups, levels of all nephrotoxic metals were well within their respective JCEFA permissible limits suggesting no cause for renal concern of heavy metal exposure via dietary route.

Moreover as shown in **Figures 3.2.1**; **3.2.2; 3.2.3 and 3.2.4** & **Tables 3.2.2; 3.2.3; 3.2.4 and 3.2.5,** estimated mean levels (calculated for each food sample collected during three different seasons) of all heavy metals (i.e. Pb, Cd, As and Hg) were comparable between all three study groups for each food sample type analysed and were well below the respective JECFA permissible limits suggesting a common food procurement source by subjects in all study groups/area (Achary et al., 2017; Bandara et al.,2010a; Jayatiilake et al., 2013; Noli and Tsamos, 2016). From, demographic study (Chapter 2) it was established that farming and fishing are not prevalent occupations in whole of Canacona but are restricted to few villages such as Molorem, Endrem which in the current study also constitutes healthy controls villages (study area 3) with complete exclusion of these occupations in CKDu affected villages i.e. Chaudi and Ponsulem. Mass production of cereals, vegetable & fishing are generally carried out in these healthy villages wherein produce i.e. food crops and fish catch are further marketed and distributed to rest of Canacona. As such farming & fishing does not occur in each taluka's villages. Since production source of food crops (i.e. cereals & vegetables) & fish catching and their distribution to 3 study areas is common i.e. arising from healthy villages-Molorem & Endrem (study area 3), this explains trend of comparable heavy-metal levels noted in all food samples types (plant, animal) in these study areas (Achary et

al.,2017; Bandara et al.,2010a; Chandrajith et al.,2011; Jayatiilake et al., 2013; Noli and Tsamos, 2016). Furthermore minimal heavy metals levels (i.e. Pb, Cd, As, Hg) noted in all foods to be below their respective JECFA permissible limits **(Figures 3.2.1**; **3.2.2; 3.2.3 and 3.2.4 & Tables 3.2.2; 3.2.3; 3.2.4 and 3.2.5)** could be possibly attributed to negligible anthropogenic influence of mining or discharge of untreated heavy metal laden effluents from industries (battery, smelting, paint) or municipal treatment plants or domestic sewage into aquatic and terrestrial environments (Chandrajith et al., 2011; Jayatiilake et al., 2013) that could have caused minimal contamination of inhabiting food plant and animals(established from Demographic survey in Chapter 2 & Statistical Hand Book of Goa Government, 2016). The heavy-metals levels distribution in various food samples in three study areas at three different time-points (seasons) are detailed below.

3.2.2.3.1.1.1 Concentration of lead in food samples consumed in the study population

JEFCA has reported 42% of human exposure to lead occurs via consumption of leafy vegetables, bony-fish and cereals contaminated by human activities like discharge of untreated effluents from batteries, mining, paints, smelting industries in terrestrial and aquatic environments. Hence lead levels were estimated in above mentioned foods in the current study to assess lead's dietary contribution (if any) in CKDu etiology of Canacona (Flora et al., 2012; Sankhla, et al., 2016; Weaver and Jaar, 2015).According to JECFA, maximum permissible limit of lead was described to be 0.3 $mgkg^{-1}$ (Weaver and Jaar, 2015). As depicted in **Figure 3.2.1** & **Table 3.2.2**, in cereals and pulses category of food samples analysed, mean lead levels over three seasons in particular food type) in the study group 1, 2 and 3 were similar and estimated to be as 0.048, 0.044 and 0.05 mg/kg respectively with maximum mean values of 0.061, 0.062, and 0.070 mg/kg noted in wheat and minimum values of 0.029, 0.020, and 0.033 mg/kg noted in ragi food type respectively. The lead levels in rice samples collected from all study groups during three seasons were below detectable levels (BDL) suggesting no dietary lead contribution from staple food i.e. rice intake in this taluka. BDLs of lead in rice could be attributed to non-usage of lead based agrochemicals and use of natural compost for better crop production due to economic constraints preventing them from affording costly agrochemicals (Jayatilake et al.,2013).100% of cereals analysed in 3 study areas possessed lead levels prevalent below JECFA limit-0.2mg/kg (Paranagama et al.,2018; Swaddiwudhipong et al.,2015).Wherein 65, 72 and 68% of samples were in lowest range of 0.020-0.040 mg/kg and 35, 28 and 32% were in slightly higher range but within legislative limits i.e.0.041-0.07 mg/kg from study areas 1, 2 and 3 respectively.

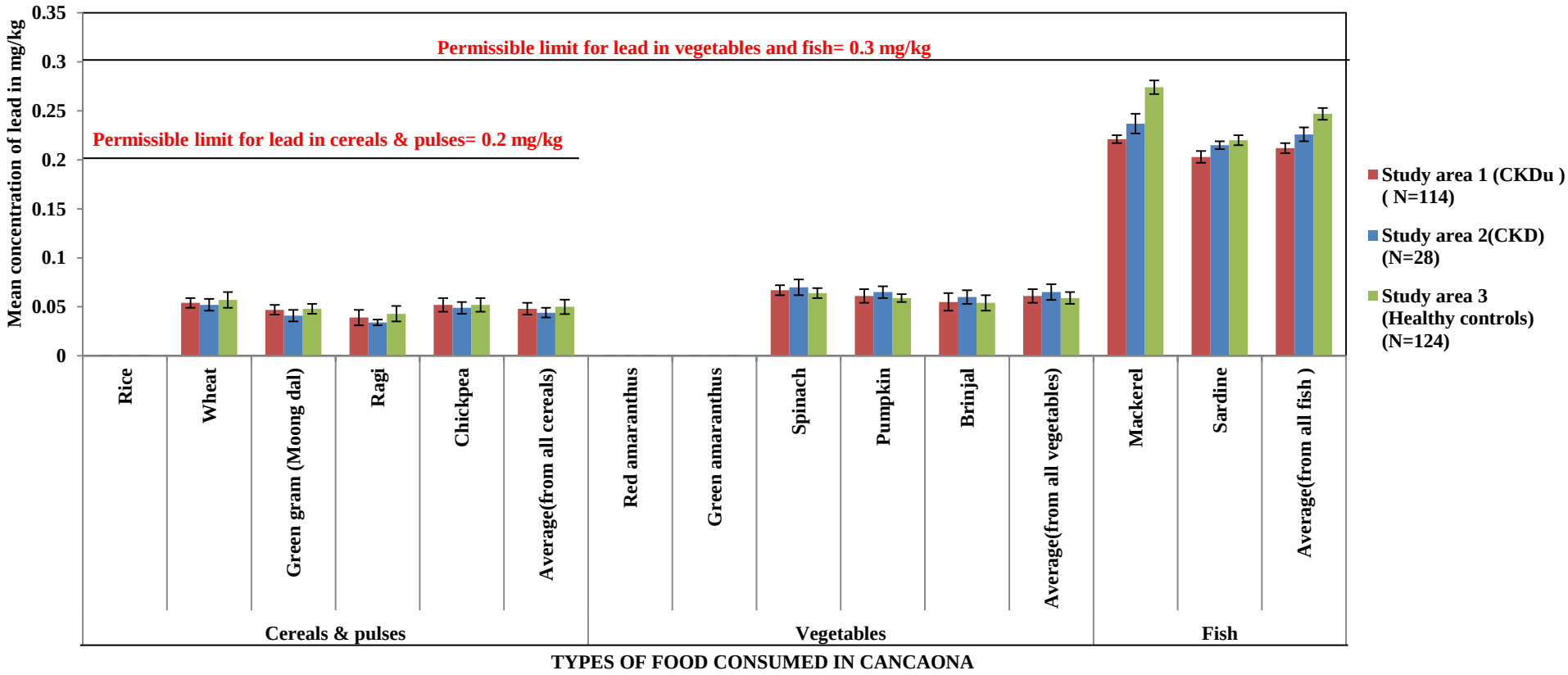

Figure 3.2.1. Mean concentrations of nephrotoxic heavy metal- 'lead' in several sub-types of three major food categories (i.e. cereals or pulses ,vegetables and fish) consumed by the study population of Canacona during three different seasons

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; Data are derived from two independent experiments with the lead levels of an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which samples of each sub-type of the three food categories viz. cereals& pulses, vegetables and fish were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons viz. Pre -Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal variations in the levels of lead in the food. Values are represented as mean ± SE (standard error) of lead levels estimated in each food sample sub-type (of the three aforesaid food categories) over three different sampling seasons or time points viz. PRM,POM,MON in the three study areas. Moreover the mean levels of lead in each food category (i.e. cereals and pulses or vegetables or fish) were calculated from the average of the levels in their respective sub-types(for example average of the lead levels in mackerel and sardines sub-types were reported as mean lead levels in the fish category). All the values are in mg/kg. Below detectable levels of lead were noted in rice of the cereals category and in red and green amaranthus of the vegetables category, hence are not represented by bars in the figure. The mean lead levels were found to be well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established permissible limits and were comparable[with no significant difference($p>0.05$)] in all three study areas for each food category suggesting a common source of origin of these foods in the three study areas. The details on the seasonal distribution of the lead levels and the minimum to maximum range of lead concentrations are listed in **Table 3.2.2**.

Table 3.2.2: Mean & range concentrations (in mg/kg) of nephrotoxic heavy metal- 'lead' during three different seasons in various sub-types of three major categories of food (i.e. cereals/ pulses ,vegetables & fish) consumed by study population of Canacona taluka and their comparison with respective JCEFA permissible limits in food.

CKD status	CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				JCEFA established permissible limits (in mg/kg)
	Study region or group 1(N=114)				Study region or group 2(N=28)				Study region or group 3(N=124)				
Food category with sub-types/ sampling time period	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	
Cereals and pulses (in mg/kg)													
Rice	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Wheat	0.059± 0.006	0.049± 0.004	0.054± 0.006	**0.054± 0.005**	0.057± 0.009	0.046± 0.010	0.053± 0.07	**0.052± 0.006**	0.066± 0.004	0.044± 0.009	0.061± 0.010	**0.057± 0.008**	
	(0.50-0.061)	(0.042-0.057)	(0.052-0.059)	**(0.042-0.061)**	(0.050-0.062)	(0.040-0.051)	(0.048-0.057)	**(0.040-0.062)**	(0.061-0.070)	(0.041-0.048)	(0.055-0.064)	**(0.041-0.070)**	
Green gram (Moong dal)	0.051± 0.009	0.042± 0.004	0.048± 0.006	**0.047± 0.005**	0.048± 0.005	0.035± 0.004	0.04± 0.009	**0.041± 0.006**	0.058± 0.009	0.034± 0.007	0.052± 0.006	**0.048± 0.005**	**For Cereals and pulses= 0.2**
	(0.049-0.055)	(0.038-0.048)	(0.046-0.052)	**(0.038-0.055)**	(0.046-0.050)	(0.032-0.039)	(0.038-0.044)	**(0.032-0.050)**	(0.054-0.060)	(0.033-0.037)	(0.049-0.058)	**(0.037-0.060)**	
Ragi	0.043± 0.011	0.034± 0.009	0.04± 0.004	**0.039± 0.008**	0.042± 0.009	0.021± 0.004	0.039± 0.006	**0.034± 0.003**	0.048± 0.005	0.037± 0.010	0.044± 0.007	**0.043± 0.008**	
	(0.040-0.047)	(0.029-0.038)	(0.035-0.044)	**(0.029-0.045)**	(0.039-0.047)	(0.020-0.025)	(0.037-0.043)	**(0.020-0.043)**	(0.043-0.050)	(0.033-0.039)	(0.041-0.047)	**(0.033-0.047)**	
Chickpea	0.057±0.005	0.045±0.009	0.054± 0.008	**0.052± 0.007**	0.058± 0.005	0.038± 0.007	0.051± 0.006	**0.049± 0.006**	0.057± 0.010	0.047± 0.008	0.052± 0.04	**0.052± 0.007**	
	(0.052-0.059)	(0.039-0.046)	(0.052-0.057)	**(0.039-0.057)**	(0.056-0.063)	(0.035-0.041)	(0.049-0.054)	**(0.035-0.063)**	(0.054-0.062)	(0.042-0.049)	(0.050-0.058)	**(0.042-0.062)**	
Average (from all cereal sub-types)				**0.048±0.06**				**0.044±0.052**				**0.05±0.0074**	
				(0.029-0.061)				**(0.020-0.062)**				**(0.033-0.070)**	
Vegetables (in mg/kg)													
Red amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Green amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Spinach	0.074± 0.007	0.062± 0.005	0.065± 0.004	**0.067± 0.005**	0.075± 0.009	0.066± 0.007	0.069± 0.008	**0.07± 0.008**	0.072± 0.009	0.051± 0.004	0.069± 0.003	**0.064± 0.005**	
	(0.071-0.076)	(0.059-0.066)	(0.062-0.069)	**(0.059-0.076)**	(0.072-0.079)	(0.061-0.069)	(0.067-0.071)	**(0.061-0.079)**	(0.069-0.074)	(0.049-0.055)	(0.067-0.072)	**(0.050-0.074)**	**For vegetables= 0.3**
Pumpkin	0.066± 0.008	0.055± 0.005	0.062± 0.006	**0.061± 0.007**	0.069± 0.004	0.062± 0.007	0.064± 0.009	**0.065± 0.006**	0.064± 0.009	0.055± 0.010	0.058± 0.004	**0.059± 0.004**	
	(0.063-0.071)	(0.052-0.058)	(0.060-0.066)	**(0.052-0.071)**	(0.066-0.072)	(0.057-0.066)	(0.061-0.067)	**(0.057-0.072)**	(0.061-0.068)	(0.050-0.059)	(0.055-0.063)	**(0.049-0.068)**	
Brinjal	0.063± 0.009	0.043± 0.007	0.059± 0.011	**0.055± 0.009**	0.064± 0.007	0.056± 0.010	0.06± 0.004	**0.06± 0.007**	0.059± 0.004	0.05± 0.014	0.053± 0.008	**0.054± 0.008**	
	(0.060-0.067)	(0.041-0.047)	(0.057-0.063)	**(0.041-0.067)**	(0.062-0.066)	(0.052-0.058)	(0.057-0.062)	**(0.052-0.066)**	(0.057-0.063)	(0.048-0.053)	(0.051-0.057)	**(0.048-0.063)**	
Average (from all vegetable sub-types)				**0.061±0.007**				**0.065±0.008**				**0.059±0.006**	
				(0.041-0.076)				**(0.052-0.079)**				**(0.048-0.074)**	
Fish (in mg/kg)													
Mackerel	0.226± 0.004	0.215± 0.008	0.222± 0.006	**0.221± 0.004**	0.249± 0.010	0.226± 0.005	0.236± 0.006	**0.237± 0.010**	0.281± 0.008	0.267± 0.006	0.274± 0.008	**0.274± 0.007**	
	(0.213-0.246)	(0.210-0.218)	(0.219-0.229)	**(0.210-0.246)**	(0.226-0.254)	(0.224-0.234)	(0.229-0.248)	**(0.224-0.254)**	(0.275-0.284)	(0.264-0.271)	(0.270-0.278)	**(0.264-0.284)**	
Sardine	0.212± 0.007	0.192± 0.003	0.205± 0.004	**0.203± 0.006**	0.227± 0.007	0.206± 0.010	0.212± 0.008	**0.215± 0.004**	0.226± 0.003	0.215± 0.006	0.219± 0.005	**0.22± 0.005**	**For Fish= 0.3**
	(0.207-0.214)	(0.184-0.199)	(0.201-0.209)	**(0.184-0.214)**	(0.223-0.231)	(0.199-0.210)	(0.207-0.219)	**(0.199-0.231)**	(0.221-0.237)	(0.212-0.220)	(0.214-0.226)	**(0.212-0.237)**	
Average (from all fish sub-types)				**0.212±0.005**				**0.226±0.007**				**0.247±0.006**	
				(0.184-0.246)				**(0.199-0.254)**				**(0.212-0.284)**	

Abbreviations: BDL- below detectable levels; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; JECFA- Joint FAO/WHO Expert Committee on Food Additives (JECFA). Data are derived from two independent experiments with the lead levels of an individual experiment measured in triplicates. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured. Values are represented as mean±SE with the range of minimum to maximum concentrations (shown in parenthesis) of lead, estimated in each food sample sub-type (of the three respective food categories over three different sampling time points viz. PRM,POM,MON in the three study groups or regions . The overall concentration of lead in a particular food sub-type (for example rice of the cereals category) was calculated by taking the average of the concentrations estimated during the aforementioned time-points. Moreover the mean concentrations of lead in each food category (i.e. cereals and pulses or vegetables or fish) were estimated from the average of the concentrations in their respective sub-types. No statistically significant ($p>0.05$) differences were noted in the concentrations of lead between the three study regions for each food category and were well below the JECFA established permissible limits.

Among vegetables analysed, mean lead levels (estimated over three seasons in particular food type) in study group 1, 2 and 3 **(Figure 3.2.1 & Table 3.2.2)** were comparable and estimated to be 0.061, 0.065 & 0.059 mg/kg respectively with maximum mean values of 0.076, 0.079 and 0.074 mg/kg respectively noted in spinach and minimum values of 0.041, 0.052 & 0.048 mg/kg noted in brinjal food type respectively. The lead levels in red & green amaranthus collected from all three study groups during different seasons were significantly BDL suggesting no dietary lead contribution from consumption of local and organically grown vegetables i.e. red and green amaranthus in this taluka, possibly attributed to their organic mode of farming wherein there is no usage of lead-based agrochemicals (Jayatilake et al., 2013) and only use natural compost for crop productivity improvement(as established from our demographic survey and Personal communication with Canacona zonal agricultural officer, Mr. Shivram Gaonkar). 100% of all vegetable sample types analysed in 3 study areas possessed lead levels prevalent below JECFA permissible limits-0.30 mg/kg. Wherein 71, 74 and 79% of samples were in lowest range of 0.04-0.07 mg/kg and 29, 25 and 21% in higher range but within legislative limit i.e.0.07-0.08 mg/kg in 1, 2 and 3 respectively.

The average lead levels in fish in study group 1, 2 and 3 **(Figure 3.2.1 & Table 3.2.2)** were comparable and determined to be as 0.212, 0.226 and 0.247 mg/kg respectively with maximum mean values of 0.246, 0.254 and 0.284 mg/kg noted in mackerel fish and minimum values of 0.184, 0.199, 0.212 mg/kg noted in sardine fish respectively. 100% of fish samples analysed in 3 study areas possessed lead levels prevalent below JECFA permissible limit of 0.3 mg/kg. Wherein78, 83 and74% of samples were in lowest range of 0.15-0.25 mg/kg and 22, 17,and 26% of samples were in slightly higher range but within legislative limit i.e.0.25-0.35 mg/kg collected from study areas 1, 2 and 3 respectively.

In totality for all food types of all study regions analysed for lead, highest values of 0.212, 0.226, 0.247 mg/kg were noted in fish i.e. mackerel with lowest levels of 0.039, 0.034, 0.043 mg/kg noted in ragi cereal of study groups 1, 2, 3 respectively. However lead content in these foods were comparable and well below JEFCA permissible limits in all 3 regions. Overall, these results were in agreement with lead levels noted in similar food consumed in homologous CKDu affected countries like Sri Lanka and Central America wherein no association with observed renal toxicity was noted on chronic intake of food containing such JCEFA set safe lead levels (Banadara et al., 2008; Bandara et al., 2010a; Correa-Rotter., 2017; Gunatilake et al., 2015; Jayatilake et al., 2013; Levine et al., 2016; Rajapakse et al., 2016; Ramirez-Rubio et al., 2013;Wanigasuriya et al., 2011;Wesseling et al., 2013;

Wimalawansa, 2016). Thus, suggesting lack of prevalent dietary lead role in taluka's CKDu causation that was backed by similar levels noted in CKDu hit and healthy regions.

3.2.2.3.1.1.2 Concentration of cadmium in food consumed in the study population

JEFCA reported 61% of human exposure to Cd occurs via consumption of foods like cereal (rice), vegetables, fish polluted by human activities like untreated effluents discharge from batteries, smelting in terrestrial and aquatic environments or usage of Cd laden agrochemical. Hence cadmium levels were determined in these foods in the current study as well to assess dietary cadmium contribution (if any) in CKDu development in Canacona (Swaddiwudhipong et al., 2015; Paranagama et al., 2018).

As indicated in **Figure 3.2.2** & **Table 3.2.3**, mean Cd levels(estimated over 3 seasons in particular food type) in cereals and pulses in study group 1, 2 and 3 were similar and estimated to be 0.037, 0.033, and 0.039 mg/kg respectively with maximum mean values of 0.050, 0.046 and 0.051 mg/kg noted in green gram (moong dal) & minimum values of 0.027, 0.020 and 0.026 mg/kg noted in ragi food type respectively. Cd levels in rice and wheat in all study groups were BDL possibly attributed to non-usage of Cd laden agrochemicals owing to organic farming practiced signifying no dietary Cd contribution from staple food crop consumption (rice, wheat) in this taluka (Jayatilake et al., 2013).100% of cereal contained Cd levels prevalent at JECFA permissible limits of 0.03 mg/kg. From which 61, 68, 63% of samples were in lowest range of 0.02-0.04 mg/kg and 39, 32 and 37% of samples were in slightly higher range but within limits i.e.0.04-0.06 mg/kg in study areas 1, 2, 3 respectively. Mean Cd levels in vegetables in study group 1, 2 and 3 were comparable and estimated to be 0.052, 0.056 & 0.054mg/kg with maximum mean values of 0.061,0.066 & 0.062mg/kg noted in pumpkin and minimum values of 0.042, 0.047 & 0.051 mg/kg noted in brinjal respectively. Cd levels in red and green amaranthus, spinach in all study groups during 3 seasons were BDL owing to organic farming (no usage of Cd rich agrochemicals). 100% of all vegetable types in 3 study areas contained Cd levels prevalent below JECFA permissible limits-0.2mg/kg. From which 63, 67, 74% samples were in lowest range of 0.04-0.06 mg/kg and 37, 33, 26% samples in higher but within limits i.e.0.06-0.07 mg/kg in areas 1, 2, 3 respectively. Average Cd levels in 100% fish of all study areas were found to be BDL. In totality for all food types of all 3 study regions analysed for Cd, highest values of 0.055, 0.059, 0.057 mg/kg were noted in vegetable (pumpkin) with lowest levels of 0.032, 0.024, 0.031 mg/kg in ragi cereal of study groups 1, 2, 3 respectively. However Cd level in these foods were similar and below JEFCA limits in all 3 regions **(Figure 3.2.2 & Table 3.2.3)**.

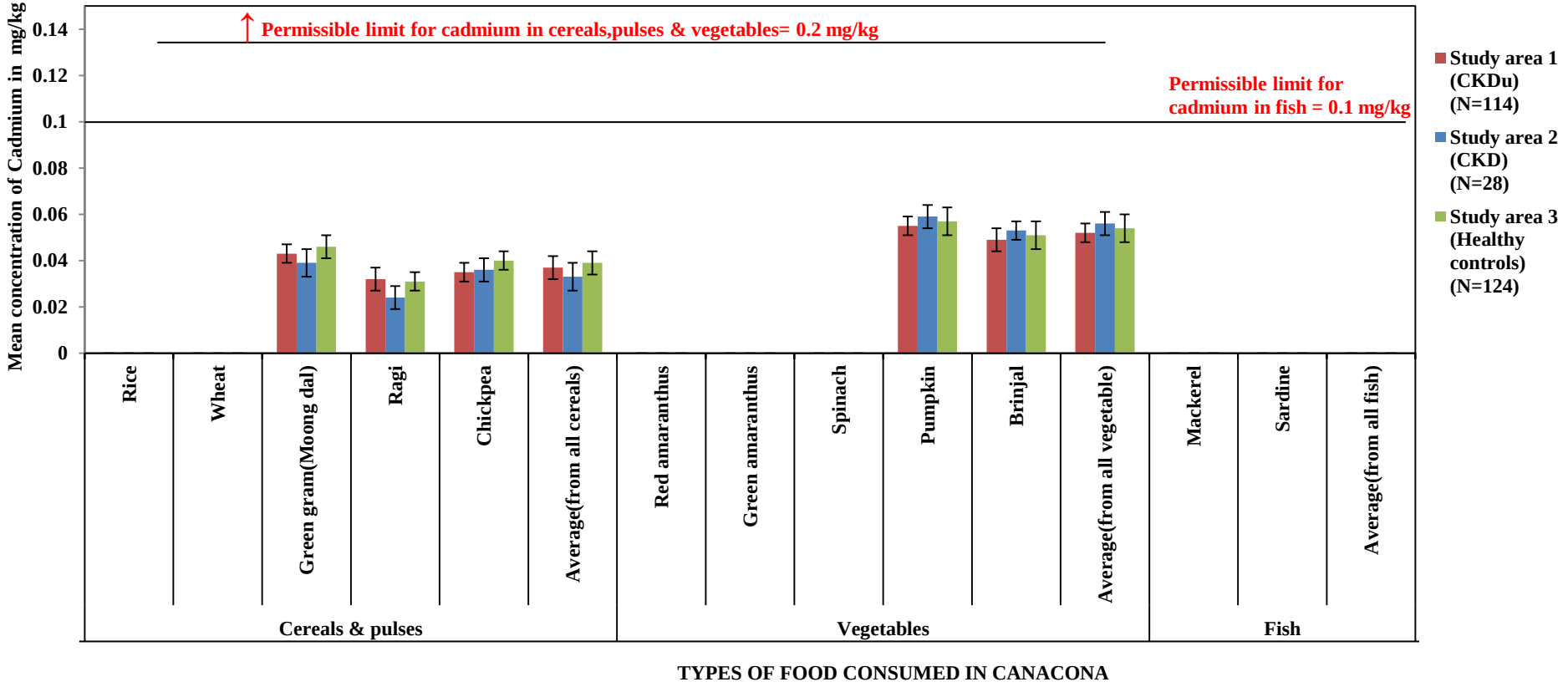

Figure 3.2.2. Mean concentrations of nephrotoxic heavy metal- 'cadmium' in several sub-types of three major food categories (i.e. cereals or pulses ,vegetables and fish) consumed by the study population of Canacona during three different seasons

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; Data are derived from two independent experiments with the cadmium levels of an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which samples of each sub-type of the three food categories viz. cereals& pulses, vegetables and fish were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons viz. Pre-Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal differences in the levels of cadmium in the food. Values are represented as mean ± SE (standard error) of cadmium levels assessed in each food sample sub-type (of the three aforesaid food categories) over three different sampling seasons or time points viz. PRM,POM,MON in the three study areas. Moreover the mean levels of cadmium in each food category (i.e. cereals and pulses or vegetables or fish) were calculated from the average of the levels in their respective sub-types(for example average of the cadmium levels in pumpkin and brinjal sub-types were reported as mean cadmium levels in the vegetables category). All the values are in mg/kg. Below detectable levels of cadmium were noted in rice,wheat and green gram of the cereals category; red cum green amaranthus & spinach of the vegetables category, and both sub-types of fish; hence are not represented by bars in the figure. The mean cadmium levels were found to be well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established permissible limits and were similar [with no significant difference($p>0.05$)] in all three study areas for each food category suggesting a common source of origin of these foods in the three study areas. The details on the seasonal distribution of the cadmium levels and the minimum to maximum range of cadmium concentrations are listed in **Table 3.2.3**.

Table 3.2.3: Mean & range concentrations (in mg/kg)of nephrotoxic heavy metal- 'cadmium' during three different seasons in various sub-types of three major categories of food (i.e. cereals or pulses ,vegetables, fish) consumed by study population of Canacona and their comparison with the respective JECFA permissible limits in food.

CKD status	CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				JCEFA established permissible limits (in mg/kg)
	Study region or group 1(N=114)				Study region or group 2(N=28)				Study region or group 3(N=124)				
Food category with sub-types/ sampling time period	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	
Cereals and pulses (in mg/kg)													
Rice	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
Wheat	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
Green gram (Moong dal)	0.048± 0.009	0.039± 0.006	0.042± 0.004	0.043± 0.006	0.043± 0.010	0.034± 0.007	0.04± 0.003	0.039± 0.010	0.049± 0.009	0.043± 0.007	0.046± 0.004	0.046± 0.010	
	(0.041-0.050)	(0.036-0.042)	(0.039-0.043)	(0.036-0.050)	(0.041-0.046)	(0.033-0.038)	(0.039-0.041)	(0.033-0.046)	(0.047-0.051)	(0.039-0.045)	(0.042-0.048)	(0.039-0.051)	For Cereals and pulses= 0.2
Ragi	0.036± 0.005	0.028± 0.010	0.032± 0.004	0.032± 0.008	0.027± 0.005	0.021± 0.007	0.024± 0.008	0.024± 0.006	0.034± 0.011	0.028± 0.004	0.031± 0.008	0.031± 0.007	
	(0.033-0.039)	(0.027-0.030)	(0.029-0.033)	(0.027-0.039)	(0.025-0.034)	(0.018-0.024)	(0.021-0.026)	(0.020-0.034)	(0.032-0.037)	(0.026-0.030)	(0.029-0.033)	(0.026-0.037)	
Chickpea	0.039± 0.008	0.032± 0.006	0.037± 0.005	0.035± 0.004	0.038± 0.009	0.033± 0.003	0.037± 0.010	0.036± 0.007	0.044± 0.008	0.037± 0.004	0.039± 0.005	0.04± 0.006	
	(0.036-0.041)	(0.031-0.033)	(0.035-0.039)	(0.031-0.040)	(0.036-0.041)	(0.029-0.035)	(0.034-0.040)	(0.029-0.041)	(0.041-0.046)	(0.036-0.039)	(0.037-0.041)	(0.036-0.046)	
Average (from all cereal sub-types)				0.037±0.007				0.033±0.008				0.039±0.006	
				(0.027-0.050)				(0.020-0.046)				(0.026-0.051)	
Vegetables (in mg/kg)													
Red amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
Green amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
Spinach	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
Pumpkin	0.059± 0.006	0.051± 0.004	0.055± 0.011	0.055± 0.007	0.064± 0.010	0.055± 0.008	0.058± 0.006	0.059± 0.008	0.06± 0.011	0.054± 0.005	0.057± 0.004	0.057± 0.007	
	(0.056-0.061)	(0.049-0.053)	(0.052-0.057)	(0.049-0.061)	(0.062-0.066)	(0.052-0.057)	(0.056-0.060)	(00052-0.066)	(0.057-0.062)	(0.051-0.056)	(0.055-0.060)	(0.051-0.062)	For vegetables= 0.2
Brinjal	0.053± 0.008	0.046± 0.009	0.048± 0.011	0.049± 0.009	0.056± 0.006	0.049± 0.004	0.054± 0.003	0.053± 0.005	0.056± 0.011	0.047± 0.009	0.05± 0.004	0.051± 0.008	
	(0.051-0.056)	(0.042-0.049)	(0.047-0.050)	(0.042-0.056)	(0.054-0.059)	(0.047-0.051)	(0.051-0.056)	(0.047-0.059)	(0.055-0.057)	(0.044-0.049)	(0.047-0.052)	(0.044-0.057)	
Average (from all vegetable sub-types)				0.052±0.008				0.056±0.006				0.054±0.009	
				(0.042-0.061)				(0.047-0.066)				(0.051-0.062)	
Fish (in mg/kg)													
Mackerel	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
Sardine	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	For Fish = 0.1
Average (from all fish sub-types)													

Abbreviations: BDL- below detectable levels; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; JECFA- Joint FAO/WHO Expert Committee on Food Additives (JECFA). Data are derived from two independent experiments with cadmium levels of an individual experiment measured in triplicates. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured. Values are represented as mean±SE with the range of minimum to maximum concentrations (shown in parenthesis) of cadmium estimated in each food sample sub-type (of the three respective food categories) over three different sampling time points viz. PRM, POM, MON in the three study groups or regions. The overall concentration of cadmium in a particular food sub-type (for example rice of the cereals category) was calculated by taking the average of the concentrations estimated during the aforementioned time-points. Moreover the mean concentrations of cadmium in each food category (i.e. cereals and pulses or vegetables or fish) were estimated from the average of the concentrations in their respective sub-types. No statistically significant ($p>0.05$) differences were observed in the concentrations of cadmium between the three study regions for each food category and were well below the JECFA established permissible limits.

Overall, these results **(Figure 3.2.2 & Table 3.2.3)** were comparable with Cd levels noted in similar food consumed in CKDu affected countries like Sri Lanka and Central America wherein no link with observed nephrotoxicity was noted on long-term intake of food containing such JCEFA safe Cd levels. Thus, signifying prevalent dietary Cd in taluka's CKDu induction can be ruled out, backed by similar Cd levels noted in CKDu hit and healthy regions (Almaguer et al.,2014; Chandrajith et al.,2011; Correa-Rotter, 2017; Jayatilake et al., 2013; McClean et al., 2012; Siriwardhana et al., 2014; Wesseling et al., 2013).

3.2.2.3.1.1.3 Concentration of arsenic in food collected from the study population

As per JECFA, 50% and 41% human exposure to As arises from consuming water and food mainly fish respectively contaminated with As by activities like untreated industrial effluents discharge or application of As based agrochemicals in terrestrial and aquatic environments. Hence As levels were determined in these foods in this study to assess dietary As contribution (if any) in CKDu development in Canacona (Hsu et al., 2017; Jayasumana et al, 2011; Jayasumana et al, 2014; Jayasumana et al., 2015c; Wanigasuriya et al.,2012).

As indicated in **Figure 3.2.3 & Table 3.2.4**, mean As levels in cereals and pulses in study group 1,2,3 were similar and found to be 0.032, 0.036 and 0.034 mg/kg with maximum values of 0.039, 0.044 and 0.041 mg/kg noted in ragi and minimum values of 0.023,0.29 and 0.027 mg/kg noted in chickpea respectively. As levels in remaining cereals were BDL attributed to organic farming (no use of As rich agrochemicals). 100% of cereals in all study areas possessed As levels prevalent below JECFA permissible limits of 1 mg/kg, wherein 54, 59, 62% samples were in lowest range of 0.02-0.04 mg/kg & 46, 41, 38% samples in slightly higher range but within limits i.e.0.04-0.05 mg/kg in study areas 1, 2, 3 respectively. Average As levels in vegetables of study group 1,2,3 were comparable and found to be 0.013, 0.017 & 0.015mg/kg with maximum values of 0.019, 0.025 & 0.023 mg/kg noted in pumpkin & minimum values of 0.005, 0.010, 0.007 mg/kg noted in brinjal respectively. As contents in remaining vegetables were BDL.100% of all vegetable types of 3 study areas possessed As contents prevalent below JECFA permissible limits-5mg/kg, wherein 65, 59, 60% samples were in lowest range of 0.005-0.020 mg/kg and 35, 41, 40% samples were in higher range but within limit i.e.0.020-0.030 mg/kg in areas 1, 2, 3 respectively. Mean As levels in 100% of fish of all groups were found to be BDL**.** In entirety for all food types of all 3 study regions analysed for As, highest values of 0.035, 0.038, 0.037 mg/kg were noted in ragi cereal with lowest levels of 0.011, 0.012, 0.014 mg/kg in brinjal vegetable of groups 1, 2, 3 respectively. However As level in these foods were similar and below JEFCA limits in all 3 regions.

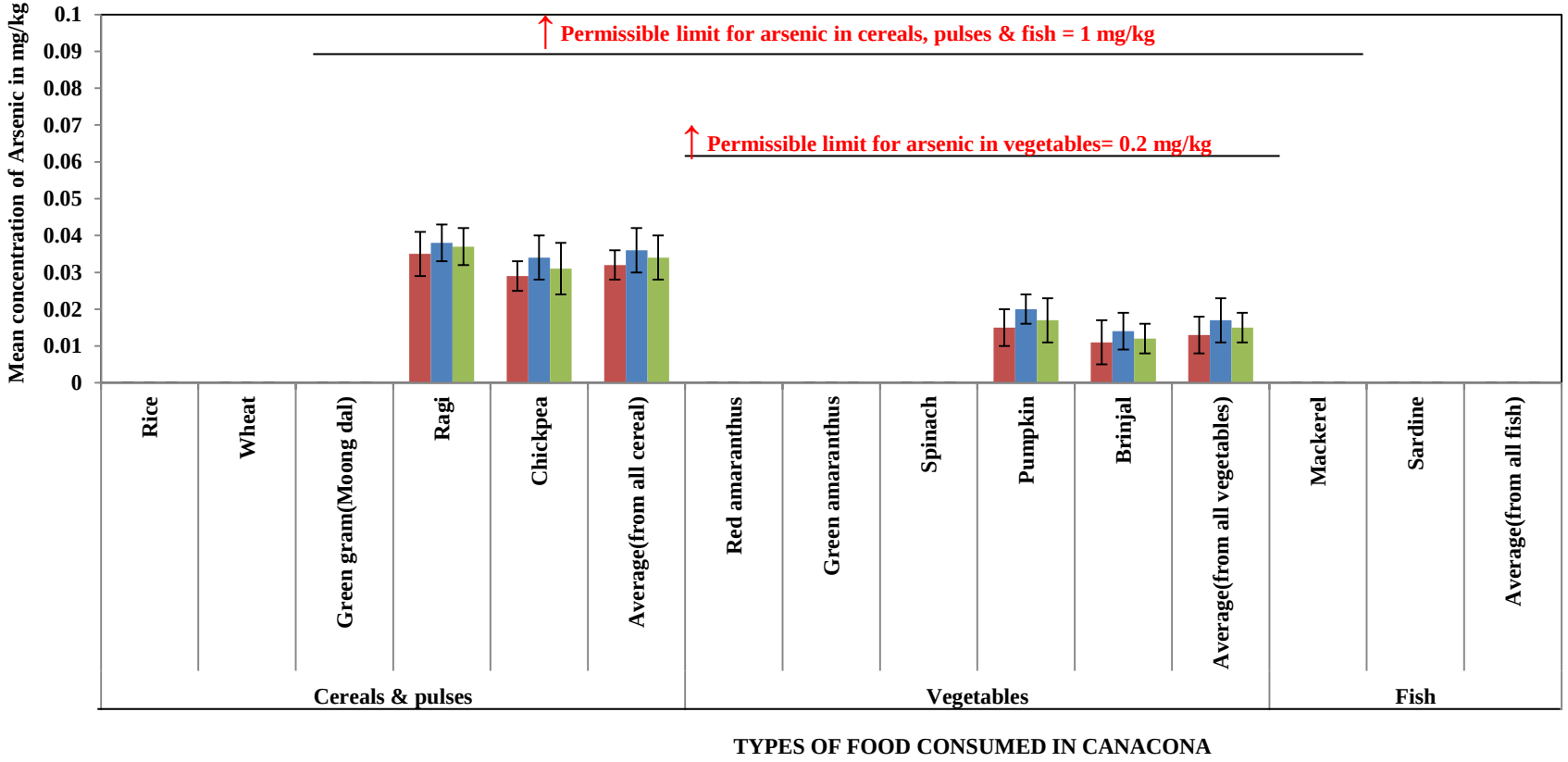

Figure 3.2.3. Mean concentrations of nephrotoxic heavy metal- 'arsenic' in several sub-types of three major food categories (i.e. cereals or pulses ,vegetables and fish) consumed by the study population of Canacona during three different seasons

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; Data are derived from two independent experiments with the arsenic levels of an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which samples of each sub-type of the three food categories viz. cereals& pulses, vegetables and fish were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons viz. Pre-Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal differences in the levels of arsenic in the food. Values are represented as mean ± SE (standard error) of arsenic levels assessed in each food sample sub-type (of the three aforesaid food categories) over three different sampling seasons or time points viz. PRM,POM,MON in the three study areas. Moreover the mean levels of arsenic in each food category (i.e. cereals and pulses or vegetables or fish) were calculated from the average of the levels in their respective sub-types(for example average of the arsenic levels in pumpkin and brinjal sub-types were reported as mean arsenic levels in the vegetables category). All the values are in mg/kg. Below detectable levels of arsenic were noted in rice,wheat and green gram of the cereals category; red cum green amaranthus & spinach of the vegetables category, and both sub-types of fish; hence are not represented by bars in the figure. The mean arsenic levels were found to be well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established permissible limits and were comparable [with lack of significant difference($p>0.05$)] in all three study areas for each food category suggesting a common source of origin of these foods in the three study areas. The details on the seasonal distribution of the arsenic levels and the minimum to maximum range of arsenic concentrations are listed in **Table 3.2.4**.

Table 3.2.4: Mean & range concentrations (in mg/kg)of nephrotoxic heavy metal- 'arsenic' during three different seasons in various sub-types of three major categories of food (i.e. cereals/ pulses ,vegetables, fish) consumed by study population of Canacona & their comparison with the respective JECFA permissible limits in food.

CKD status	CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				JCEFA established permissible limits (in mg/kg)
	Study region or group 1(N=114)				Study region or group 2(N=28)				Study region or group 3(N=124)				
Food category with sub-types/ sampling time period	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	
Cereals and pulses (in mg/kg)													
Rice	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Wheat	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Green gram (Moong dal)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Ragi	0.038± 0.004	0.031± 0.009	0.036± 0.007	**0.035± 0.006**	0.042± 0.005	0.035± 0.008	0.037± 0.003	**0.038± 0.005**	0.04± 0.010	0.034± 0.008	0.037± 0.011	**0.037± 0.009**	
	(0.036-0.039)	(0.029-0.033)	(0.035-0.038)	**(0.029-0.039)**	(0.040-0.044)	(0.033-0.036)	(0.035-0.040)	**(0.033-0.44)**	(0.038-0.041)	(0.031-0.036)	(0.033-0.039)	**(0.031-0.041)**	
Chickpea	0.033± 0.006	0.025± 0.005	0.029± 0.008	**0.029± 0.007**	0.037± 0.009	0.03± 0.010	0.035± 0.008	**0.034± 0.009**	0.035± 0.005	0.028± 0.009	0.03± 0.006	**0.031± 0.007**	
	(0.031-0.036)	(0.023-0.027)	(0.028-0.031)	**(0.023-0.036)**	(0.035-0.040)	(0.029-0.033)	(0.034-0.036)	**(0.029-0.040)**	(0.033-0.038)	(0.027-0.030)	(0.029-0.033)	**(0.027-0.038)**	
Average (from all cereal sub-types)				**0.032±0.006**				**0.036±0.008**				**0.034±0.008**	For Cereals and pulses = 1
				(0.023-0.039)				**(0.029-0.044)**				**(0.027-0.041)**	
Vegetables (in mg/kg)													
Red amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Green amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Spinach	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Pumpkin	0.018± 0.005	0.013± 0.008	0.014± 0.009	**0.015± 0.007**	0.023± 0.006	0.016± 0.004	0.021± 0.009	**0.02± 0.006**	0.02± 0.010	0.013± 0.008	0.018± 0.005	**0.017± 0.007**	
	(0.015-0.019)	(0.009-0.015)	(0.013-0.016)	**(0.009-0.019)**	(0.020-0.025)	(0.014-0.018)	(0.019-0.024)	**(0.014-0.025)**	(0.018-0.023)	(0.011-0.016)	(0.016-0.021)	**(0.011-0.023)**	
Brinjal	0.013± 0.009	0.008± 0.005	0.012± 0.010	**0.011± 0.008**	0.016± 0.011	0.011± 0.009	0.015± 0.006	**0.014± 0.008**	0.015± 0.006	0.008± 0.005	0.01± 0.009	**0.012± 0.007**	
	(0.012-0.015)	(0.005-0.009)	(0.010-0.013)	**(0.005-0.015)**	(0.014-0.018)	(0.010-0.013)	(0.013-0.017)	**(0.010-0.018)**	(0.014-0.016)	(0.007-0.009)	(0.009-0.012)	**(0.007-0.017)**	
Average (from all vegetable sub-types)				**0.013±0.007**				**0.017±0.008**				**0.015±0.007**	For vegetables= 0.2
				(0.005-0.019)				**(0.010-0.025)**				**(0.007-0.023)**	
Fish (in mg/kg)													
Mackerel	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Sardine	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Average (from all fish sub-types)													For Fish= 1.0

Abbreviations: BDL- below detectable levels; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; JECFA- Joint FAO/WHO Expert Committee on Food Additives (JECFA). Data are derived from two independent experiments with arsenic levels of an individual experiment measured in triplicates. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured. Values are represented as mean±SE with the range of minimum to maximum concentrations(shown in parenthesis) of arsenic estimated in each food sample sub-type (of the three respective food categories) over three different sampling time points viz. PRM,POM,MON in the three study groups or regions. The overall concentration of arsenic in a particular food sub-type (for example rice of the cereals category) was calculated by taking the average of the concentrations estimated during the aforementioned time-points. Moreover the mean concentrations of arsenic in each food category (i.e. cereals and pulses or vegetables or fish) were estimated from the average of the concentrations in their respective sub-types. No statistically significant ($p>0.05$) differences were noted in the concentrations of arsenic between the three study regions for each food category and were well below the JECFA established permissible limits.

Overall, these results **(Figure 3.2.3 & Table 3.2.4)** were consistent with As levels noted in similar food consumed in CKDu hit country like Sri Lanka wherein no relation with noted nephrotoxicity was found on long-term intake of food containing such JCEFA safe As levels. Thus, indicating prevalent dietary As in taluka's CKDu induction can be negated, backed by similar As levels noted in CKDu hit and healthy regions (Bandara et al.,2008; Bandara et al., 2010a; Chandrajith et al., 2011; Jayatilake et al.,2013; Levine et al.,2016; Siriwardhana et al., 2014; Wanigasuriya et al., 2011).

3.2.2.3.1.1.4 Concentration of mercury in food samples collected in the study population

JECFA reported 32% and 63% human exposure to Hg occurs via consumption of water and food mainly fish or shellfish polluted by Hg from activities like discharge of untreated effluents from industries (like batteries, smelting) or municipal/domestic sewage enriched in medical waste like thermometer Hg, dental amalgam etc. in terrestrial and aquatic ecosystems. Hence Hg levels were measured in these foods in this study to assess its dietary contribution in taluka's CKDu causation (Barone et al., 2015; Faragher and DeHaan, 2018; Ferreira et al.,2015; Gundacker et al.,2010; Kim et al.,2016).As indicated in **Figure 3.2.4 & Table 3.2.5**, Hg levels in cereals and pulses specifically ragi were comparable in study areas 1,2,3 possessing values of 0.008, 0.011 and 0.010 mg/kg respectively. 100% ragi samples analysed in all study areas possessed Hg levels prevalent below JECFA permissible limits-0.5 mg/kg wherein 67, 63, 70% of samples were in lowest range of 0.005-0.010 mg/kg & 33, 37, 30% samples were in higher but within permissible limit i.e. 0.010-0.020 mg/kg in areas 1, 2, 3 respectively. Hg levels in remaining analysed cereal and pulses and vegetable types in all 3 study areas during different seasons were BDL attributed to the organic mode of farming (i.e. non-usage of Hg based agrochemicals).Hg levels in fish of study areas 1, 2, 3 were comparable and found to be 0.075, 0.078, 0.073 mg/kg with maximum values of 0.082, 0.081, 0.079 mg/kg noted in mackerel and minimum values of 0.068, 0.072, 0.066 mg/kg noted in sardine respectively.100% of fish in all study areas contained Hg levels prevalent below JECFA permissible limits-5mg/kg wherein 72, 76, 71% of samples were in lowest range of 0.065-0.075 mg/kg and 11, 14, 9% of samples were in higher but permissible range i.e. 0.075-0.085 mg/kg in study areas 1, 2, 3 respectively. In totality for all food types of all 3 study regions analysed for Hg, highest values of 0.077, 0.080, 0.074 mg/kg were noted in mackerel fish with lowest levels of 0.008, 0.011, 0.010 mg/kg in ragi cereal of study areas 1, 2, 3 respectively. However Hg level in these foods were comparable and below JEFCA limits in all 3 regions.

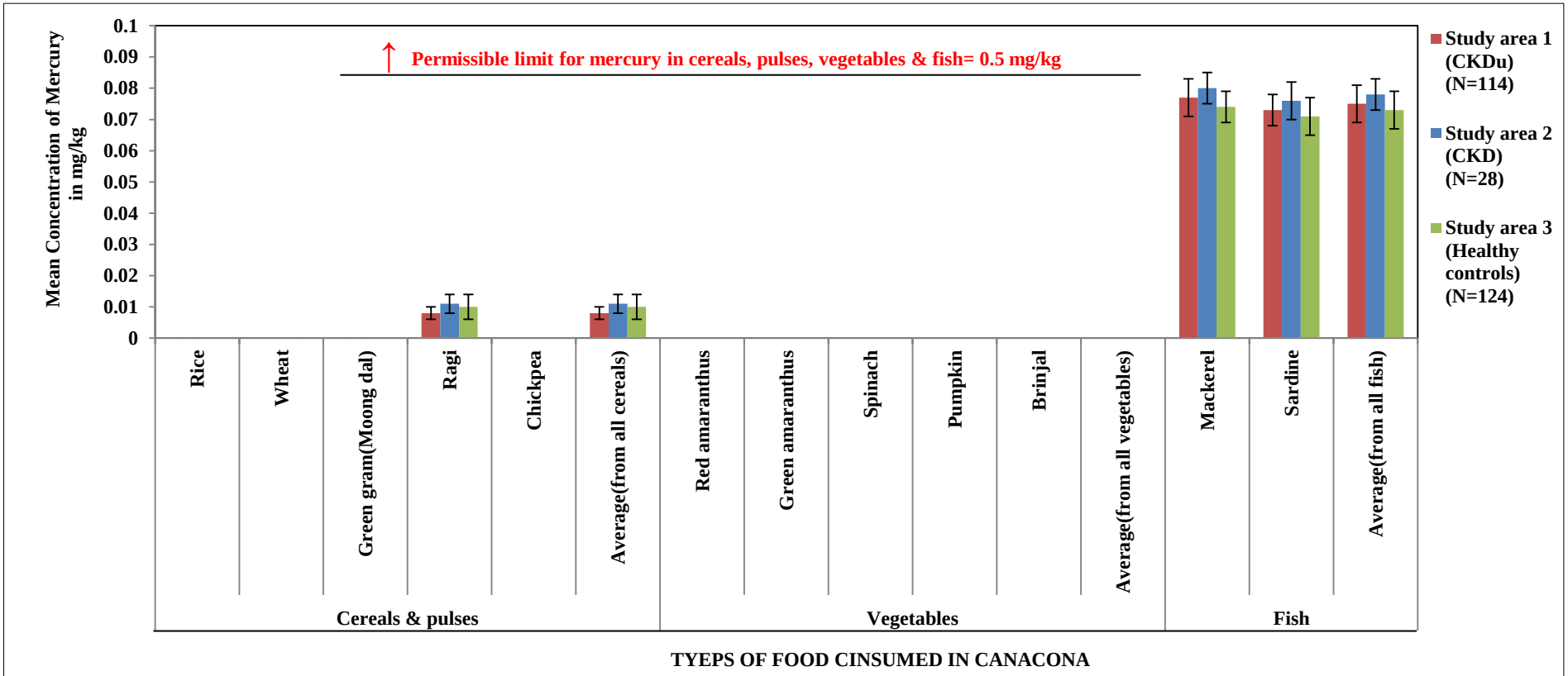

Figure 3.2.4. Mean concentrations of nephrotoxic heavy metal- 'mercury' in several sub-types of three major food categories (i.e. cereals or pulses ,vegetables and fish) consumed by the study population of Canacona during three different seasons

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; Data are derived from two independent experiments with the mercury levels of an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which samples of each sub-type of the three food categories viz. cereals& pulses, vegetables and fish were collected.These samples were collected repeatedly for analysis from each of the study areas over three different seasons viz. Pre-Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal differences in the levels of mercury in the food. Values are represented as mean ± SE (standard error) of mercury levels estimated in each food sample sub-type (of the three aforesaid food categories) over three different sampling seasons or time points viz. PRM,POM,MON in the three study areas. Moreover the mean levels of mercury in each food category (i.e. cereals and pulses or vegetables or fish) were calculated from the average of the levels in their respective sub-types(for example average of the mercury levels in mackerel and sardine sub-types were reported as mean mercury levels in the fish category). All the values are in mg/kg. Below detectable levels of mercury were noted all the sub-types of the cereals category except for ragi and in all the types of vegetables, hence are not represented by bars in the figure.The mean mercury levels were found to be well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established permissible limits and were comparable [with lack of significant difference($p>0.05$)] in all three study areas for each food category suggesting a common source of origin of these foods in the three study areas. The details on the seasonal distribution of the mercury levels and the minimum to maximum range of mercury concentrations are listed in **Table 3.2.5**.

Table 3.2.5 : Mean & range concentrations (in mg/kg) of nephrotoxic heavy metal- 'mercury' during three different seasons in various sub-types of three major categories of food (i.e. cereals or pulses ,vegetables, fish) consumed by study population of Canacona & their comparison with respective JECFA permissible limits in food.

CKD status	CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				JCEFA established permissible limits (in mg/kg)
	Study region or group 1(N=114)				Study region or group 2(N=28)				Study region or group 3(N=124)				
Food category with sub-types/ sampling time period	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	
Cereals and pulses (in mg/kg)													
Rice	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Wheat	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Green gram (Moong dal)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Ragi	0.01± 0.003	0.005± 0.001	0.009± 0.002	**0.008± 0.002**	0.013± 0.003	0.009± 0.001	0.011± 0.002	**0.011± 0.003**	0.013± 0.009	0.006± 0.004	0.011± 0.006	**0.01± 0.006**	
	(0.008-0.012)	(0.004-0.006)	(0.008-0.011)	**(0.004-0.012)**	(0.012-0.014)	(0.008-0.011)	(0.010-0.013)	**(0.008-0.014)**	(0.009-0.014)	(0.005-0.007)	(0.009-0.013)	**(0.005-0.013)**	
Chickpea	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Average (from all cereal sub-types)				**0.008± 0.002**				**0.011± 0.003**				**0.01± 0.006**	
				(0.004-0.012)				**(0.008-0.014)**				**(0.005-0.013)**	
Vegetables (in mg/kg)													
Red amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Green amaranthus	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Spinach	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Pumpkin	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Brinjal	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	
	-	-	-	-	-	-	-	-	-	-	-	-	
Average (from all vegetable sub-types)													
Fish (in mg/kg)													
Mackerel	0.081± 0.004	0.074± 0.009	0.076± 0.010	**0.077± 0.007**	0.084± 0.009	0.077± 0.008	0.079± 0.005	**0.08± 0.007**	0.077± 0.006	0.07± 0.003	0.075± 0.009	**0.074± 0.006**	
	(0.078-0.082)	(0.073-0.075)	(0.075-0.077)	**(0.073-0.082)**	(0.081-0.085)	(0.075-0.078)	(0.078-0.081)	**(0.075-0.081)**	(0.074-0.079)	(0.069-0.071)	(0.073-0.076)	**(0.069-0.079)**	
Sardine	0.077± 0.007	0.07± 0.006	0.072± 0.003	**0.073± 0.005**	0.079± 0.004	0.073± 0.009	0.076± 0.006	**0.076± 0.007**	0.075± 0.005	0.068± 0.008	0.07± 0.004	**0.071± 0.008**	
	(0.075-0.078)	(0.068-0.071)	(0.070-0.073)	**(0.068-0.078)**	(0.077-0.080)	(0.072-0.075)	(0.075-0.078)	**(0.072-0.080)**	(0.073-0.077)	(0.066-0.069)	(0.069-0.072)	**(0.066-0.077)**	
Average (from all fish sub-types)				**0.075± 0.006**				**0.078± 0.007**				**0.073± 0.007**	
				(0.068-0.082)				**(0.072-0.081)**				**(0.066-0.079)**	

Abbreviations: BDL- below detectable levels; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; JECFA-Joint FAO/WHO Expert Committee on Food Additives (JECFA). Data are derived from two independent experiments with mercury levels of an individual experiment measured in triplicates. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured. Values are represented as mean±SE with the range of minimum to maximum concentrations(shown in parenthesis) of mercury estimated in each food sample sub-type (of the three respective food categories) over three different sampling time points viz. PRM,POM,MON in the three study groups or regions. The overall concentration of mercury in a particular food sub-type (for example rice of the cereals category) was calculated by taking the average of the concentrations estimated during the aforementioned time-points. Moreover the mean concentrations of mercury in each food category (i.e. cereals and pulses or vegetables or fish) were estimated from the average of the concentrations in their respective sub-types. No statistically significant ($p>0.05$) differences were noted in the concentrations of mercury between the three study regions for each food category and were well below the JECFA established permissible limits.

Overall, these results **(Figure 3.2.4 & Table 3.2.5)** were consistent with Hg levels noted in similar food consumed in CKDu countries like Sri Lanka and Central America wherein no link with noted renal-toxicity was found on chronic intake of food containing such JCEFA safe Hg levels. Thus, indicating lack of prevalent dietary Hg role in taluka's CKDu development, supported by similar Hg levels noted in CKDu hit and healthy regions (Almaguer et al.,2014; Bandara et al.,2008; Bandara et al.,2010a; Chandrajith et al.,2011; Jayatilake et al.,2013; Levine et al.,2016; McClean et al.,2012; Wanigasuriya et al.,2011; Wesseling et al., 2014).

On the whole, contents of all four heavy metals viz. Pb, Cd, As and Hg analysed in frequently consumed foods in Canacona were observed to be comparable among taluka's CKDu affected (study region 1), diabetes & hypertensive CKD affected (study region 2) & healthy regions (study region 3). Additionally, these levels were well below their respective JECFA permissible limits with some food types displaying BDL in a similar fashion in all 3 regions. The significant absence of non-toxic heavy metal levels (i.e. Pb, Cd, As, Hg) in regularly consumed food of Canacona suggests lack of role of dietary or food consumption based heavy-metal exposure in etiological development of high CKDu incidence in the taluka.

3.2.2.3.1.2 Concentrations of ochratoxin in cereals and pulses of study population.

JECFA stated human exposure to ochratoxin is dietary i.e. from food wherein 95% and 5% exposure emerges from consumption of developing country's staple cereals (like rice, wheat) and uncommon cereals and pulses (like green-gram, ragi, chickpea) respectively. Exposures through contaminated drinking water, vegetables or fish is unlikely. Ochratoxin contamination in cereals arises from improper cereal storage (like high moisture, low temperatures) that favor contaminating fungal growth, thus increased OTA production. Based on OTA's nephrotoxic effects, JECFA has set a permissible limit of 5 µg/kg in cereals and pulses (Duarte et al., 2010; Gifford et al.,2017; Malir et al.,2016). Hence OTA levels in above said cereals and pulses were measured in this study to assess OTA contribution in taluka's CKDu causation **(Figure 3.2.5 and Table 3.2.6)**.

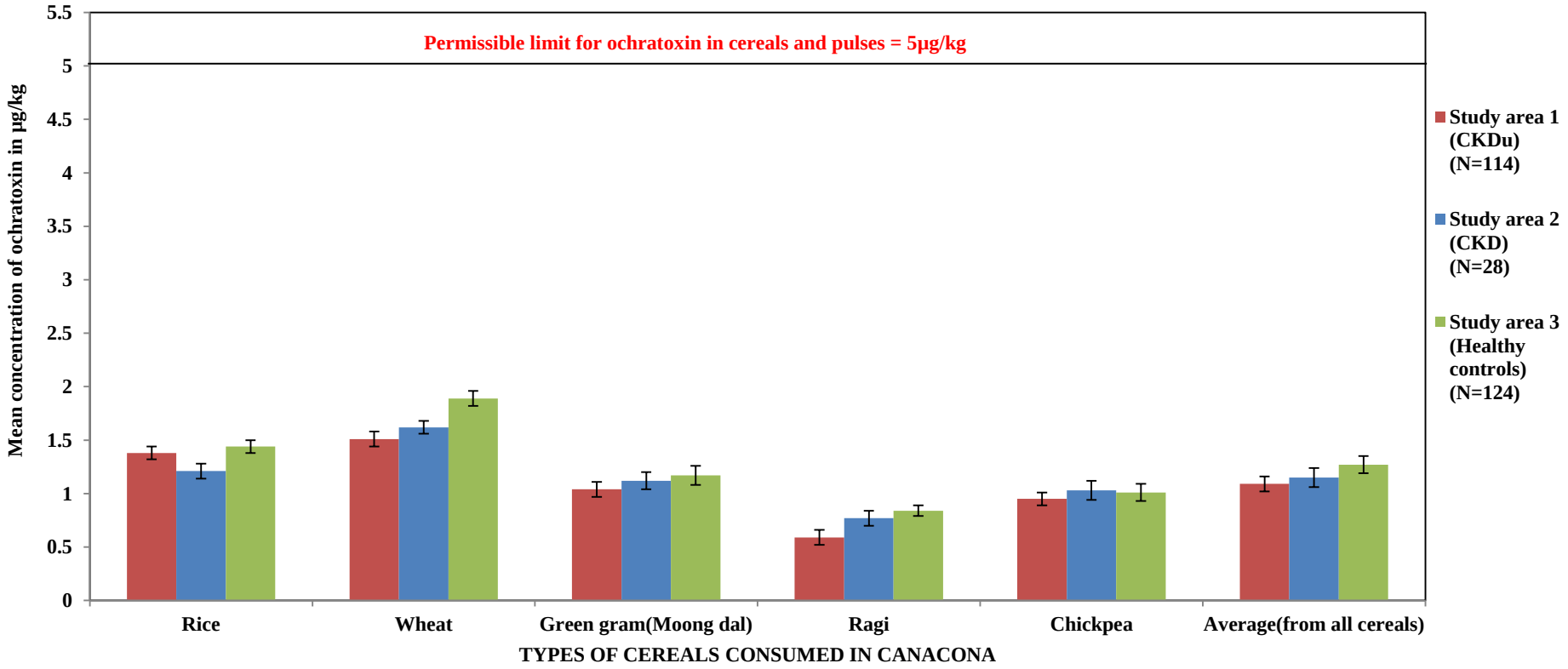

Figure 3.2.5 : Mean concentrations of nephrotoxic mycotoxin-'ochratoxin' in different types of cereals and pulses consumed by the study population of Canacona during three different seasons
Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; Data are derived from two independent experiments with ochratoxin levels of an individual experiment measured in triplicates. 'N' in each study area depicts the number of subjects in that study area or group from which samples of different types of cereals and pulses were obtained.These samples were collected repeatedly for analysis from each of the study areas over three different seasons viz. Pre-Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal differences in the levels of ochratoxin in the food. Values are represented as mean ± SE (standard error) of ochratoxin levels estimated in each type of cereals and pulses over three different sampling seasons or time points viz. PRM,POM,MON in the three study areas. Moreover the overall mean concentrations of ochratoxin in the cereals & pulses food category was calculated from the average of the concentrations in its sub-types(for example average of the ochratoxin levels in rice,wheat,ragi,green gram and chickpea sub-types were reported as mean ochratoxin levels in the cereals & pulses category).Ochratoxin levels were solely analysed in cereals & pulses food samples only as it is a contaminant of the same and does not occur in vegetables and fish. All the values are in µg/kg. The mean ochratoxin levels were found to be well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established permissible limits and were comparable [with no significant difference($p>0.05$)] in the cereals & pulses consumed by all three study areas suggesting a common source from where these cereals are procured in the three study areas. The details on the seasonal distribution of the ochratoxin levels and the minimum to maximum range of ochratoxin concentrations are listed in **Table 3.2.6**.

Table 3.2.6 : Mean and range concentrations(in µg/kg)of nephrotoxic mycotoxin-'ochratoxin' during three different seasons in various types of cereals and pulses commonly consumed by study population of Canacona taluka and their comparison with the respective JECFA established permissible limits in food.

CKD status	CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				JCEFA established permissible limits (in µg/kg)
	Study region or group 1(N=114)				Study region or group 2(N=28)				Study region or group 3(N=124)				
Food category with sub-types/sampling time period	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	
Cereals and pulses (in µg/kg)													
Rice	1.35± 0.03	1.42± 0.09	1.37± 0.08	**1.38± 0.06**	1.13± 0.10	1.28± 0.09	1.22± 0.08	**1.21± 0.07**	1.39± 0.04	1.49± 0.05	1.44± 0.09	**1.44± 0.06**	
	(1.22-1.41)	(1.34-1.55)	(1.33-1.47)	**(1.22-1.55)**	(1.10-1.23)	(1.26-1.49)	(1.15-1.31)	**(1.10-1.49)**	(1.32-1.45)	(1.37-1.60)	(1.35-1.53)	**(1.32-1.60)**	
Wheat	1.23± 0.07	1.89± 0.09	1.41± 0.04	**1.51± 0.07**	1.45± 0.05	1.89± 0.10	1.52± 0.03	**1.62± 0.06**	1.83± 0.05	1.93± 0.09	1.91± 0.03	**1.89± 0.07**	
	(1.20-1.31)	(1.71-1.91)	(1.34-1.52)	**(1.20-1.91)**	(1.42-1.55)	(1.82-1.95)	(1.47-1.69)	**(1.42-1.95)**	(1.70-1.91)	(1.89-2.03)	(1.85-1.99)	**(1.70-2.03)**	
Green gram (Moong dal)	0.91± 0.04	1.13± 0.07	1.08± 0.08	**1.04± 0.07**	1.07± 0.10	1.18± 0.09	1.11± 0.04	**1.12± 0.08**	1.14± 0.12	1.21± 0.07	1.16± 0.09	**1.17± 0.09**	For Cereals and pulses = 5
	(0.90-0.99)	(1.03-1.25)	(1.04-1.21)	**(0.90-1.25)**	(1.03-1.14)	(1.15-1.30)	(1.09-1.19)	**(1.03-1.30)**	(1.05-1.21)	(1.19-1.37)	(1.14-1.28)	**(1.05-1.37)**	
Ragi	0.39± 0.07	0.78± 0.06	0.6± 0.09	**0.59± 0.07**	0.72± 0.09	0.81± 0.05	0.78± 0.06	**0.77± 0.07**	0.81± 0.04	0.87± 0.03	0.84± 0.09	**0.84± 0.05**	
	(0.37-0.49)	(0.68-0.88)	(0.59-0.69)	**(0.37-0.88)**	(0.61-0.83)	(0.78-1.01)	(0.75-0.89)	**(0.61-1.01)**	(0.67-0.91)	(0.82-1.09)	(0.81-0.96)	**(0.67-1.09)**	
Chickpea	0.92± 0.06	0.97± 0.09	0.96± 0.04	**0.95± 0.06**	0.96± 0.07	1.09± 0.10	1.04± 0.09	**1.03± 0.09**	0.98± 0.06	1.05± 0.09	1± 0.08	**1.01± 0.08**	
	(0.89-0.97)	(0.93-1.15)	(0.92-1.03)	**(0.89-1.15)**	(0.92-1.05)	(1.03-1.21)	(1.01-1.15)	**(0.92-1.21)**	(0.95-1.05)	(1.01-1.23)	(0.98-1.11)	**(0.95-1.23)**	
Average (from all cereal sub-types)				**1.09± 0.07**				**1.15±0.09**				**1.27± 0.08**	
				(0.37-1.91)				**(0.61-1.95)**				**(0.67-2.03)**	

Abbreviations:- CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; JECFA-Joint FAO/WHO Expert Committee on Food Additives (JECFA). Data are derived from two independent experiments with ochratoxin levels of an individual experiment measured in triplicates. 'N' in each study region depicts the number of subjects in that study region or group from which the different types of cereal food samples were obtained. Values are represented as mean±SE with the range of minimum to maximum concentrations (shown in parenthesis) of ochratoxin estimated in each type of cereal over three different sampling time points viz. PRM,POM,MON in the three study groups or regions. The overall concentration of ochratoxin in each type of cereals was calculated by taking the average of the concentrations estimated during the aforementioned time-points. Moreover the general mean concentrations of ochratoxin in the cereals food category was estimated from the average of the concentrations in its sub-types. No statistically significant ($p>0.05$) differences were noted in the concentrations of ochratoxin estimated in each cereal type between the three study regions/groups and were well below the JECFA established permissible limits.

Owing to seasonal changes affecting OTA levels, seasonal variations of OTA were evaluated in all cereals and pulses during 3 different seasons i.e. Pre-monsoon (PRM), monsoon (MON) and post-monsoon (POM) in the current study (**Table 3.2.6).** As indicated in **Table 3.2.6**, the trend in seasonal variations of OTA levels was comparable in all cereals and pulses of all 3 study regions. In all study regions, OTA content in MON was slightly higher followed by POM with lowest values noted in PRM, with statistically insignificant ($p>0.05$) differences. Higher levels in MON could be attributed to incessant precipitation that reduces environment's temperature and enhances humidity or moisture content thus supporting fungal growth resulting in high OTA production. Reduced OTA content in POM (winter) could be ascribed to rainfall cessation and winter induced temperature and humidity stabilization, thus not supporting fungal growth. Highly reduced levels in PRM (summer) could be linked to higher temperatures and related raised evaporation that reduces moisture content to below-optimal levels which retards fungal growth resulting in lowest OTA levels (Erkekoglu et al., 2010; Jonsyn-Ellis, 2001; Sabuncuoglu et al., 2015). However, despite insignificant seasonal changes in OTA contents in cereals and pulses of all study regions, levels were below permissible limit indicating no concern for renal injury by dietary OTA exposure.

Moreover mean OTA levels (calculated for a particular food type over 3 different seasons) in all cereals and pulses of study groups 1, 2, 3 were similar & estimated to be 1.09, 1.15, 1.27 µg/kg with maximum values of 1.91, 1.95, 2.03 µg/kg noted in wheat and minimum values of 0.37, 0.60, 0.67µg/kg noted in ragi respectively with a ranking order (in terms of OTA level) of Wheat>Rice>Green gram>Chickpea>Ragi **(Figure 3.2.5 &Table 3.2.6)**. 100% of cereals & pulses in all study areas possessed OTA levels prevalent significantly below JECFA permissible limits-5 µg/kg wherein 65, 68, 72% samples were present in lowest range of 0.1-1.0 µg/kg and remaining 35, 32, 28% were in higher but permissible range of 1.0-2.0 µg/kg in areas 1, 2, 3 respectively. Similar OTA levels noted in cereals and pulses of all 3study regions indicates a common food distribution source (Khlifa et al., 2012) i.e. healthy villages (no CKD prevalence)- Molorem and Endrem to entire studied regions of Canacona owing to farming prevalence in these villages (as established in CKDu demographic survey-Chapter 2). These results were consistent with OTA level ranges noted in similar cereals/pulses consumed in CKDu hit countries like Sri Lanka & Central America wherein no link with reported nephrotoxicity was noted on chronic intake of these foods containing such safe OTA contents (Almaguer et al., 2014; Correa-Rotter et al., 2014; García-Trabanino et al., 2015; McClean et al., 2012; Rajapakse et al., 2016; Wanigasuriya et al., 2008; Wesseling et al., 2013). Thus indicating lack of contribution of prevalent dietary OTA at JCEFA safe levels in

Canacona CKDu causation, backed by similar OTA levels noted in CKDu hit and healthy-regions.

3.2.2.3.1.3 Concentrations of aristolochic acid in cereals and pulses of study population.

JECFA stated human exposure to aristolochic acid (AA) is dietary only i.e. from food wherein 96% and 4% of exposure arises from consumption of developing country's staple cereals (wheat, rice) and uncommon cereals and pulses (viz. green-gram, ragi, chickpea) respectively. Water, vegetables or fish are unlikely AA exposure sources. Cereal pollution with AA arises from incorrect cereal storage (high moisture, low temperatures) that favors polluting plant growth. Based on AA induced renal toxicity JECFA has set permissible limit of 1.5µg/kg in cereals/pulses (Michl et al.,2013; Jadot et al.,2017).Hence AA levels in abovesaid cereals were measured in this study to assess AA role in taluka's CKDu causation **(Figure 3.2.6 and Table 3.2.7)**. As indicated in **Table 3.2.7**, pattern of seasonal changes in AA levels were similar in all cereals of all 3 study regions wherein AA levels in monsoon (MON) were slightly greater trailed by post-monsoon (POM) with lowest values in pre-monsoon (PRM), having statistically insignificant differences ($p>0.05$).Higher levels in MON could be accredited to continuous rainfall which increases soil's moisture content and humidity with simultaneous temperature reduction in soil and air supporting increased plant germination and growth resulting in larger AA production. Reduced levels in POM could be ascribed to rainfall cessation & winter linked stabilization of soil's & air's temperature and moisture content to optimal levels which equilibrates plant growth rate, eventually progressing into dormancy preventing flowering and further plant growth due to plateaued growth conditions. Highly reduced levels in PRM could be explained by increased temperatures linked enhanced atmospheric & soil evaporation causing reduction in respective moisture content to below-optimal levels, which simultaneously triggers amplified plant dehydration via excessive transpiration that ultimately retards plant metabolism manifesting in decreased AA production (Dhouioui et al., 2016; Prinsloo and Nogemane,2018; Sime et al.,2000). However, despite insignificant seasonal changes in AA levels in cereals/pulses of all study regions, contents were below permissible limit indicating no concern for renal damage by alimentary AA exposure (**Figure 3.2.6 and Table 3.2.7).**

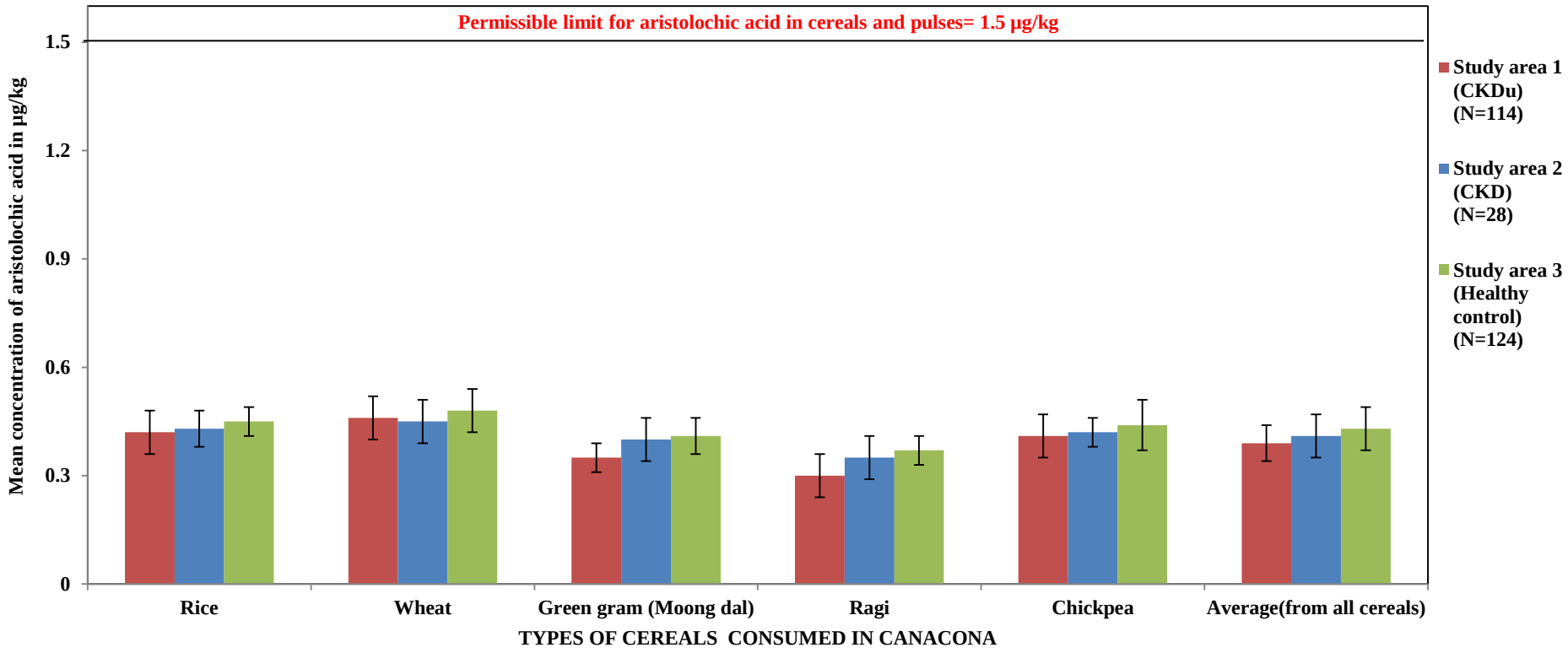

Figure 3.2.6 : Mean concentrations of nephrotoxic phytotoxin-'aristolochic acid' in different types of cereals and pulses consumed by the study population of Canacona during three different seasons
Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; Data are derived from two independent experiments with aristolochic acid levels of an individual experiment measured in triplicates.'N' in each study area depicts the number of subjects in that study area or group from which samples of different types of cereals and pulses were obtained.These samples were collected repeatedly for analysis from each of the study areas over three different seasons viz. Pre-Monsoon (PRM), Monsoon (MON) and Post-Monsoon (POM) to assess for seasonal differences in the levels of aristolochic acid in the food. Values are represented as mean ± SE (standard error) of aristolochic acid levels estimated in each type of cereals and pulses over three different sampling seasons or time points viz. PRM,POM,MON in the three study areas. Moreover the overall mean concentrations of aristolochic acid in the cereals & pulses food category was estimated from the average of the concentrations in its sub-types(for example average of the aristolochic acid levels in rice,wheat,ragi,green gram and chickpea sub-types were reported as mean aristolochic acid levels in the cereals & pulses category). Aristolochic acid levels were exclusively analysed in cereals & pulses food samples only as it is a contaminant of the same and does not occur in vegetables and fish. All the values are in µg/kg. The mean aristolochic acid levels were found to be well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established permissible limits and were similar [with lack of significant difference($p>0.05$)] in the cereals & pulses consumed by all three study areas suggesting a common source from where these cereals are procured in the three study areas. The details on the seasonal distribution of the aristolochic acid levels and the minimum to maximum range of aristolochic acid concentrations are listed in **Table 3.2.7**.

Table 3.2.7 : Mean and range concentrations (in µg/kg)of nephrotoxic phytotoxin-'aristolochic acid' during three different seasons in various cereals and pulses commonly consumed by study population of Canacona taluka and their comparison with the respective JECFA established permissible limits in food.

CKD status	CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				JCEFA established permissible limits (in µg/kg)
	Study region or group 1(N=114)				Study region or group 2(N=28)				Study region or group 3(N=124)				
Food category with sub-types/sampling time period	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	PRM	MON	POM	MEAN (of all seasons)	
Cereals and pulses (in µg/kg)													For Cereals and pulses = 1.5
Rice	0.41± 0.06	0.44± 0.07	0.42± 0.08	**0.42± 0.07**	0.39± 0.09	0.46± 0.04	0.44± 0.05	**0.43± 0.06**	0.42± 0.07	0.49± 0.03	0.44± 0.05	**0.45± 0.05**	
	(0.39-0.49)	(0.42-0.50)	(0.40-0.48)	**(0.39-0.50)**	(0.35-0.47)	(0.44-0.56)	(0.41-0.49)	**(0.35-0.56)**	(0.39-0.50)	(0.45-0.59)	(0.41-0.48)	**(0.39-0.59)**	
Wheat	0.43± 0.10	0.5± 0.04	0.45± 0.09	**0.46± 0.08**	0.42± 0.04	0.49± 0.11	0.44± 0.06	**0.45± 0.07**	0.44± 0.03	0.51± 0.08	0.49± 0.06	**0.48± 0.07**	
	(0.38-0.48)	(0.47-0.61)	(0.42-0.53)	**(0.38-0.61)**	(0.41-0.51)	(0.45-0.64)	(0.42-0.57)	**(0.41-0.64)**	(0.44-0.58)	(0.50-0.69)	(0.47-0.59)	**(0.44-0.69)**	
Green gram (Moong dal)	0.32± 0.06	0.39± 0.04	0.34± 0.05	**0.35± 0.05**	0.37± 0.09	0.43± 0.04	0.4± 0.08	**0.4± 0.07**	0.4± 0.04	0.44± 0.08	0.41± 0.05	**0.41± 0.06**	
	(0.31-0.38)	(0.35-0.49)	(0.31-0.40)	**(0.31-0.49)**	(0.34-0.47)	(0.42-0.55)	(0.39-0.51)	**(0.34-0.55)**	(0.38-0.45)	(0.42-0.53)	(0.39-0.48)	**(0.38-0.53)**	
Ragi	0.27± 0.09	0.34± 0.10	0.29± 0.04	**0.3± 0.08**	0.32± 0.08	0.39± 0.09	0.34± 0.07	**0.35± 0.08**	0.34± 0.05	0.39± 0.06	0.38± 0.09	**0.37± 0.07**	
	(0.25-0.33)	(0.31-0.43)	(0.27-0.35)	**(0.25-0.35)**	(0.30-0.41)	(0.36-0.49)	(0.33-0.44)	**(0.30-0.49)**	(0.27-0.39)	(0.37-0.53)	(0.35-0.50)	**(0.27-0.53)**	
Chickpea	0.38± 0.03	0.44± 0.10	0.41± 0.06	**0.41± 0.07**	0.4± 0.06	0.45± 0.04	0.41± 0.05	**0.42± 0.05**	0.41± 0.11	0.48± 0.09	0.43± 0.08	**0.44± 0.09**	
	(0.36-0.41)	(0.43-0.54)	(0.39-0.50)	**(0.36-0.54)**	(0.38-0.43)	(0.42-0.57)	(0.40-0.54)	**(0.38-0.57)**	(0.40-0.52)	(0.45-0.60)	(0.41-0.58)	**(0.40-0.60)**	
Average (from all cereal sub-types)				**0.39± 0.06**				**0.41± 0.08**				**0.43± 0.07**	
				(0.25-0.61)				**(0.30-0.64)**				**(0.27-0.69)**	

Abbreviations:- CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; PRM-pre-monsoon; MON-monsoon; POM-post-monsoons; JECFA- Joint FAO/WHO Expert Committee on Food Additives (JECFA). Data are derived from two independent experiments with aristolochic acid levels of an individual experiment measured in triplicates. 'N' in each study region depicts the number of subjects in that study region or group from which the different types of cereal food samples were obtained. Values are represented as mean±SE with the range of minimum to maximum concentrations (shown in parenthesis) of aristolochic acid estimated in each type of cereal over three different sampling time points viz. PRM, POM, MON in the three study groups or regions. The overall concentration of aristolochic acid in each type of cereals was calculated by taking the average of the concentrations estimated during the aforementioned time-points. Moreover the general mean concentrations of aristolochic acid in the cereals food category was estimated from the average of the concentrations in its sub-types. No statistically significant ($p>0.05$) differences were noted in the concentrations of aristolochic acid estimated in each cereal type between the three study regions/groups and were well below the JECFA established permissible limits.

Moreover mean AA levels (computed for a particular food type over 3 different seasons) in all cereals and pulses of study groups 1, 2, 3 were comparable and found to be 0.39, 0.41, 0.43 µg/kg with maximum values of 0.61, 0.64, 0.69µg/kg noted in wheat and minimum values of 0.25,0.30,0.27µg/kg noted in ragi respectively with a ranking order (in terms of AA level) of Wheat>Rice>Green gram>Chickpea>Ragi **(Figure 3.2.6 and Table 3.2.7)**. 100% of cereals & pulses in all study regions contained AA levels prevalent below JECFA permissible limits-1.5 µg/kg wherein 59, 63, 65% of samples were present in lowest range of 0.25-0.45 µg/kg and 41, 37, 35% of samples in higher but permissible range of 0.46-0.65 µg/kg in area 1, 2, 3 respectively. Comparable AA levels noted in cereals & pulses of all 3 study areas signifies a common food distribution source (Khlifa et al., 2012) i.e. healthy villages-Molorem and Endrem to entire studied areas of Canacona ascribed to farming prevalence in these villages (established in CKDu demographic survey-Chapter 2). These results were consistent with AA level ranges noted in similar cereals/pulses consumed in CKDu hit countries like Sri Lanka & Central America wherein no relation with observed nephrotoxicity was noted on chronic intake of these foods containing such non-toxic AA contents (Almaguer et al.,2014; Abiola, 2017; Correa-Rotter et al.,2014; García-Trabanino et al.,2015; McClean et al.,2012; Rajapakse et al., 2016; Wesseling et al.,2013). Thus indicating lack of contribution of prevalent dietary AA at JCEFA safe levels in Canacona's CKDu causation, backed by comparable AA levels noted in CKDu hit and healthy-regions.

3.2.2.3.2 Association between nephrotoxins in food samples via correlation assessments

The relationship between measured nephrotoxins in each food category were analysed by calculation of Pearson's correlation coefficients (r) with their respective p-values. The correlation results in each food category i.e. cereal/pulses or vegetables or fish between mean of all nephrotoxins (with each nephrotoxin mean calculated from average of levels present in sub-types of a given food category) estimated over 3 different seasons (i.e. PRM, MON, POM) in all 3 study groups/areas are presented in **Table 3.2.8.**The correlations in each food category (i.e. cereals & pulses or vegetables or fish) between various nephrotoxins were similar in all 3 study groups indicating a common origin of food sources (Bandara et al., 2010a; Jayatilake et al.,2013) in all 3 studied regions i.e. food is distributed from healthy control villages-Molorem and Endrem (study area3) that is occupationally dominated by farming& fishing to rest of the study regions. Moreover, seasonal trend did not effect correlations as analysed nephrotoxins levels varied to similar extents based on respective season (Chandrajith et al., 2011).

Table 3.2.8: Correlation assessments between the estimated mean levels of various nephrotoxins analysed over three different time-points/seasons in various food categories(i.e. cereals and pulses or vegetables or fish) regularly consumed by the study population of Canacona taluka for identification of an association (if any) between these nephrotoxins.

Type of Nephrotoxin	Study group (total number of subjects)	Food category	Correlation coefficients (r)																			
			Pb				Cd				As				Hg				Ochratoxin				Aristolochic acid			
			PRM	MON	POM	MEAN (from all seasons)	PRM	MON	POM	MEAN (from all seasons)	PRM	MON	POM	MEAN (from all seasons)	PRM	MON	POM	MEAN (from all seasons)	PRM	MON	POM	MEAN (from all seasons)	PRM	MON	POM	MEAN (from all seasons)
Pb	1	C and P	1	1	1	1																				
		V	1	1	1	1																				
		F	1	1	1	1																				
	2	C and P	1	1	1	1																				
		V	1	1	1	1																				
		F	1	1	1	1																				
	3	C and P	1	1	1	1																				
		V	1	1	1	1																				
		F	1	1	1	1																				
Cd	1	C and P	0.713	0.657	0.689	0.699	1	1	1	1																
		V	0.757	0.712	0.746	0.723	1	1	1	1																
		F	NC				NC																			
	2	C and P	0.671	0.614	0.623	0.637	1	1	1	1																
		V	0.781	0.748	0.752	0.767	1	1	1	1																
		F	NC				NC																			
	3	C and P	0.74	0.723	0.731	0.737	1	1	1	1																
		V	0.699	0.645	0.657	0.669	1	1	1	1																
		F	NC				NC																			
As	1	C and P	0.693	0.623	0.651	0.687	0.614	0.603	0.607	0.608	1	1	1	1												
		V	0.514	0.495	0.503	0.509	0.599	0.599	0.607	0.607	1	1	1	1												
		F	NC				NC				NC															
	2	C and P	0.713	0.698	0.701	0.704	0.703	0.689	0.693	0.695	1	1	1	1												
		V	0.617	0.608	0.612	0.612	0.625	0.613	0.618	0.622	1	1	1	1												
		F	NC				NC				NC															
	3	C and P	0.654	0.643	0.647	0.649	0.628	0.618	0.621	0.621	1	1	1	1												
		V	0.497	0.468	0.472	0.468	0.619	0.614	0.617	0.617	1	1	1	1												
		F	NC				NC				NC															
Hg	1	C and P	-0.015	-0.006	-0.008	-0.009	-0.011	-0.009	-0.01	-0.01	-0.012	-0.0071	-0.0078	-0.089	1	1	1	1								
		V	NC				NC				NC				NC											
		F	0.417	0.401	0.409	0.41	NC				NC				NC											
	2	C and P	-0.021	-0.015	-0.017	-0.019	-0.021	-0.015	-0.016	-0.018	-0.018	-0.014	-0.016	-0.017	1	1	1	1								
		V	NC				NC				NC				NC											
		F	0.457	0.421	0.436	0.446	NC				NC				NC											
	3	C and P	-0.02	-0.011	-0.009	-0.016	-0.018	-0.013	-0.015	-0.016	-0.017	-0.012	-0.014	-0.015	1	1	1	1								
		V	NC				NC				NC				NC											
		F	0.448	0.416	0.438	0.431	NC				NC				NC											
Ochratoxin	1	C and P	-0.008	-0.009	-0.002	-0.006	-0.007	-0.009	-0.008	-0.009	-0.009	-0.012	-0.01	-0.01	-0.008	-0.012	-0.007	-0.009	1	1	1	1				
		V	NC				NC				NC				NC				NC							
		F	NC				NC				NC				NC				NC							
	2	C and P	-0.01	-0.008	-0.006	-0.008	-0.005	-0.006	-0.004	-0.005	-0.013	-0.015	-0.011	-0.011	-0.009	-0.001	-0.009	-0.008	1	1	1	1				
		V	NC				NC				NC				NC				NC							
		F	NC				NC				NC				NC				NC							
	3	C and P	-0.012	-0.01	-0.013	-0.011	-0.012	-0.008	-0.007	-0.009	-0.011	-0.02	-0.01	-0.005	-0.013	-0.015	-0.012	-0.01	1	1	1	1				
		V	NC				NC				NC				NC				NC							
		F	NC				NC				NC				NC				NC							
Aristolochic acid	1	C and P	-0.0005	-0.0006	-0.0003	-0.0005	-0.0001	-0.0005	-0.0004	-0.0003	-0.0001	-0.0003	-0.0002	-0.0002	-0.0003	-0.0006	-0.0004	-0.0004	-0.55	-0.596	-0.555	-0.589	1	1	1	1
		V	NC				NC				NC				NC				NC				NC			
		F	NC				NC				NC				NC				NC				NC			
	2	C and P	-0.0007	-0.0008	-0.0005	-0.0006	-0.0003	-0.0006	-0.0008	-0.0006	-0.0004	-0.0006	-0.0005	-0.0005	-0.0005	-0.0008	-0.0003	-0.0006	-0.61	-0.064	-0.619	-0.619	1	1	1	1
		V	NC				NC				NC				NC				NC				NC			
		F	NC				NC				NC				NC				NC				NC			
	3	C and P	-0.0003	-0.0006	-0.0004	-0.0005	-0.0002	-0.0007	-0.0006	-0.0009	-0.001	-0.0008	-0.0009	-0.0009	-0.0004	-0.0006	-0.0004	-0.0005	-0.66	-0.678	-0.653	-0.659	1	1	1	1
		V	NC				NC				NC				NC				NC				NC			
		F	NC				NC				NC				NC				NC				NC			

Abbreviations: C and P- cereals and pulses; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; F- fish; PRM- pre-monsoon; MON-monsoon; POM-post-monsoons; NC:- not calculated correlation coefficient owing to the absence of one of the heavy metals in the food sample; V-vegetables. The total number of subjects in all the three study regions(viz. study region 1, 2 and 3) from which the various categories of food samples were procured was 114; 28 and 124 respectively. Values are represented as Pearson's correlation coefficients (r) with their respective p-values also calculated for identification of statistically significant associations (if any) present between any given two nephrotoxins. Differences at $p<0.05$ were considered to be statistically significant correlations, which are highlighted in bold. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, positive and statistically significant($p<0.05$) associations were noted between Pb and Cd, Cd and As and Pb and As present in the cereals, pulses and vegetables consumed by the study population signifying a common source of origin of these heavy metals in these two food categories. Similarly, statistically significant ($p<0.05$) positive correlations were noted between Pb and Hg in the fish consumed by the three study groups, denoting a common source of contamination of the fish with these heavy metals. For units of each individual nephrotoxin concentrations, kindly refer Tables 3.2.2 to 3.2.7 of this chapter.

In cereals and vegetables food categories (**Table 3.2.8)**, significant and positive mean correlations (r) of 0.699(p=0.083)/0.723(p=0.28); 0.637(p=0.5)/0.767(p=0.16) and 0.737 (p=0.066)/0.669(p=0.073) were found between Pb and Cd; of 0.608(p=0.16)/0.607(p=0.058); 0.695(p=0.51)/0.622(p=0.079) and 0.621(p=0.075)/0.617(p=0.064) between Cd and As and of 0.687(p=0.078)/0.509(p=0.156); 0.704(0.059)/0.612(0.057) and 0.649(p=0.071)/0.468 (p=0.125) between Pb and As in study groups 1,2,3 respectively suggesting a common origin source of these nephrotoxins measured in all cereals possibly emerging from heavy-metal enriched untreated industrial effluents discharge (from batteries, paint, smelting) into terrestrial environment that directly contaminates cereal & vegetable crops by direct metal uptake and accumulation from soil (Bandara et al.,2008; Bandara et al.,2010a; Jayatilake et al.,2013; Levine et al.,2016; Qureshi et al.,2016). Parallely in same abovesaid food categories, negative and non-significant (p>0.05) associations were noted between Hg and other heavy metals i.e Pb, Cd and As in each study group indicating different metals origin source with Pb, Cd and As possibly arising from industrial effluents and Hg from municipal sewage containing medical waste like dental amalgam/broken Hg-thermometers released into terrestrial environment (Almaguer et al.,2014; Abiola,2017; Barone et al.,2015;Correa-Rotter, 2017;Giri and Singh,2017;Levine et al.,2016;Siriwardhana et al.,2014;Wesseling et al.,2014).
Moreover in cereals and pulses food category, complete lack of relationships were noted between OTA or AA with heavy metals in each study groups owing to different origin source of these nephrotoxins with OTA and AA arising from natural polluting organism (i.e. fungal and plant sp. respectively) and heavy metals from anthropogenic activities like smelting, battery production etc. (Almaguer et al., 2014; Correa-Rotter, 2017; García-Trabanino et al., 2015; Ma et al., 2016a; McClean et al., 2012; Pepeljnjak and Segvkic Klanc, 2010; Pfohl-Leszkowicz,2009; Rajapakse et al., 2016; Wanigasuriya et al., 2008 Wesseling et al., 2013).
Furthermore, although OTA and AA are major cereal contaminants, insignificant & negative correlations(p>0.05) were noted between them signifying varied sources of these nephrotoxin with former arising from fungus & latter from plant (Almaguer et al.,2014; Correa-Rotter et al., 2014;García-Trabanino et al.,2015;McClean et al.,2012;Wesseling et al., 2013).
In fish category, strong and significant (p<0.05) correlations (r) of 0.41(p=0.069), 0.446 (p=0.078) and 0.431(p=0.096) between Pb and Hg in study groups 1,2,3 respectively were noted between Pb and Hg, signifying a common nephrotoxin origin source, possibly arising from discharge of Pb and Hg enriched untreated effluents from fossil fuel industries, paper/mining industries etc. into aquatic ecosystems that detoriates inhabiting marine life on metal uptake and bioaccumulation. Moreover, lack of correlations were noted between Pb &

As, Pb and Cd, As and Cd, As &Hg, Cd and Hg in all groups owing to absence of significant detectable As and Cd levels in fish owing to lack of Cd or As contaminating source (McClean et al.,2012; Javed and Usmani,2016; Jayatilake et al.,2013; Wanigasuriya et al.,2011).

3.2.2.3.3 Human health risk (specifically nephrotoxicity) assessment

3.2.2.3.3.1 Estimated daily intake (EDI) of various nephrotoxins in the commonly consumed foodstuffs of the study population

Although the levels of analysed nephrotoxin in various commonly consumed foods in Canacona did not surpass JCEFA regulatory limits, toxic potential of these nephrotoxins cannot be ignored as it depends on exposure dosages. Thus JECFA has formulated a standard index called estimated daily intake (EDI) that enables determination of renal toxicity risk by estimation of daily dietary intake (through various foods) of targeted nephrotoxins. EDI is an accurate indicator of nephrotoxin transfer from food crops (cereals, vegetables) and animals (fish) in terrestrial and aquatic ecosystem respectively to humans in order to assess extent of exposure of the latter to specific nephrotoxins and risk of developing nephrotoxicity in analysed study population (Ahmed et al.,2015; Islam et al.,2015; Javed and Usmani,2016; Jelaković et al.,2015; Oueslati et al.,2018; Shaheen et al.,2016). EDI is dependent on nephrotoxin concentration in food and daily rate of contaminated food consumption. The food's nephotoxin concentration is dependent on biological and environmental conditions such as nephrotoxin levels in aquatic and terrestrial environments, feeding habits, degree of accumulation in food chain members, food chain position (with top orders possessing larger nephrotoxins levels), age etc. Population eating habits (such as food types consumed, daily intake rate [in g/day] of various foods) also influences nephrotoxin uptake or exposure dosage rate in humans.These eating habits vary within population, hence a demographic survey of daily consumption rate of each food type (in g/day) of the study population is mandatory for EDI calculation,hence determined in current study (described in section 3.2.2.2). Body weight of targeted human being can influence tolerance to nephrotoxins. In this study, nephrotoxin EDI was calculated according to JECFA guidelines & using Goa's food legislative authorities stated daily intake rate (in g/day) for each food type as described in Materials and methods (Ahmed et al.,2015; BorteySam et al.,2015; Chammanejadian et al.,2013; Chandrajith et al.,2011; Nuapia et al., 2018; Shaheen et al., 2016; Ullah et al. 2017). The computed EDI was validated by comparison with JCEFA formulated nephrotoxin's daily intake reference limit called tolerable daily intake (TDI), used for chronic diseases analysis. TDI was designed to show safe exposure levels that do not induce significant nephrotoxicity

and used to estimate nephrotoxin amount that can be ingested over a lifetime from food without appreciable risk of developing expected health effect i.e.Chronic tubulointerstitial nephritis (Abdullah et al.,2017; Chen et al.,2018; Mitchell et al.,2017; Giri and Singh,2017).
EDI values for all nephrotoxins (from each food type intake) in comparison with their respective TDI are represented in **Figure 3.2.7 & Table 3.2.9.**EDI values of all nephrotoxins (Pb, Cd, As, Hg, OTA, AA) in all 3 study regions were comparable signifying a common source of distribution of these food s (Bandara et al.,2010a; Chandrajith et al.,2011; Levine et al., 2016; Klifa et al.,2012) i.e. from healthy villages-Molorem and Endrem to all studied areas of Canacona owing to occupational dominance of farming and fishing in these villages.

3.2.2.3.3.1.1 EDI of lead in the study population

As indicated in **Figure 3.2.7A & Table 3.2.9**, mean EDI values of lead from cereals were comparable in study groups1, 2, 3 & determined to be 0.104, 0.096, 0.109 µg/day kg bw with a maximum intake of 0.125, 0.138, 0.154 µg/day kg bw noted from wheat and a minimum intake of 0.083, 0.070 and 0.072 µg/day kg bw noted from ragi respectively. No intake was noted from staple food crop i.e.rice.100% of all cereals and pulses analysed in all 3 study areas displayed EDI values to be prevalent below JECFA set TDI limits of 3.6 µg/day kg bw. From this, 65, 72, 68% of samples and remaining 35, 28, 32% were present in corresponding lowest range of 0.05-0.10 & higher range of 0.10-0.20 µg/day kgbw for area 1,2,3 respectively. The mean lead intake from cereal consumption in groups 1,2, 3 were found to be 0.104, 0.096,0.109 µg/day kg bw respectively which was below JECFA TDI of 3.6 µg/day kgbw (Chammanejadian et al.,2013; Chen et al.., 2018; Islam et al.,2015) with minimal contribution of 2.8, 2.6, 3.0% to TDI from these respective groups. In vegetables analysed, mean EDI values of lead were similar in study groups 1, 2, 3 and estimated to be 0.207, 0.22, 0.206 µg/day kg bw respectively with maximum intake of 0.259, 0.269, 0.262 µg/day kg bw from spinach and minimum intake of 0.14, 0.17, 0.16 µg/day kg bw from brinjal respectively. No intake noted from majorly consumed vegetables i.e. red & green amaranthus. 100% of vegetables in all regions displayed EDI values to be prevalent below JECFA set TDI limits of 3.6 µg/day kg bw wherein 71, 74, 79% of samples and residual 29, 26, 21% were present in their corresponding lowest range of 0.15-0.20 and higher ranges of 0.20-0.30 µg/day kg bw for area 1, 2, 3 respectively. Mean lead intake from vegetable consumption in groups 1,2,3 were reported to be 0.207, 0.22, 0.206 µg/day kg bw respectively which was below TDI of 3.6µg/day kg bw (Islam et al., 2015; Qureshi et al.,2016; Shaheen et al.,2016) with a minimal contribution of 5.7%, 6.1%, 5.8% to TDI from each respective groups.

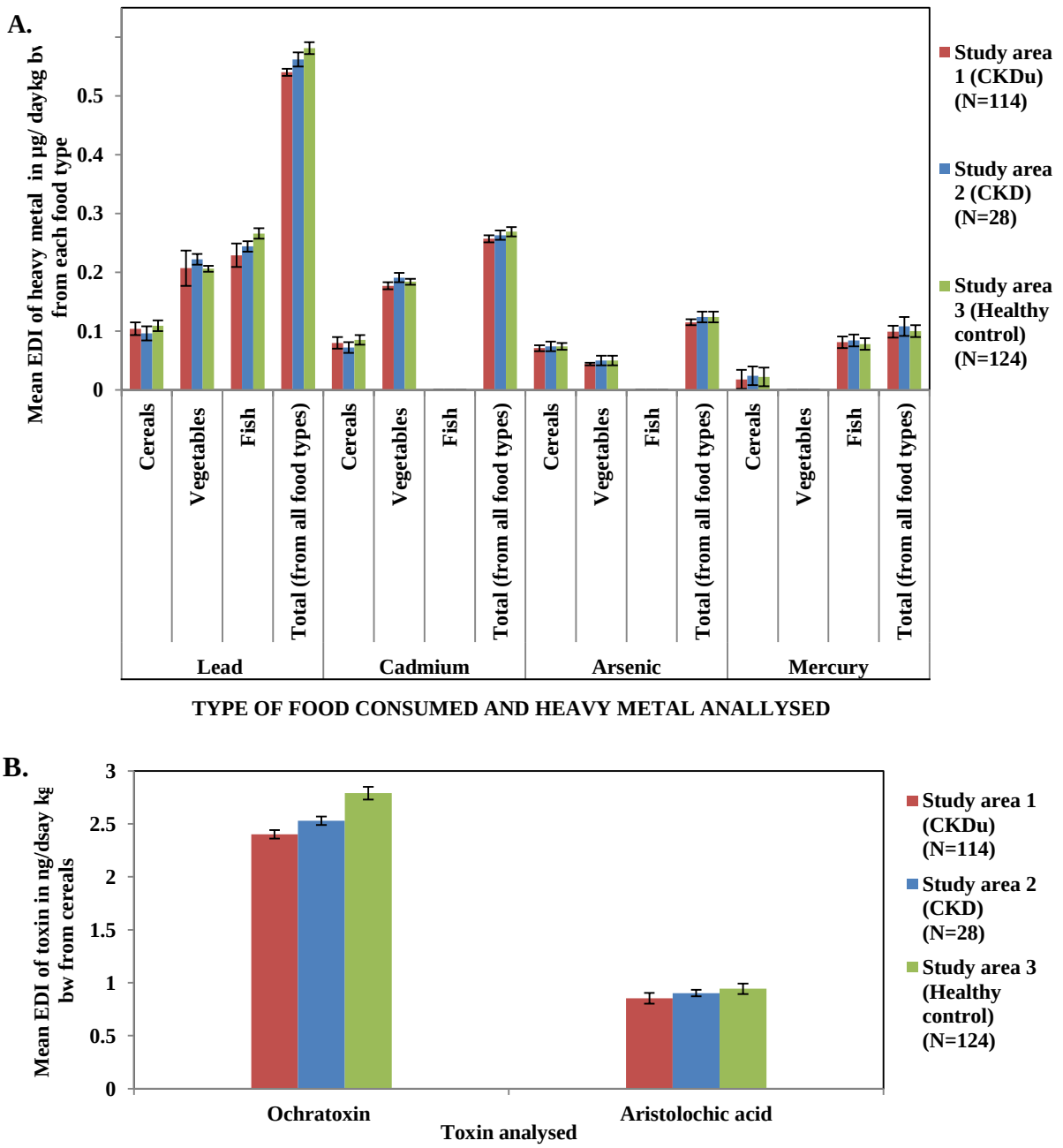

Figure 3.2.7. Mean estimated daily intake (EDI) of various nephrotoxins from consumption of three main food categories(viz. cereals & pulses,vegetables and fish) by the study population of Canacona

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; EDI-estimated daily intake. 'N' in each study area depicts the number of subjects in that study area or group from which samples of each sub-type of the three food categories viz. cereals& pulses, vegetables and fish were collected.(A)Mean estimated daily intake(EDI) levels of heavy metals viz.lead,cadmium,arsenic and mercury from consumption of three categories of food i.e. cereals & pulses,vegetables and fish.(B) Mean EDI levels of ochratoxin and aristolochic acid from consumption of cereals. Values of an individual nephrotoxin are represented as mean±SE of the intake levels in each food category (i.e. cereals & pulses,vegetables and fish) with mean calculated from the average of the intake levels from consumption of respective sub-types of a given food category in the three study areas(for example,mean intake of lead from cereals & pulses was obtained from the average of the lead intake levels from wheat,green gram,ragi and chickpea).The total(cumulative intake) of an individual heavy metal from food is also depicted(A) and was estimated from summation of the individual average intake levels from each of the three food categories[i.e.Total EDI of heavy metal(from all food types)= mean EDI (from cereals or pulses), mean EDI (from vegetables) and mean EDI (from fish)].The total intake of ochratoxin and aristolochic acid was obtained from consumption of cereals & pulses exclusively(B) as they are contaminants of only the same. The EDI levels for the heavy metals viz. lead(Pb), cadmium(Cd), arsenic(As) and mercury(Hg) are indicated in µg/ day kg bw and for ochratoxin and aristolochic acid in ng/ day kg bw. The JECFA established tolerable daily intake(TDI) levels for lead=3.6µg/ day kg bw; cadmium=1µg/ day kg bw; arsenic=2.2µg/ day kg bw and mercury=0.5µg/ day kg bw; ochratoxin= 14ng/ day kg bw; aristolochic acid=3ng/ day kg bw.No intake levels of mercury from vegetables & cadmium and arsenic from fish was noted,hence are not represented by bars.The mean intake levels of each individual nephrotoxin from consumption of each food category and total intake levels of the same from all food were found to be well below the JECFA established TDI limits and were comparable [with no significant difference($p>0.05$)] in all three study areas suggesting lack of hazardous exposure levels to these nephrotoxins via the food exposure route in the CKDu endemic region of Canacona due to occurrence of JECFA established safe levels of toxins in the food.The details of the mean intake levels of the individual nephrotoxin from each sub-type of a given food category(viz.cereals & pulses,vegetables and fish) and the range of minimum to maximum intake levels in each sub-type,broad food categories and cumulatively from all food are listed in **Table 3.2.9**.

Table 3.2.9: Mean and range of estimated daily intake (EDI) of various nephrotoxins from the consumption of different food categories by the study population of Canacona taluka and their comparison with the respective JECFA recommended tolerable daily intake (TDI) allowances

Type of Nephrotoxin	Study group (total number of subjects)/ Food category with their sub-types	EDI of nephrotoxin from Cereals and pulses						EDI of nephrotoxin form Vegetables						EDI of nephrotoxin from Fish			Total EDI of a nephrotoxin from all possible food source categories [Total EDI = mean EDI(cereals)+ mean EDI(vegetables)+ mean EDI(fish)]	JECFA-Tolerable daily intake (TDI) of the nephrotoxin from all food source categories
		Rice	Wheat	Green gram (Moong dal)	Ragi	Chickpea	*Mean EDI (from all cereals)*	Red amaranthus	Green amaranthus	Spinach	Pumpkin	Brinjal	*Mean EDI (from all vegetables)*	Mackerel	Sardine	*Mean EDI (from all fish)*		
Heavy metals (in µg/ day kg bw)																		
Pb	**1**	NI	0.118± 0.016	0.103± 0.010	0.085± 0.009	0.113± 0.010	0.104± 0.011	NI	NI	0.228± 0.011	0.208± 0.012	0.187± 0.015	0.207± 0.012	0.239± 0.010	0.219± 0.009	0.229± 0.009	0.54± 0.009	**Pb= 3.6µg/ day kg bw**
	(N=114)	-	(0.092-0.134)	(0.083-0.121)	(0.063-0.099)	(0.085-0.125)	(0.083-0.125)	-	-	(0.201-0.259)	(0.177-0.242)	(0.14-0.228)	(0.14-0.259)	(0.227-0.266)	(0.199-0.231)	(0.199-0.266)	(0.422-0.65)	
	2	NI	0.114± 0.012	0.09± 0.009	0.074± 0.008	0.107± 0.005	0.096± 0.008	NI	NI	0.239± 0.010	0.222± 0.009	0.205± 0.007	0.222± 0.009	0.256± 0.005	0.232± 0.006	0.244± 0.005	0.562± 0.0047	
	(N=28)	-	(0.088-0.136)	(0.070-0.11)	(0.066-0.094)	(0.077-0.138)	(0.070-0.138)	-	-	(0.208-0.269)	(0.194-0.246)	(0.177-0.225)	(0.177-0.269)	(0.242-0.275)	(0.215-0.250)	(0.215-0.275)	(0.462-0.682)	
	3	NI	0.125± 0.009	0.105± 0.010	0.094± 0.007	0.114± 0.006	0.109± 0.009	NI	NI	0.235± 0.010	0.201± 0.008	0.184± 0.009	0.206± 0.009	0.296± 0.008	0.237± 0.010	0.266± 0.009	0.581± 0.009	
	(N=124)	-	(0.09-0.154)	(0.072-0.13)	(0.072-0.103)	(0.092-0.136)	(0.072-0.154)	-	-	(0.198-0.252)	(0.170-0.232)	(0.167-0.215)	(0.167-0.252)	(0.286-0.307)	(0.229-0.256)	(0.229-0.307)	(0.468-0.713)	
Cd	**1**	NI	NI	0.094± 0.005	0.07± 0.004	0.077± 0.009	0.08± 0.006	NI	NI	NI	0.187± 0.011	0.167± 0.013	0.177± 0.012	NI	NI	NI	0.257± 0.010	**Cd= 1µg/ day kg bw**
	(N=114)	-	-	(0.079-0.11)	(0.059-0.085)	(0.068-0.088)	(0.059-0.088)	-	-	-	(0.167-0.208)	(0.143-0.191)	(0.143-0.208)	-	-	-	(0.202-0.296)	
	2	NI	NI	0.085± 0.004	0.052± 0.007	0.079± 0.009	0.072± 0.010	NI	NI	NI	0.202± 0.009	0.181± 0.010	0.191± 0.009	NI	NI	NI	0.263± 0.008	
	(N=28)	-	-	(0.070-0.101)	(0.044-0.074)	(0.063-0.090)	(0.044-0.101)	-	-	-	(0.177-0.225)	(0.16-0.201)	(0.16-0.225)	-	-	-	(0.204-0.326)	
	3	NI	NI	0.101± 0.005	0.068± 0.007	0.088± 0.006	0.085± 0.006	NI	NI	NI	0.194± 0.007	0.174± 0.010	0.184± 0.008	NI	NI	NI	0.269± 0.005	
	(N=124)	-	-	(0.085-0.112)	(0.057-0.081)	(0.079-0.101)	(0.057-0.112)	-	-	-	(0.174-0.211)	(0.15-0.194)	(0.15-0.211)	-	-		(0.207-0.323)	
As	**1**	NI	NI	NI	0.077± 0.007	0.064± 0.010	0.071± 0.008	NI	NI	NI	0.051± 0.005	0.037± 0.008	0.044± 0.006	NI	NI	NI	0.115± 0.007	**As= 2.2 µg/ day kg bw**
	(N=114)	-	-	-	(0.063-0.079)	(0.05-0.077)	(0.05-0.079)	-	-	-	(0.030-0.064)	(0.017-0.051)	(0.017-0.064)	-	-	-	(0.067-0.143)	
	2	NI	NI	NI	0.083± 0.008	0.074± 0.005	0.078± 0.006	NI	NI	NI	0.068± 0.007	0.051± 0.009	0.059± 0.008	NI	NI	NI	0.137± 0.008	
	(N=28)	-	-	-	(0.072-0.088)	(0.063-0.088)	(0.063-0.088)	-	-	-	(0.047-0.085)	(0.034-0.061)	(0.034-0.085)	-	-	-	(0.097-0.173)	
	3	NI	NI	NI	0.081± 0.004	0.068± 0.008	0.074± 0.005	NI	NI	NI	0.058± 0.006	0.041± 0.009	0.05± 0.008	NI	NI	NI	0.124± 0.006	
	(N=124)	-	-	-	(0.068-0.083)	(0.059-0.083)	(0.059-0.083)	-	-	-	(0.037-0.078)	(0.023-0.058)	(0.023-0.078)	-	-	-	(0.082-0.161)	
Hg	**1**	NI	NI	NI	0.018± 0.002	NI	0.018± 0.002	NI	NI	NI	NI	NI	NI	0.083± 0.007	0.079± 0.009	0.081± 0.008	0.099± 0.006	**Hg= 0.5µg/ day kg bw**
	(N=114)	-	-	-	(0.008-0.026)	-	(0.008-0.026)	-	-	-	-	-	-	(0.079-0.088)	(0.068-0.078)	(0.068-0.088)	(0.076-0.114)	
	2	NI	NI	NI	0.024± 0.008	NI	0.024± 0.008	NI	NI	NI	NI	NI	NI	0.086± 0.005	0.082± 0.003	0.084± 0.004	0.108± 0.005	
	(N=28)	-	-	-	(0.017-0.030)	-	(0.017-0.030)	-	-	-	-	-	-	(0.081-0.087)	(0.072-0.080)	(0.072-0.087)	(0.089-0.117)	
	3	NI	NI	NI	0.022± 0.005	NI	0.022± 0.005	NI	NI	NI	NI	NI	NI	0.08± 0.010	0.076± 0.008	0.078± 0.009	0.1± 0.007	
	(N=124)	-	-	-	(0.011-0.028)	-	(0.011-0.028)	-	-	-	-	-	-	(0.074-0.085)	(0.066-0.077)	(0.066-0.085)	(0.077-0.113)	
Ochratoxin (in ng/ day kg bw)	**1**	3.036± 0.016	3.322± 0.018	2.288± 0.022	1.298± 0.015	2.09± 0.017	2.4± 0.016	NA(Not applicable) for EDI calculations as ochratoxin is not a contaminant of vegetables						NA (Not applicable) for EDI calculations as ochratoxin is not a contaminant of fish			2.4± 0.016	**Ochratoxin= 14 ng/ day kg bw**
	(N=114)	(2.684-3.41)	(2.64-4.20)	(1.98-2.75)	(0.81-1.93)	(1.95-2.53)	(0.81-3.41)										(0.81-3.41)	
	2	2.662± 0.019	3.564± 0.009	2.464± 0.017	1.694± 0.018	2.266± 0.011	2.53± 0.017										2.53± 0.017	
	(N=28)	(2.42-3.278)	(3.12-4.29)	(2.26-2.86)	(1.34-2.22)	(2.02-2.66)	(1.34-4.29)										(2.02-4.29)	
	3	3.168± 0.015	4.158± 0.014	2.574± 0.012	1.848± 0.009	2.222± 0.011	2.79± 0.012										2.79± 0.012	
	(N=124)	(2.90-3.52)	(3.74-4.46)	(2.31-2.79)	(0.83-2.39)	(2.09-2.70)	(0.83-4.46)										(0.81-4.46)	
Aristolochic acid (in ng/ day kg bw)	**1**	0.924± 0.018	1.012± 0.011	0.77± 0.010	0.66± 0.009	0.902± 0.015	0.853± 0.012	NA(Not applicable) for EDI calculations as aristolochic acid is not a contaminant of vegetables						NA(Not applicable) for EDI calculations as aristolochic acid is not a contaminant of fish			0.853± 0.012	**Aristolochic acid = 3 ng/day kg bw**
	(N=114)	(0.836-1.342)	(0.836-1.34)	(0.682-1.07)	(0.55-0.77)	(0.55-0.77)	(0.55-1.34)										(0.55-1.34)	
	2	0.946± 0.006	0.99± 0.007	0.88± 0.011	0.77± 0.012	0.924± 0.009	0.902± 0.011										0.902± 0.011	
	(N=28)	(0.90-1.408)	(0.902-1.41)	(0.74-1.21)	(0.66-1.07)	(0.66-1.07)	(0.66-1.41)										(0.66-1.41)	
	3	0.975± 0.009	1.056± 0.007	0.902± 0.003	0.814± 0.010	0.968± 0.008	0.943± 0.008										0.943± 0.008	
	(N=124)	(0.968-1.518)	(0.968-1.49)	(0.83-1.156)	(0.59-1.16)	(0.59-1.06)	(0.59-1.51)										(0.59-1.518)	

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; JECFA- Joint FAO/WHO Expert Committee on Food Additives (JECFA); NI-no intake; NA-not applicable for EDI calculation owing to its absence in the food sample. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured. Values are represented as mean±SE with the range of minimum to maximum daily intake levels (shown in parenthesis) of the nephrotoxin estimated in each food sample sub-type (of the three respective food categories) in the three study groups or regions. The EDI levels for the heavy metals viz. lead(Pb), cadmium(Cd), arsenic(As) and mercury(Hg) are indicated in µg/ day kg bw and for ochratoxin and aristolochic acid in ng/ day kg bw. The average concentrations of an individual nephrotoxin(refer Tables 3.2.2 to 3.2.7) in a particular sub-type of the respective food categories (i.e. cereals and pulses or vegetables or fish) estimated over three different sampling time points i.e. pre-monsoon, monsoon and post-monsoon were used for EDI calculations of the nephrotoxin. The EDI was calculated by considering the state's Food legislative authorities viz. Directorate of Agriculture and Directorate of Fisheries (2016) established average rates of daily consumption (in g/day) of each food category (i.e. cereals and pulses or vegetables or fish) for an average person bearing a body weight of 60kg (applicable for the study population of Canacona taluka as well). The state's Food legislative authorities established average daily consumption of cereals and pulses, vegetables and fish were reported to be 132g, 205g, and 65 g respectively. The general EDI of a nephrotoxin from each food category (i.e. cereals and pulses or vegetables or fish) was estimated by taking the average of the EDI's from their respective food sub-types. Thereafter, the total EDI of a nephrotoxin from consumption of all possible food categories in the study population was obtained by the summation of the nephrotoxin's average EDI from each individual food category [i.e. mean EDI (cereals or pulses), mean EDI (vegetables) and mean EDI (fish)], which was then compared with the JECFA established tolerable dietary intake (TDI) levels for assessment of danger of developing renal disease. No statistically significant ($p>0.05$) differences were noted in total estimated daily intake(EDI) levels of each nephrotoxin between three study groups or regions & were well below the JECFA TDI limits

In fish analysed, mean lead EDI values were comparable in study groups 1, 2, 3 **(Figure 3.2.7A & Table 3.2.9)** which were estimated to be 0.22,0.24,0.26 µg/day kg bw with highest intake of 0.266, 0.275, 0.307 µg/day kg bw reported from mackerel and lowest intake of 0.199, 0.215, 0.229 µg/day kg bw reported from sardine respectively.100% of all fish in all 3 study areas displayed EDI values to be below JECFA set TDI limits of 3.6 µg/day kg bw (Islam et al.,2015; Javed and Usmani,2016; Ullah et al.,2017) wherein 78, 83, 74% of samples were present in their respective lowest range of 0.15-0.25µg/day kg bw and remaining 22, 17, 26% in upper range of 0.25-0.35 µg/day kgbw for area 1, 2, 3 respectively. Average lead intake from fish in groups 1, 2, 3 were noted as 0.266, 0.275, 0.307 µg/day kg bw which was below TDI of 3.6 µg/day kg bw with a marginal contribution of 7.3%, 7.6%, 8.5% to TDI from each respective groups.

Overall, total cumulative lead intake (total EDI) in Canacona from all foods (calculated from sum of all EDI values from cereals, vegetables, fish) were comparable in all 3 study areas and estimated to be 0.54, 0.56, 0.58 µg/day kg bw contributing to 14.9, 15.45, 15.8% of TDI limit (of 3.6 µg/day kg bw) respectively; which were well below JECFA set safe TDI levels. Maximum but safe Pb intake in Canacona was noted from fish consumption and minimum intake from cereals ingestion being comparable in all 3 groups but below JECFA set safe TDI limits. These results were consistent with similar lead intakes noted from consumption of homologous foods (cereals, vegetables and fish) in CKDu hit countries like Sri Lanka and Central America wherein no significant link to observed renal toxicity was noted at such Pb ingestion levels (Banadara et al.,2008; Ramirez-Rubio et al.,2013; Gunatilake et al., 2015; Levine et al., 2016; Jayatilake et al.,2016; Saha et al.,2016; Wanigasuriya et al., 2011; Wimalawansa,2016; Wesseling et al., 2013). Thus indicating lack of contribution of prevalent dietary Pb exposure at safe intake levels (through food consumption) in Canacona's CKDu development, backed by similar Pb intake levels noted in CKDu hit and healthy regions.

3.2.2.3.3.1.2 EDI of cadmium in the study population

As indicated in **Figure 3.2.7A & Table 3.2.9**, mean Cd EDI values from cereals were similar in groups 1, 2, 3 and determined to be 0.08, 0.072, 0.085 µg/day kg bw with highest intake of 0.088, 0.10, 0.112 µg/day kg bw noted from green gram (moong dal) and lowest intake of 0.059, 0.044, 0.057 µg/day kg bw noted from ragi respectively. No intake was noted from taluka's two major staple food crops i.e. rice and wheat.100% of all cereals & pulses in all 3 study regions displayed EDI values to be prevalent below JECFA set TDI limit of 1.0 µg/day kg bw (Chammanejadian et al.,2013; Chen et al.,2018; Islam et al.,2015) wherein 61, 68,

63% of cereal & pulses were prevalent in their respective lowest range of 0.05-0.10 μg/day kg bw and 39, 32, 37% of pulses were present in higher ranges of 0.101-0.12 μg/day kg bw for areas 1, 2 ,3 respectively. Mean Cd intake from cereals intake in groups 1, 2, 3 were reported 0.088, 0.10, 0.112 μg/day kg bw respectively which was below TDI of 1.0 μg/day kg bw with minimal contribution of 8.8%, 10%, 11.2% to TDI from each respective groups.

In vegetables analysed, mean Cd EDI values were similar in groups 1, 2, 3 and ranged from 0.177, 0.191, 0.184 μg/day kg bw with maximum intake of 0.208, 0.225, 0.211 μg/day kg bw reported from pumpkin & minimum intake of 0.14,0.16 and 0.15 μg/day kg bw reported from brinjal respectively. No intake noted from majorly consumed vegetables i.e. red cum green and amaranthus and spinach.100% of all vegetable in all 3 study areas displayed EDI values to be prevalent below JECFA set permissible TDI of 1.0 μg/day kg bw (Islam et al., 2015; Qureshi et al., 2016; Shaheen et al., 2016) wherein 63,67, 74% of samples were present in lowest range of 0.15-0.18 μg/day kg bw and residual 11, 14, 9% in higher ranges of 0.18-0.22 μg/day kg bw for areas 1, 2, 3 respectively. Average Cd intake from vegetable consumption in groups 1, 2, 3 were noted as 0.177, 0.191, 0.184 μg/day kg bw which was below TDI of 1.0μg/day kg bw with minimal contribution of 17.7, 19.1, 18.4% to TDI from each respective groups. No Cd intake was noted from fish intake as BDL of the same was detected in them negating its contribution to Cd's total dietary intake. Overall, total cumulative Cd intake (total EDI) from all food sources(i.e. sum of EDI's from cereals, pulses and vegetables) in Canacona were comparable in all three study groups and estimated to be 0.257,0.263,0.269 μg/day kg bw respectively contributing to 25.7, 26.3, 26.9% of TDI (of 3.6 μg/day kg bw) respectively; being well below JECFA set safe Cd TDI (Barone et al., 2015; Islam et al., 2015; Ullah et al. 2017).Maximum but safe Cd intake in Canacona was noted from vegetable consumption and minimum intake from cereals ingestion being comparable in all 3 groups but below JECFA set safe TDI limits. These results were in agreement with similar Cd intakes noted from ingestion of similar foods (cereals, vegetables) in CKDu hit countries like Sri Lanka and Central America wherein no significant relation to noted nephrotoxicity was noted at such Cd ingestion levels (Almaguer et al., 2014; Chandrajith et al., 2011; McClean et al., 2012; Levine et al., 2016; Siriwardhana et al.,2014; Wesseling et al., 2013). Thus indicating that prevalent Cd intakes (through food consumption) in Canacona do not significantly contribute to its CKDu causation backed by similar Cd intake levels noted in CKDu hit and healthy regions and presence at JCEFA safe intake levels.

3.2.2.3.3.1.3 EDI of arsenic in the study population

As indicated in **Figure 3.2.7A & Table 3.2.9,** mean arsenic EDI values from cereals were comparable in groups 1, 2, 3 and noted to be 0.071,0.078 and0.074 μg/day kg bw with a maximum intake of 0.079, 0.088, 0.073 μg/day kg bw noted from ragi and lowest intake of 0.05, 0.063, 0.059 μg/day kg bw noted from chickpea respectively. No intake was noted from taluka's staple foods i.e. rice, wheat, green gram.100% of all cereals & pulses in all regions displayed EDI to be prevalent below TDI limits of 2.2 μg/day kgbw (Chammanejadian et al., 2013; Chen et al.,2018; Islam et al.,2015) wherein 54, 59, 62% of samples were present in their lowest range of 0.05-0.07μg/day kg bw and residual 46, 41, 38% in upper range of 0.07-0.09μg/day kg bw for areas 1, 2, 3 respectively. Mean As intake from cereals in groups 1,2,3 were noted as 0.071, 0.078, 0.074 μg/day kg bw respectively which was below TDI of 2.2 μg/day kg bw with minimal contribution of 3.2,3.5,3.3% to TDI from each respective groups. In vegetables analysed, mean As EDI values were similar in groups 1, 2, 3 &estimated to be 0.044, 0.059, 0.05 with highest intake of 0.064, 0.085, 0.078 μg/day kg bw from pumpkin and lowest intake of 0.017, 0.034, 0.023 μg/day kg bw from brinjal respectively. No intake was noted from majorly consumed vegetables i.e. red cum green amaranthus, spinach. 100% of all vegetables in all 3 study areas displayed EDI values to be prevalent below JECFA set TDI limit of 2.2 μg/day kg bw (Islam et al.,2015; Qureshi et al.,2016; Shaheen et al.,2016) wherein 65, 59, 60% of samples were present in lowest range of 0.01-0.05 μg/day kg bw and residual 35, 41, 40% in upper ranges of 0.05-0.08 μg/day kg bw for study area1, 2, 3 respectively. Mean As intake from vegetable consumption in groups 1, 2, 3 were found to be 0.044, 0.059, 0.05μg/day kg bw respectively which was below safe TDI of 2.2 μg/day kg bw with a minimal contribution of 2, 2.6, 2.2% to TDI from each respective groups. No daily As intake was observed from fish consumption as BDL of the same were detected in them thereby negating its contribution to total As dietary intake**.** Overall,total cumulative As intake (total EDI) from all food sources (i.e. sum of all EDIs from cereals, pulses, vegetables) were comparable in all 3 study groups and estimated to be 0.11,0.13,0.12 μg/day kg bw contributing to 5, 5.9, 5.4% of TDI limit (2.2 μg/day kg bw) respectively; which were below JECFA set safe TDI (Javed and Usmani,2016;Ullah et al. 2017). Higher but safe As intake was noted from cereal consumption and lowest intake from vegetable ingestion, being comparable in all 3 groups but below JECFA set safe TDI. These results were in agreement with comparable As intakes noted from consumption of similar foods (cereals, fish) in CKDu hit countries like Sri Lanka wherein no significant link to noted nephrotoxicity was found at such As intake levels (Bandara et al.,2008;Bandara et al., 2010a;Chandrajith et al.,2011;

Levine et al.,2016; Jayatilake et al.,2013; Rajapakse et al.,2016;Siriwardhana et al.,2014; Wanigasuriya et al.,2011). Thus signifying that prevalent As intakes (through food ingestion) in Canacona do not significantly contribute to its CKDu causation, backed by similar As intake noted in CKDu and healthy areas and presence at JCEFA safe intake levels.

3.2.2.3.3.1.4 EDI of mercury in the study population

As indicated in **Figure 3.2.7A & Table 3.2.9,** the mean Hg daily intake from cereals was solely contributed from ragi food type. EDI values from ragi cereal were found to be comparable within all 3 groups and below JECFA permissible TDI(0.5µg/day kg).EDI values from ragi consumption in groups 1, 2, 3 were noted to be 0.018, 0.024, 0.022 µg/daykg bw which minimally contributed to 3.6, 4.8, 4.4% of TDI respectively. 100% of all ragi cereal samples in all study groups displayed EDI values at levels below JECFA set TDI limits (Chammanejadian et al., 2013; Chen et al.., 2018; Islam et al., 2015) wherein 67, 63, 70% of samples were present in lowest range of 0.005-0.020 µg/day kg bw and remaining 33, 37, 30% in higher ranges of 0.020-0.030 µg/day kg for respective areas1, 2, 3. No intake was noted from taluka's other consumed staple food crops i.e. rice, wheat, green gram, chickpea. Similarly, complete lack of contribution in Hg's dietary intake from vegetables was noted due to BDL of Hg detected in these samples. In fish samples analysed, Hg EDI values were comparable in groups 1, 2, 3 and ranged from 0.081, 0.084, 0.088µg/day kg bw with highest intake of 0.088, 0.087, 0.085 µg/day kg bw reported from mackerel and lowest intake of 0.068, 0.072, 0.066 µg/day kg bw from sardines respectively. 100% of all fish in all 3 study areas displayed EDI values at levels below JECFA set TDI limit of 0.5 µg/day kg bw (Barone et al., 2015; Islam et al., 2015; Ullah et al. 2017) wherein 72, 76, 71% of samples were present in lowest range of 0.06-0.08 µg/day kg bw and remaining 28, 24, 29% in higher ranges of 0.08-0.09 µg/day kg bw for respective study areas 1, 2, 3.Mean Hg intake from fish consumption in study groups 1, 2, 3 were noted to be 0.081, 0.084, 0.088µg/day kg bw respectively which was below safe TDI levels of 0.5 µg/day kg bw with minimal contribution of 16.2, 16.8, 17.6% to TDI from each respective groups. Overall, total cumulative Hg intake (total EDI) from all food sources (i.e. sum of all EDIs from cereals, pulses, vegetables) were comparable in all 3 study groups and estimated to be 0.099, 0.108, 0.10µg/day kg bw contributing to19.8, 21.6 and 20% of TDI limit (0.5 µg/day kg bw) respectively; which were below JECFA set safe TDI level. Maximum but safe Hg intake was noted from fish ingestion & lowest intake from ragi cereal ingestion, being comparable in all 3 study groups but below JECFA set safe TDI. These results were in agreement with similar Hg intakes noted from

intake of similar foods (cereals, fish) in CKDu hit countries like Sri Lanka, Central America wherein no link to noted nephrotoxicity was found at such Hg intake levels (Bandara et al., 2008 & 2010a; Correa-Rotter,2017; Jayatilake et al.,2013; Levine et al.,2016; McClean et al., 2012;Wanigasuriya et al.,2011;Wesseling et al.,2014).Thus denoting that prevalent Hg intake (via food) in Canacona do not significantly contribute to its CKDu induction,backed by similar Hg intake noted in CKDu & healthy areas & occurrence of JCEFA safe intake levels.

3.2.2.3.3.1.5 EDI of ochratoxin in the study population

As indicated in **Figure 3.2.7B & Table 3.2.9**, mean daily OTA intake was majorly contributed by cereals and pulses as OTA contaminates cereals and pulses only. OTA mean EDI values from cereals were comparable in groups 1, 2, 3 and observed to be 2.4,2.53,2.79 ng/day kg bw with maximum intake of 3.41, 4.29, 4.66 ng/day kg bw noted from wheat and minimum intake of 0.81, 1.34, 0.83 ng/day kg bw from ragi respectively. 100% of all cereals & pulses in all 3 study areas displayed EDI values below JECFA set TDI of 14ng/day kg bw (Mitchell et al.,2017; Oueslati et al.,2018; Soto et al.,2016) wherein 65, 68, 72% of samples were present in lowest range of 0-3.8 ng/day kg bw and remaining 35,32.28% in higher ranges of 3.8-5.0 ng/daykg bw for respective study area 1, 2, 3.Overall,total cumulative OTA intake (total EDI) from all food sources (i.e. sum of all EDIs from cereals and pulses only) were comparable in all 3 study groups and estimated to be 2.4, 2.53 and 2.79 ng/day kg bw contributing to 17.1, 18.0, 19.9% of TDI limit (14 ng/day kg bw) respectively; which were below JECFA set safe TDI level. These results were consistent with similar OTA intakes noted from ingestion of similar cereals/pulses in CKDu hit countries like Sri Lanka, Central America wherein no significant link to noted nephrotoxicity was found at such OTA intake levels (Bandara et al.,2008 & 2010a; McClean et al., 2012; Wanigasuriya et al., 2008; Wanigasuriya et al.,2011; Wesseling et al.,2014).Thus denoting that prevalent OTA intakes (via food) in Canacona do not contribute to its CKDu induction, backed by similar OTA intakes noted in CKDu & healthy regions and presence at JCEFA safe intake levels.

3.2.2.3.3.1.6 EDI of aristolochic acid in the study population

As indicated in **Figure 3.2.7B & Table 3.2.9**, average AA daily intake was majorly contributed by cereals and pulses as this nephrotoxin contaminates cereals and pulses only. AA EDI values from cereals were comparable in groups 1, 2, 3 and estimated to be 0.85, 0.94, 0.90 ng/day kg bw with highest intake of 1.34, 1.41, 1.51 ng/day kg bw noted from wheat and lowest intake of 0.55, 0.66, 0.59 ng/day kg bw from ragi respectively. 100% of all

cereals and pulses in all 3 study areas depicted EDI values below JECFA set TDI limits of 3 ng/day kg bw (Abdullah et al.,2017; Gokmen et al.,2013; Jadot et al.,2017; Jelaković et al.,2015) wherein 59, 63, 65% of samples were present in lowest range of 0-1.0 ng/day kg bw and remaining 41,37,35% in higher ranges of 1.0-2.0 ng/day kg bw for respective areas 1,2 and 3. Overall, total cumulative AA intake (total EDI) from all food sources (i.e. sum of all EDIs from cereals and pulses only) were comparable in all 3 study groups and estimated to be 0.85, 0.94 and 0.90 ng/day kg bw contributing to 28.3,31.3,30% of TDI limit (3 ng/day kg bw) respectively; which were below JECFA set safe TDI level. These results were in agreement with similar AA intakes noted from ingestion of homologous cereals/pulses in CKDu hit countries like Central America wherein no significant relation to noted nephrotoxicity was found at such AA intake levels (Almaguer et al.,2014; Abiola, 2017; Correa-Rotter et al.,2014; García-Trabanino et al.,2015; McClean et al.,2012; Rajapakse et al.,2016; Wesseling et al., 2013).Thus denoting that prevalent AA intakes (through food intake) in Canacona do not significantly contribute to its CKDu induction, backed by similar AA intakes noted in CKDu hit & healthy regions and prevalence at JCEFA safe intake levels. Overall, daily intake of all analysed nephrotoxins through food consumption were noted to be comparable among CKDu affected (study area 1), diabetes/hypertensive CKD affected (study area 2) and healthy (study area 3) of the taluka. Moreover each nephrotoxin's dietary intake were noted to be well below their respective JECFA set TDI limits. Thus strongly indicating occurrence of no appreciable risk to study population in developing renal disease on regular intake of these nephrotoxins through consumption of common foods viz. cereals, vegetables, fish over a lifetime. Additionally, highlighting that these nephrotoxin intake at JECFA proved safe & non-toxic levels averts dietary contribution of these nephrotoxins in etiological development of high incidence of CKDu in Canacona (**Figure 3.2.7 and Table 3.2.9).**

3.2.2.3.3.2 Target hazard quotient (THQ) of nephrotoxins anlaysed in study population

THQ helps to predict nephrotoxicity risk that can be inflicted due to dietary consumption (i.e via food) of the toxin. As per JECFA, health risk (nephrotoxicity risk) through food consumption route exhibits a threshold below which no adverse effect is expected and is always set at 1.Hence, THQ of less than 1 indicates no risk of developing nephrotoxicity on oral consumption of the toxin analysed/target food species. In the current study, THQ values **(Figure 3.2.8 & Table 3.2.10**) for analysed nephrotoxins due to consumption of different food types in Canacona was calculated according to JECFA set formula (described in materials and methods section)which is the ratio of EDI to oral reference dose (specific for

food consumption (Abdullah et al.,2017; Barone et al.,2015; Bortey-Sam et al.,2015; Chandrajith et al.,2011; Chen et al., 2018; Erkekoğlu et al., 2010;Oueslati et al., 2018; Shaheen et al., 2016; Ullah et al.,2017).THQ allows to assess contribution of nephrotoxin's EDI as a fraction of reference dose to give an estimate of probable nephrotoxic risk that can be induced during an individual's lifespan since reference dose is the representation of maximum permissible risk that can be induced in lifetime on nephrotoxin consumption via food route (Bandara et al., 2010; Chammanejadian et al., 2013; Mitchell et al., 2017; Noli and Tsamos, 2016; Saha et al.,2016; Qureshi et al., 2016).

3.2.2.3.3.2.1 THQ of lead in the study population

As indicated in **Figure 3.2.8A & Table 3.2.10,** for Pb, mean THQ values from cereals were similar in study groups 1, 2, 3 and estimated to be 0.025, 0.023, 0.027 respectively with maximum hazardous risk value of 0.029, 0.028, 0.031 & minimum risk value of 0.021, 0.018 and 0.023 noted from wheat & ragi respectively. No risk was noted from staple food crop i.e. rice due to BDL of Pb noted in these samples which diminishes daily Pb intake & associated nephrotoxicity risk.100% of all cereals in all 3 study areas displayed THQ values below the JECFA set safe reference threshold of 1 (Chammanejadian et al., 2013; Chen et al., 2018) wherein 65, 72, 68% of samples and remaining 35, 28,32% were present in lowest range of 0.02-0.025 & higher ranges of 0.025-0.030 for areas1,2,3 respectively. Mean nephrotoxicity risk of lead from cereals intake in groups 1,2,3 were found to be 0.025, 0.023, 0.027 respectively which was below JECFA set safe threshold of 1 with each one contributing to 2.9, 2.8, 3.1% of total nephrotoxicity hazardous risk that can be inflicted by Pb from all consumed foods. In vegetables, mean lead THQ values were similar in groups 1, 2, 3 and observed to be 0.05, 0.055, 0.051 respectively with higher risk value of 0.056, 0.059, 0.058 & minimum risk value of 0.043, 0.051, 0.046 noted from spinach & brinjal ingestion respectively. No risk was noted from majorly consumed vegetables i.e. red/green amaranthus owing to BDL of lead noted in these samples which negates daily lead intake and associated health risk through consumption of these food. 100% of all vegetable in all areas displayed THQ values below JECFA set permissible threshold of 1 (Islam et al.,2015;Shaheen et al., 2016) wherein 71, 74, 79% of samples and residual 29, 26, 21% were present in lowest range of 0.04-0.055 & higher ranges of 0.055-0.060 for respective areas1, 2, 3.Mean nephrotoxic risk of lead from vegetable intake in groups 1, 2 & 3 were 0.05,0.055,0.051 which was below JECFA safe threshold of 1, contributing to 5.0, 5.5, 5.1% of total nephrotoxic risk that can be inflicted by lead from all foods consumption (i.e. cereals, pulses, vegetables, fish).

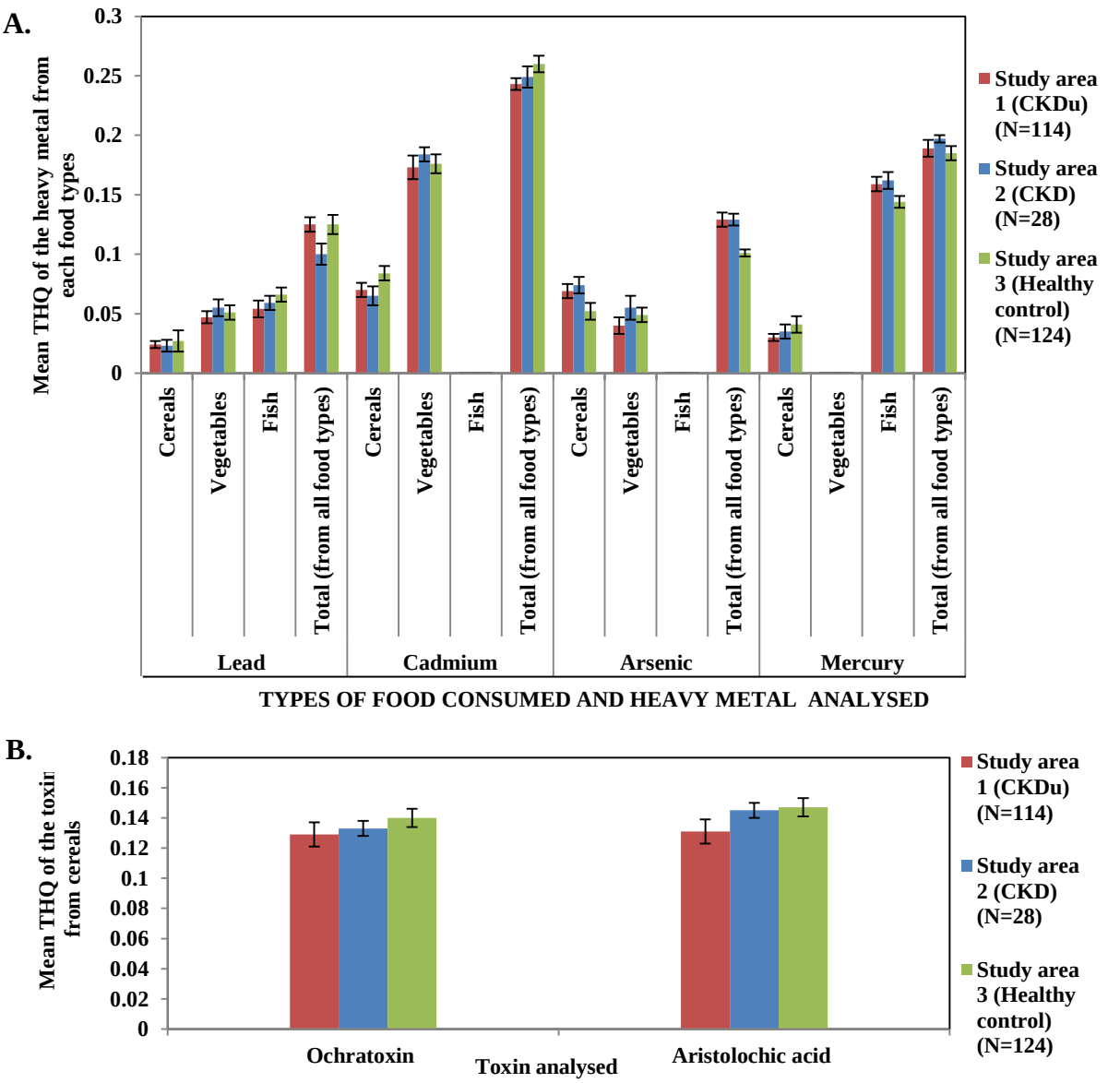

Figure 3.2.8. Mean target hazard quotients (THQ) of various nephrotoxins from consumption of three main food categories(viz. cereals & pulses,vegetables and fish) by the study population of Canacona

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; THQ-target hazard quotient. 'N' in each study area depicts the number of subjects in that study area or group from which samples of each sub-type of the three food categories viz. cereals& pulses, vegetables and fish were collected. The average EDI of an individual nephrotoxin (refer **Figure 3.2.7 and** Table 3.2.9)from the three respective food categories were used for THQ estimations of the nephrotoxin by calculating the fractional contribution of the measured nephrotoxin's EDI to the respective JECFA established oral reference doses. The JCEFA set oral reference doses for lead,cadmium,arsenic,mercury,ochratoxin and aristolochic acid are 4 µg/day kg bw, 1 µg /day kg bw, 2.5 µg /day kg bw, 3 µg /day kg bw, 17 ng/day kg bw and 4 ng/day kg bw respectively.(A)Mean target hazard quotient(THQ) values of heavy metals viz.lead,cadmium,arsenic and mercury from consumption of three categories of food i.e. cereals & pulses,vegetables and fish.(B) Mean THQ values of ochratoxin and aristolochic acid from consumption of cereals. Values of an individual nephrotoxin are represented as mean±SE of the THQ levels in each food category (i.e. cereals & pulses,vegetables and fish) with mean calculated from the average of THQ values noted from intake levels of the toxin through respective sub-types of a given food category in the three study areas(for example,mean THQ of lead from toxin intake levels through cereals & pulses was obtained from the average of the lead THQ values noted from intake levels through wheat,green gram,ragi and chickpea).The total(cumulative) THQ of an individual heavy metal from intake levels from all food types is also depicted(A) and was estimated from summation of the individual average THQ values from each of the three food categories[i.e.Total THQ of heavy metal(from all food types)= mean THQ(from cereals or pulses), mean THQ(from vegetables) and mean THQ(from fish)].The total THQ of ochratoxin and aristolochic acid was calculated solely from toxin intake levels through consumption of cereals & pulses exclusively (B) as they are contaminants of only the same.Since THQ is a ratio,it does not possess units. The JECFA established safe and non-toxic threshold of THQ of each individual nephrotoxin is 1,below which no risk of developing nephrotoxicity is noted if the estimated intake levels of the toxin equals the oral reference dose(i.e. maximum amount of toxin that can be ingested daily without inducing nephrotoxicity over a lifetime).No THQ values of the following toxins i.e. cadmium and arsenic from intake levels through fish and mercury from intake levels through vegetables were noted,hence are not represented by bars. The mean THQ values of each nephrotoxin from intake levels through consumption of every food category and total THQ values of the toxin from combined intake levels through all food types were found to be well below the JECFA established safe threshold of 1 and were similar [with no significant difference($p>0.05$)] in all three study areas.Thus suggesting the lack of risk of developing nephrotoxicity (as evident from lower THQ values) in the susceptible population of the CKDu endemic region at the prevalent JECFA set safe and non-toxic intake levels of these nephrotoxin via the food exposure route. The details of the mean THQ of the individual nephrotoxin from intake levels through each sub-type of a given food category(viz.cereals & pulses,vegetables and fish) and the range of minimum to maximum THQ values from intake through each sub-type,broad food categories and cumulatively from all food types are listed in **Table 3.2.10**.

Table 3.2.10: Mean and range of target hazard quotients (THQ) of various nephrotoxins from the consumption of different food categories by the study population of Canacona taluka and their comparison with the respective JECFA recommended safe and non-toxic threshold.

Type of Nephrotoxin	Study group (total number of subjects)/ Food category with their sub-types	THQ of the nephrotoxin from Cereals and pulses						THQ of the nephrotoxin from Vegetables						THQ of the nephrotoxin from Fish			Total THQ of a nephrotoxin from all possible food source categories [Total THQ =THQ(cereals)+ THQ(vegetables)+ THQ(fish)]	Hazard index(HI)= Total [THQ(Pb)+ THQ(Cd)+ THQ(As)+ THQ(Hg)+ THQ(Ochratoxin)+ THQ(Aristolochic acid)]
		Rice	Wheat	Green gram (Moong dal)	Ragi	Chickpea	*Mean THQ (from all cereals)*	Red amaranthus	Green amaranthus	Spinach	Pumpkin	Brinjal	*Mean THQ (from all vegetables)*	Mackerel	Sardine	*Mean THQ (from all fish)*		
THQ of Heavy metals																		
Pb	1 (N=114)	NR	0.029± 0.003	0.025± 0.006	0.021± 0.009	0.028± 0.004	0.024± 0.003	NR	NR	0.056± 0.003	0.05± 0.005	0.043± 0.007	0.047± 0.005	0.057± 0.006	0.052± 0.009	0.054± 0.007	0.125± 0.006	HI(study group 1) =0.901; HI(study group 2) =0.923; HI(study group 3) =0.937
	2 (N=28)	NR	0.028± 0.006	0.022± 0.007	0.018± 0.005	0.026± 0.002	0.023± 0.005	NR	NR	0.059± 0.009	0.055± 0.005	0.051± 0.006	0.055± 0.007	0.061± 0.004	0.058± 0.009	0.059± 0.006	0.1± 0.009	
	3 (N=124)	NR	0.031± 0.008	0.026± 0.003	0.023± 0.006	0.028± 0.003	0.027± 0.009	NR	NR	0.058± 0.004	0.05± 0.009	0.046± 0.006	0.051± 0.006	0.074± 0.004	0.059± 0.009	0.066± 0.006	0.125± 0.008	
Cd	1 (N=114)	NR	NR	0.093± 0.002	0.07± 0.008	0.077± 0.007	0.07± 0.006	NR	NR	NR	0.185± 0.003	0.167± 0.008	0.173± 0.010	NR	NR	NR	0.243± 0.005	
	2 (N=28)	NR	NR	0.083± 0.006	0.053± 0.008	0.073± 0.007	0.065± 0.008	NR	NR	NR	0.192± 0.006	0.181± 0.004	0.184± 0.006	NR	NR	NR	0.249± 0.009	
	3 (N=124)	NR	NR	0.101± 0.007	0.068± 0.006	0.088± 0.005	0.084± 0.006	NR	NR	NR	0.186± 0.006	0.174± 0.003	0.176± 0.008	NR	NR	NR	0.26± 0.007	
As	1 (N=114)	NR	NR	NR	0.076± 0.004	0.063± 0.008	0.069± 0.06	NR	NR	NR	0.051± 0.003	0.037± 0.008	0.04± 0.007	NR	NR	NR	0.109 ± 0.006	
	2 (N=28)	NR	NR	NR	0.083± 0.008	0.073± 0.007	0.074± 0.007	NR	NR	NR	0.067± 0.006	0.051± 0.006	0.055± 0.010	NR	NR	NR	0.129± 0.005	
	3 (N=124)	NR	NR	NR	0.061± 0.006	0.048± 0.008	0.052± 0.007	NR	NR	NR	0.058± 0.009	0.041± 0.008	0.049± 0.006	NR	NR	NR	0.101± 0.003	
Hg	1 (N=114)	NR	NR	NR	0.03± 0.003	NR	0.03± 0.003	NR	NR	NR	NR	NR	NR	0.167± 0.006	0.157± 0.009	0.159± 0.0064	0.189± 0.007	
	2 (N=28)	NR	NR	NR	0.35± 0.002	NR	0.035± 0.006	NR	NR	NR	NR	NR	NR	0.165± 0.008	0.160± 0.006	0.162± 0.007	0.197 ± 0.003	
	3 (N=124)	NR	NR	NR	0.041± 0.009	NR	0.041± 0.007	NR	NR	NR	NR	NR	NR	0.149± 0.009	0.141± 0.002	0.144± 0.005	0.185± 0.006	
Ochratoxin	1 (N=114)	0.176± 0.005	0.184± 0.007	0.132± 0.010	0.074± 0.006	0.121± 0.004	0.129± 0.016	NA(Not applicable) for THQ calculations as ochratoxin is not a contaminant of vegetables						NA (Not applicable) for THQ calculations as ochratoxin is not a contaminant of fish			0.129± 0.016	
	2 (N=28)	0.155± 0.003	0.208± 0.010	0.143± 0.006	0.09± 0.005	0.131± 0.003	0.133± 0.008										0.133± 0.008	
	3 (N=124)	0.185± 0.004	0.243± 0.003	0.15± 0.005	0.107± 0.009	0.129± 0.003	0.140± 0.007										0.140± 0.007	
Aristolochic acid	1 (N=114)	0.168± 0.009	0.184± 0.006	0.14± 0.005	0.12± 0.003	0.164± 0.010	0.131± 0.008	NA(Not applicable) for THQ calculations as aristolochic acid is not a contaminant of vegetables						NA(Not applicable) for THQ calculations as aristolochic acid is not a contaminant of fish			0.131± 0.008	
	2 (N=28)	0.172± 0.006	0.18± 0.008	0.16± 0.002	0.14± 0.003	0.168± 0.009	0.145± 0.005										0.145± 0.005	
	3 (N=124)	0.17± 0.003	0.19± 0.006	0.164± 0.008	0.142± 0.003	0.171± 0.005	0.147± 0.0066										0.147± 0.0066	

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; JECFA- Joint FAO/WHO Expert Committee on Food Additives (JECFA); NR-no risk (of nephrotoxicity); NA-not applicable for THQ calculations owing to the absence of intake of the nephrotoxin from that particular food sample category. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured. Values are represented as mean±SE with the range of minimum to maximum THQ values (shown in parenthesis) of the nephrotoxin estimated in each food sample sub-type (of the three respective food categories) in the three study groups or regions. The THQ value being a ratio possesses no units. The average EDI of an individual nephrotoxin(refer Table 3.2.9) in a particular sub-type of the respective food categories (i.e. cereals and pulses or vegetables or fish) were used for THQ estimations of the nephrotoxin by calculating the fractional contribution of the measured nephrotoxin's EDI to the respective JECFA established oral reference doses. The JCEFA set oral reference doses for Pb, Cd, As, Hg, ochratoxin and aristolochic acid are 4 µg/day kg bw, 1 µg /day kg bw, 2.5 µg /day kg bw, 0.5 µg /day kg bw, 17 ng/day kg bw and 4 ng/day kg bw respectively. The general THQ of a nephrotoxin from consumption of each food category(i.e. cereals and pulses or vegetables or fish) was estimated by taking the average of the THQ's from their respective food sub-types. Thereafter, the total THQ of a nephrotoxin from consumption of all possible food categories in the study population was obtained by the summation of the nephrotoxin's average THQ from each individual food category [i.e. mean THQ (cereals or pulses), mean THQ (vegetables) and mean THQ (fish)], which was then compared with the JECFA established safe and non-toxic threshold of 1 for assessment of the risk of developing renal damage through the daily consumption of the contaminated food source. Additionally the cumulative risk of developing renal toxicity (i.e. Hazard index, HI) by the combined effects of all the nephrotoxins by exposure at the current study's levels of contamination in the food was calculated by the summation of the total THQ's of each individual nephrotoxin[i.e. Total [THQ (Pb)+ THQ (Cd)+ THQ (As)+ THQ (Hg)+ THQ (Ochratoxin)+ THQ (Aristolochic acid)]. THQ and HI values which are well below the JECFA established safe threshold of 1 indicates no risk of developing the health hazard i.e. nephrotoxicity in this case. As indicated in the results, no statistically significant ($p>0.05$) differences were noted in the total THQ levels of each individual nephrotoxin and the cumulative THQ values of all the nephrotoxins(i.e. HI) between the three study groups or regions and were also found to be well below the JECFA established safe threshold of 1, thereby indicating that individual and combined nephrotoxins exposure in the current study via contaminated food consumption confers no risk of developing CKDu disease in the taluka.

In fish samples analysed, mean THQ values for lead were similar in groups 1, 2 & 3 **(Figure 3.2.8A & Table 3.2.10)** and observed to be 0.054, 0.059, 0.066 with highest risk value of 0.057, 0.061, 0.074 reported from mackerel and lowest risk value of 0.052, 0.058 and 0.059 from sardine respectively. 100% of all fish displayed THQ values below JECFA threshold of 1 (Barone et al.,2015; Bortey-Sam et al.,2015;Ullah et al., 2017) wherein 78, 83,74% of samples were present in their lowest range of 0.05-0.06 and remaining 22, 17, 26% in higher ranges of 0.06-0.075 for respective areas 1, 2, 3.Mean nephrotoxic risk of lead (i.e. average THQ of lead) from fish ingestion in groups 1, 2 and 3 were noted to be 0.054, 0.059, 0.066 respectively which were below JECFA safe levels of 1 with each one respectively contributing to 5.4, 5.9, 6.6% of total nephrotoxicity risk that can be inflicted by lead from ingestion of all food sources. Overall,total cumulative nephrotoxic risk of lead (total THQ of lead) in Canacona from intake of all food sources(i.e. calculated from sum of all THQ values from cereals, vegetables, fish) were comparable in all 3 study groups and estimated to be 0.129, 0.1, 0.125 respectively which were below JECFA safe threshold of 1 for the hazard index (HI, cumulative total of all nephrotoxins THQs) & correspondingly contributed to 12.9, 10, 12.5% of the cumulative nephrotoxicity risk that can be inflicted from exposure to all anlaysed nephrotoxins via consumption of major foods. Maximum but safe nephrotoxic risk of Pb in Canacona taluka was noted from fish consumption and minimum risk noted from cereals ingestion being comparable in all 3 study groups. From these results it was evident that the cumulative nephrotoxic risk of lead from all food sources (TTHQ (lead) in all 3 study groups were similar and established to be below JECFA established safe HI threshold of 1, signifying no risk of developing nephrotoxicity in Canacona taluka on exposure to lead through consumption of common foods viz. fish, vegetable, cereal (Islam et al,2015; Nuapia et al., 2018). These observations were in agreement with similar TTHQ (lead) values noted on consumption of similar foods in CKDu hit countries like Central America and Sri Lanka, wherein no significant link to noted renal toxicity was observed on exposure to such lead levels via food consumption (Banadara et al.,2008; Ramirez-Rubio et al., 2013; Gunatilake et al., 2015; Levine et al., 2016; Jayatilake et al., 2016; Saha et al., 2016; Wanigasuriya et al., 2011; Wimalawansa, 2016; Wesseling et al., 2013).Thus our results indicate that dietary exposure to Pb at such levels through food consumption are not sufficient in inducing severe and chronic nephrotoxicity over a lifetime in Canacona taluka. This was further supported by observance of similar Pb exposure levels in CKDu hit and healthy areas thus negating contribution of dietary Pb exposure in etiological development of CKDu in the taluka.

3.2.2.3.3.2.2 THQ of cadmium in the study population

As indicated in **Figure 3.2.8A & Table 3.2.10**, mean THQ values for cadmium from cereals were similar in the study groups 1, 2, 3 and ranged from 0.07, 0.065 and 0.084 respectively with a higher hazardous risk value of 0.093, 0.083 and 0.101 noted from green gram (moong dal) samples and a minimum risk value of 0.07, 0.53 and 0.068 noted from ragi variety respectively. Absolutely no risk was noted from the staple food crop in the taluka i.e. rice and wheat due to BDL levels of cadmium noted in these samples which diminishes the daily cadmium ingestion and its related health risk through these samples.100% of all the cereals and pulses analysed in the three study areas depicted prevalence ($p<0.05$) of the THQ values well below the JECFA established safe reference threshold of 1. From this, 61, 68 and 73% of the cereals and pulses were present in their corresponding lowest range category of 0.05-0.08 and the remaining 39, 32 and 27% of the same samples were prevalent in their correspondingly slightly higher ranges of 0.08-0.11 for study area 1, 2 and 3. In totality, the mean health hazardous risk (nephrotoxicity risk in this case) of cadmium from cereals consumption in study groups 1, 2 & 3 estimated by its THQ were reported to be 0.07, 0.065 & 0.084 respectively which was below the JECFA set safe threshold of 1 (Chammanejadian et al., 2013; Chen et al., 2018) with each one of them contributing to around 7.0,6.5 & 8.4% of the total hazardous risk that can be inflicted by cadmium from all consumed food types.

Moreover in the vegetable samples analysed ,mean THQ values for cadmium were similar in the study groups 1,2 & 3 and estimated to be 0.173, 0.184 and 0.176 respectively with a higher risk value of 0.185, 0.192 & 0.186 reported from pumpkin consumption and a minimum risk value of 0.167, 0.181 & 0.174 reported from brinjal ingestion respectively. No risk was noted from the majorly consumed vegetables i.e. red and green amaranthus and spinach owing to the BDL levels of cadmium observed in these samples which negates the daily cadmium ingestion and its associated health risk through these samples. 100% of all the evaluated vegetable samples for THQ values in the three study areas displayed prevalence ($p<0.05$) at levels well below the JECFA established permissible threshold of 1 (Islam et al., 2015; Shaheen et al., 2016). From this, 63, 67 and 74% of the samples were present in their corresponding lowest range category of 0.16-0.18 and the residual 37, 33 and 26% of were occurring in their respective and slightly higher ranges of 0.18-0.20 for study area 1, 2 and 3. In general, the average nephrotoxic risk of cadmium (estimated by its THQ) from vegetable consumption in study groups 1, 2 and 3 were reported to be 0.173, 0.184 and 0.176 respectively which was well below the JECFA established safe threshold of 1 with each groups correspondingly contributing around 17.3, 18.4 and 17.6% to the total nephrotoxic

risk that can be inflicted by cadmium from overall food consumption (i.e. from cereals, pulses ,vegetables). No hazardous risk of cadmium was observed from fish consumption as BDL levels of the same were detected in these samples thereby negating its contribution to the total dietary intake of cadmium and its associated potential in inducing nephrotoxic risk (Barone et al., 2015; Bortey-Sam et al., 2015; Ullah et al., 2017).

Overall, the total cumulative nephrotoxic risk of cadmium (total THQ of cadmium) in the Canacona taluka from all possible food sources (i.e. calculated from the sum of all the THQ values from cereals, pulses and vegetables) were comparable in all of the three study groups and were estimated to be 0.243, 0.249 and 0.26 respectively which were well below the JECFA established safe threshold of 1 for the hazard index (HI, cumulative total of the THQs of all nephrotoxins) and correspondingly contributed to around 24.3, 24.9 and 26% of the cumulative nephrotoxic risk that can be inflicted from exposure to all possible types of anlaysed nephrotoxins via consumption of the major food samples. Higher but safe nephrotoxic risk of Cd in the Canacona taluka was noted from cereals consumption and lowest risk was noted from vegetables ingestion being comparable in all the three study groups. From these results it was evident that the cumulative nephrotoxic risk of cadmium from all food sources (TTHQ (cadmium) in all the three study groups were similar and established to be well below the JECFA established safe HI threshold of 1, signifying no risk of developing nephrotoxicity in the Canacona taluka on exposure to cadmium through consumption of the selected food samples viz. pulses, vegetable and cereal samples. These observations were in agreement with the similar TTHQ (cadmium) values noted on daily ingestion of homologous food in various other regions across the world similarly hit by CKDu like Sri Lanka and Central America wherein no remarkable association with the predominant nephrotoxicity in these region was noted on exposure to such levels of cadmium via food ingestion (Almaguer et al., 2014; Chandrajith et al., 2011; Correa-Rotter, 2017; McClean et al., 2012; Levine et al., 2016; Siriwardhana et al., 2014; Wesseling et al., 2013).

Thus our results strongly indicate that dietary exposure to cadmium at such levels through food consumption are not sufficient in inducing severe and chronic nephrotoxicity over a lifetime in the Canacona taluka. This was further supported by the presence of similar cadmium exposure levels in the CKDu affected subjects and the healthy controls as well. Therefore refuting the contribution of dietary cadmium exposure in the etiological development of the CKDu in the taluka.

3.2.2.3.3.2.3 THQ of arsenic in the study population

As indicated in **Figure 3.2.8A & Table 3.2.10**, the mean THQ values of arsenic from cereals were similar in the study groups 1, 2 and 3 and estimated to be 0.069, 0.074 and 0.052 respectively with a higher hazardous risk value of 0.076, 0.083 and0.061noted from ragi samples and a minimum risk value of 0.063, 0.073 and 0.048 noted from chickpea variety respectively. Absolutely no risk was noted from the staple food crop in the taluka i.e. rice, wheat and green gram (moong dal) due to BDL levels of arsenic noted in these samples which diminishes the daily arsenic ingestion and its related health risk through these samples. 100% of all the cereals and pulses analysed in the three study areas depicted prevalence ($p<0.05$) of the THQ values well below the JECFA established safe reference threshold of 1 (Chammanejadian et al., 2013; Chen et al., 2018). From this, 54, 59 and 62% of the cereals and pulses were present in their corresponding lowest range category of 0.04-0.07 and the remaining 46, 41 and 38% were prevalent in their correspondingly slightly higher ranges of 0.07-0.09 for study area 1, 2 and 3.Mean nephrotoxicity risk of As from cereals consumption estimated by its THQ in groups 1, 2, 3 were reported to be 0.069, 0.074, 0.052 respectively which was well below JECFA established safe threshold of 1 with each one contributing to around 6.9, 7.4 and 5.2% of the total hazardous risk that can be inflicted by arsenic from all consumed food types.

Moreover in the vegetable samples analysed, mean THQ values for arsenic were similar in study groups 1, 2 and 3 and estimated to be 0.04, 0.055 and 0.059 respectively with a higher risk value of 0.051, 0.067, 0.058 reported from pumpkin consumption and a minimum risk value of 0.037,0.051 and 0.041 reported from brinjal ingestion respectively. No significant risk was noted from the majorly consumed vegetables i.e. red and green amaranthus and spinach owing to the BDL levels of arsenic noted in these samples which negates the daily arsenic intake and its related health risk through these samples. 100% of all the evaluated vegetable samples for THQ values in the three study areas displayed prevalence at levels well below the JECFA established permissible threshold of 1 (Islam et al., 2015; Shaheen et al., 2016). From this, 65, 59 and 60% of the samples were present in their corresponding lowest range category of 0.040-0.055 and the residual 35, 41 and 40% of were occurring in their respective and slightly higher ranges of 0.055-0.070 for study area 1, 2 and 3. In general ,the average nephrotoxic risk of arsenic from vegetable consumption in study groups 1,2 and 3 estimated by its THQ were reported to be 0.04,0.055 and 0.059 respectively which was well below the JECFA established safe threshold of 1 with each groups correspondingly contributing around 4.0,5.5 and 5.9% to the total nephrotoxic risk that can be imposed by

arsenic from overall food consumption (i.e. from cereals, pulses and vegetables). No nephrotoxic risk of arsenic exposure was observed from fish consumption as BDL levels of the same were detected in these samples thereby negating its contribution to the total dietary intake of arsenic and associated potency in inducing nephrotoxic risk (Bortey-Sam et al.,2015; Javed and Usmani,2016; Ullah et al., 2017).

Overall, the total cumulative nephrotoxic risk of arsenic (total THQ of arsenic) in the Canacona taluka from all possible food sources (i.e. calculated from the sum of all the THQ values from cereals, pulses and vegetables) were comparable in all three study groups and were estimated to be 0.109, 0.129 and 0.101 respectively which were well below the JECFA established safe threshold of 1 for the hazard index (HI, cumulative total of the THQs of all nephrotoxins) and correspondingly contributed to around 10.9, 12.9 and 10.1% of the cumulative nephrotoxic risk that can be inflicted from exposure to all possible types of anlaysed nephrotoxins via consumption of the major food samples. Higher but safe nephrotoxic risk of As in Canacona was noted from cereals consumption and lowest risk was noted from vegetables ingestion being comparable in all the three study groups. From these results it was evident that the cumulative nephrotoxic risk of arsenic from all food sources (TTHQ (arsenic) in all the three study groups were similar and established to be well below the JECFA established safe HI threshold of 1, signifying no risk of developing nephrotoxicity in the Canacona taluka on exposure to arsenic through consumption of the selected food samples viz. pulses, vegetable and cereal samples. These observations were in agreement with the comparable TTHQ (arsenic) values noted on intake of homologous food in another CKDu affected region of Sri Lanka , wherein no contribution in the nephrotoxicity endemic to the region was noted on exposure to such levels of arsenic via food consumption **(**Bandara et al., 2008; Bandara et al., 2010a; Chandrajith et al., 2011; Levine et al., 2016; Jayatilake et al., 2013; Rajapakse et al., 2016; Siriwardhana et al., 2014; Wanigasuriya et al., 2011). Thus our results strongly indicate that dietary exposure to arsenic at such levels through food consumption are not significantly sufficient in inducing severe and prolonged nephrotoxicity over a lifetime in Canacona.This was further supported by presence of similar As exposure levels in CKDu hit and healthy regions thus nullifying contribution of dietary As exposure in etiological development of CKDu in the taluka.

3.2.2.3.3.2.4 THQ of mercury in the study population

As indicated in **Figure 3.2.8A & Table 3.2.10,** the mean THQ values of mercury from the cereals samples anlaysed was solely contributed from the ragi food type only. The THQ

values from ragi cereal samples were found to be similar within the three study groups and well below the JECFA established safe permissible threshold of 1 (Chammanejadian et al., 2013; Chen et al., 2018). The THQ values of Hg from ragi consumption in the study groups 1, 2 and 3 were noted to be 0.03, 0.035 and 0.041 with each group correspondingly contributing around 3, 3.5 and 4.1% to the total nephrotoxic risk that can be imposed by mercury from overall food consumption (i.e. from cereals and fish). 100% of all the ragi cereal samples analysed in the three study groups/regions displayed a prevalence of the THQ values at levels well below the JECFA established reference threshold of 1. From this, 67, 63, 70% were present in their corresponding lowest range category of 0.03-0.035 and the remaining 33, 37 and 30% of were prevalent in their respective and somewhat higher ranges of 0035-0.045 for study area 1, 2 and 3. No mercury associated nephrotoxic risk was noted from other commonly consumed staple food crops in the taluka i.e. rice, wheat, green gram (moong dal), chickpea. Similarly a complete lack of Hg induced hazardous renal toxicity risk was noted from the commonly consumed vegetables in the taluka due to BDL levels of Hg detected in these food samples which nullifies the daily intake of Hg and its related risk of developing nephrotoxicity (Islam et al., 2015; Shaheen et al., 2016).

Furthermore among the fish samples analysed, the mean THQ values for mercury were similar in the study groups 1, 2 and 3 and found to be 0.159, 0.162, 0.144 respectively with a highest risk value of 0.167, 0.165, 0.159 reported from mackerel and a lowest risk value of 0.157, 0.160 and 0.141 reported from the sardine fish variety respectively (Barone et al., 2015; Bortey-Sam et al., 2015; Ullah et al., 2017). 100% of all the analysed fish sources for THQ values in the three study regions displayed prevalence ($p<0.05$) of their values well below the JECFA established safe threshold of 1. From this, 72, 76 and 71% of the samples were present in their respective lowest range category of 0.14-0.16 and the remaining 28, 24 and 29% of were occurring in their respective and slightly higher ranges of 0.16-0.17 for study area 1, 2 and 3. In general, the average nephrotoxic risk of mercury from fish ingestion in study groups 1, 2 and 3 estimated by its THQ were reported to be of 0.167, 0.165, 0.159 respectively which were well below the JECFA established safe levels of 1 with each one of them respectively contributing to around 16.7, 16.5 and 15.9% of the total hazardous risk that can be inflicted by mercury from all food sources (i.e. cereals and fish).

Overall, the total cumulative nephrotoxic risk of mercury (total THQ of mercury) in the Canacona taluka from all possible food sources calculated from the sum of all THQ values (from cereals and fish) were similar in all three study groups and were estimated to be 0.189, 0.197 and 0.195 respectively which were well below the JECFA established safe threshold of

1 for the hazard index (HI, cumulative total of the THQs of all nephrotoxins) and correspondingly contributed to around 18.9, 19.7 and 19.5% of the cumulative health risk that can be inflicted from exposure to all possible types of anlaysed nephrotoxins via consumption of the major food samples. Maximum but safe nephrotoxic risk of Hg in the Canacona taluka was noted from fish consumption and minimum risk was noted from cereals ingestion being comparable in all the three study groups. From these results it was evident that the cumulative nephrotoxic risk of Hg from all food sources (TTHQ (mercury) in all the three study groups were similar and established to be well below the JECFA established safe HI threshold of 1, signifying no risk of developing nephrotoxicity in the Canacona taluka on exposure to mercury through consumption of the selected food samples viz. fish and cereal samples. These observations were in consensus with the homologous TTHQ (mercury) values noted on consumption of analogous food stuff in other developing counties hit by CKDu like Sri Lanka and Central America, wherein no significant relationship with the prevalent renal toxicity was noted on exposure to the noted mercury levels via food ingestion **(**Bandara et al., 2008; Bandara et al., 2010a; Correa-Rotter, 2017; Jayatilake et al., 2013; Levine et al., 2016; McClean et al., 2012; Wanigasuriya et al.,2011; Wesseling et al., 2014). Thus our results strongly indicate that dietary exposure to mercury at such magnitudes through food consumption are not considerably sufficient in inducing severe and chronic renal toxicity over a lifetime in the Canacona taluka.This was further supported by observance of similar Hg exposure levels in CKDu hit and healthy regions as well. Therefore negating contribution of dietary Hg exposure in etiological development of CKDu in the taluka.

3.2.2.3.3.2.5 THQ of ochratoxin in the study population

As indicated in **Figure 3.2.8B & Table 3.2.10**, THQ of ochratoxin was majorly contributed by cereals & pulses as OTA contaminates cereals and pulses only. Mean THQ values from cereals were similar in groups 1, 2, 3 and noted to be 0.125, 0.133, 0.140 with maximum risk value of 0.184, 0.208, 0.243 noted from wheat and minimum risk value of 0.121, 0.131, 0.129from ragi respectively.100% of all cereals in all 3 study areas displayed THQ values below JECFA threshold of 1 (Mitchell et al.,2017) wherein 65, 68, 72% of samples and remaining 35, 32, 28% were present in lowest range of 0.1-0.2 & higher range of 0.2-0.3 for areas 1, 2, 3 respectively.

Overall, the total cumulative nephrotoxic risk of ochratoxin (estimated by calculation of the total THQ of ochratoxin) in the Canacona taluka from all possible food sources calculated from the sum of all the THQ values (from cereals and pulses) were similar in all of the three

study groups and were estimated to be 0.125, 0.133 and 0.140 respectively which were well below the JECFA established safe threshold of 1 for the hazard index (HI, cumulative total of the THQs of all nephrotoxins) and correspondingly contributed to around 12.5, 13.3 and 14.0% of the cumulative health risk that can be inflicted from exposure to all possible types of anlaysed nephrotoxins via consumption of the major food samples. Maximum but safe nephrotoxic risk of ochratoxin in the Canacona taluka was noted from cereals consumption and minimum risk was noted from pulses ingestion being comparable in all the three study groups. From these results it was evident that the cumulative nephrotoxic risk of ochratoxin from all food sources [TTHQ (ochratoxin)] in all the three study groups were similar and established to be well below the JECFA established safe HI threshold of 1, indicating that these safe and non-toxic ochratoxin levels observed in the three study groups conferred no risk of developing nephrotoxicity in the Canacona taluka on ochratoxin exposure through intake of the selected cereal samples. Analogous studies conducted in other developing countries affected by CKDu like Sri Lanka & Central America have also reported similar TTHQ levels of ochratoxin (like our current study) through consumption of similar foodstuffs viz. cereals and pulses with no significant contribution in the predominant renal injuries observed in these regions at such exposure levels (Bandara et al.., 2008; Bandara et al., 2010a; Correa-Rotter, 2017; Jayatilake et al., 2013; Levine et al., 2016; McClean et al., 2012; Wanigasuriya et al., 2008*;* Wanigasuriya et al.,2011; Wesseling et al., 2014).Thus our results strongly highlight that dietary exposure to OTA at such levels are not sufficient in inducing severe and chronic renal toxicity over lifetime in Canacona.This was supported by observance of similar OTA exposure levels in CKDu and healthy regions negating contribution of dietary OTA exposure in etiological CKDu development in the taluka.

3.2.2.3.3.2.6 THQ of aristolochic acid in the study population

As indicated in **Figure 3.2.8B & Table 3.2.10**, THQ of AA was majorly contributed by cereals and pulses as AA contaminates cereals and pulses only. Mean THQ values of AA from cereals were comparable in study groups 1, 2, 3 and found to be 0.131, 0.145, 0.147 with higher nephrotoxic risk value of 0.184, 0.18, 0.19 noted from wheat and lower risk value of 0.12, 0.14, 0.142 from ragi respectively. 100% of all cereals in all 3 areas displayed THQ values below JECFA threshold of 1 (Abdullah et al.,2017) wherein 59, 63, 65% of samples were present in lowest range of 0.10-0.15 and remaining 41, 37, 35% in higher ranges of 0.15-0.20 for respective area 1, 2, 3.

Overall, the total cumulative nephrotoxic risk of aristolochic acid (estimated by calculation of total THQ of aristolochic acid) in the Canacona taluka from all possible food sources calculated from the sum of all the THQ values (from cereals and pulses) were similar in all of the three study groups and were estimated to be 0.131, 0.145 and 0.147 respectively which were well below the JECFA established safe threshold of 1 for the hazard index (HI, cumulative total of the THQs of all nephrotoxins) and correspondingly contributed to around 13.1, 14.5 and 14.7% of the cumulative health risk that can be inflicted from exposure to all possible types of anlaysed nephrotoxins via consumption of the major food samples. Maximum but safe nephrotoxic risk of aristolochic acid in the Canacona taluka was noted from cereals consumption and minimum risk was noted from pulses intake being comparable in all the three study groups. From these results it was evident that the cumulative nephrotoxic risk of aristolochic acid from all food sources (TTHQ (aristolochic acid) in all the 3 study groups were similar and established to be well below the JECFA established safe HI threshold of 1, indicating that these safe and non-toxic aristolochic acid concentrations observed in the three study groups provided no risk of developing renal damage in the Canacona taluka on aristolochic acid exposure through intake of the selected cereal and pulses samples. Similar studies conducted in a CKDu hit region of Central America have also reported similar TTHQ levels of aristolochic acid (like this study) through analogously consumed cereals and pulses with no remarkable link with the prevalent renal damage noted in this region at such exposure levels (Almaguer et al.,2014; Abiola,2017; Correa-Rotter et al.,2014; García-Trabanino et al.,2015; McClean et al., 2012; Rajapakse et al., 2016; Wesseling et al.,2013).Thus our results strongly highlight that dietary exposure to AA at such levels are not considerably sufficient in inducing severe and chronic nephrotoxicity over a lifetime in Canacona.This was further supported by observance of comparable AA exposure levels in CKDu subjects and healthy controls as well. Therefore disproving contribution of dietary AA exposure in the etiological manifestation of CKDu in the taluka.

Therefore in general, all of the total THQ values (from all food sources) of each analysed nephrotoxin were well below the JECFA established safe permissible threshold of 1 suggesting that exposure of the Canacona taluka's study population to such toxin levels do not confer the risk of developing renal damage over an average lifetime of the populace. On evaluating the cumulative health risk (i.e. renal damage) arising from exposure to the six analysed nephrotoxins via food consumption by the measurement of the hazard index(sum of the THQ values of each nephrotoxin), depicted that the values of hazard index in all of the three study groups were comparable and significantly well below the JECFA safe threshold

of 1,again indicating no noteworthy risk of developing nephrotoxicity or CKDu throughout the lifespan of an individual despite the cumulative action of these dietary nephrotoxins (Islam et al, 2015; Nuapia et al., 2018). In cumulative nephrotoxicity risk assessment (i.e HI analysis), ranking of total THQ values of each analysed nephrotoxin (from all food consumption) & their contribution to HI (i.e. combined nephrotoxicity risk) was comparable between all groups (Almaguer et al.,2014; Levine et al.,2016; Siriwardhana et al., 2014) & established to be in the order: - Cd>Hg>Aristolochic acid>Pb>Ochratoxin >Arsenic.

Herein, the maximum contribution (i.e. around 25%) to the overall nephrotoxic hazard risk was from cadmium with minimum contribution (i.e. 11.3%) noted from arsenic (Bandara et al.,2010a; Chandrajith et al.,2011). However, these THQ (nephrotoxin-Pb/Cd/As/Hg/OTA/ AA) values were well within the JECFA set safe reference limit of 1. These observations were in agreement with the findings from similar risk assessment studies of CKDu through dietary exposure to nephrotoxins in a homologously affected region of Sri Lanka wherein the role of the aforementioned nephrotoxins through dietary route was negated. Thus highlighting that the exposure of study population to these nephrotoxins (i.e. Pb, Cd, As, Hg, OTA, AA) through the dietary consumption of regular foodstuffs contaminated with JCEFA established safe levels of these nephrotoxins in the three study regions, do not suffice as etiological agents in the development of CKDu in the Canacona taluka (Chandrajith et al., 2011; Levine et al., 2016). This was further supported by the observance of similar hazard indices noted in the CKDu affected region (study region 1), diabetes and hypertensive CKD cases (study region 2) and non-affected healthy controls regions (study region 3) as well (Almaguer et al., 2014; Chandrajith et al., 2011) (**Figure 3.2.8 & Table 3.2.10).**

3.2.2.3.4 Assessment of the role of the dietary contribution of the nephrotoxins in the induction of tubular or glomerular specific nephropathy in the study population.

The effect of daily intake (EDI, via food consumption) of analysed nephrotoxins in induction of tubular (CKDu) and glomerular (diabetes/hypertensive CKD) based nephropathies in study population was anlaysed by evaluating association between daily nephrotoxins intake levels and variations in tubular or glomerular damage specific biomarker profile via calculation of significant Pearson's correlation coefficients(r), the results of which are presented in **Table 3.2.11**. This might help in prediction of CKDu or CKD disease pattern that could be manifested on exposure to these nephrotoxins at such levels via food intake (Bandara et al.,2008; Chandrajith et al., 2016; Jayatilake et al.,2013; Levine et al.,2016).

Table 3.2.11: Correlation assessments between the total estimated daily intakes of each individual nephrotoxin and the average urinary based nephropathy specific selectivity indices of the Canacona taluka's study population for analysis of the contribution(if any) of the dietary nephrotoxin exposure in the induction of tubular or glomerular pathology.

		Correlation coefficients [r] with their (p values)					
		(p values)					
Total EDI of each nephrotoxin from all food sources	**Study group (total number of subjects)**	**Urinary nephropathy specific selectivity indices**					
		uPCR	**uACR**	**uBCR**	**uNCR**	**uAPR**	**uAlb/b2M**
Pb	1	-0.033	-0.0026	0.089	0.076	-0.0005	-0.0011
	(N=114)	*0.12*	*0.36*	*0.078*	*0.058*	*0.11*	*0.21*
	2	-0.0019	-0.009	-0.0004	-0.0005	-0.0006	-0.0008
	(N=28)	*0.058*	*0.212*	*0.102*	*0.078*	*0.102*	*0.321*
	3	-0.00002	-0.00003	-0.00009	-0.00006	-0.00005	-0.00007
	(N=124)	*0.147*	*0.156*	*0.097*	*0.41*	*0.181*	*0.132*
Cd	1	-0.027	-0.0035	0.078	0.067	-0.0007	-0.0013
	(N=114)	*0.087*	*0.1*	*0.213*	*0.354*	*0.147*	*0.156*
	2	-0.0014	-0.0006	-0.0005	-0.0002	-0.0008	-0.0009
	(N=28)	*0.147*	*0.123*	*0.213*	*0.058*	*0.097*	*0.126*
	3	-0.00008	-0.00003	-0.00002	-0.00005	-0.000004	-0.00006
	(N=124)	*0.121*	*0.059*	*0.078*	*0.197*	*0.254*	*0.312*
As	1	-0.011	-0.0013	0.042	0.039	-0.0002	-0.0007
	(N=114)	*0.101*	*0.089*	*0.047*	*0.051*	*0.067*	*0.098*
	2	-0.0013	-0.0009	-0.0002	-0.0003	-0.0005	-0.0004
	(N=28)	*0.123*	*0.165*	*0.147*	*0.214*	*0.096*	*0.147*
	3	-0.00006	-0.00004	-0.00009	-0.00005	-0.00007	-0.00003
	(N=124)	*0.247*	*0.361*	*0.147*	*0.542*	*0.214*	*0.412*
Hg	1	-0.0008	-0.0005	0.037	0.028	-0.0004	-0.0009
	(N=114)	*0.123*	*0.213*	*0.058*	*0.074*	*0.145*	*0.097*
	2	-0.0016	-0.0008	-0.0005	-0.0003	-0.0007	-0.0006
	(N=28)	*0.147*	*0.165*	*0.321*	*0.412*	*0.571*	*0.099*
	3	-0.00001	-0.00003	-0.00007	-0.0004	-0.00005	-0.00003
	(N=124)	*0.123*	*0.472*	*0.265*	*0.398*	*0.143*	*0.215*
Ochratoxin	1	-0.027	-0.0009	0.034	0.04	-0.0005	-0.0011
	(N=114)	*0.117*	*0.289*	*0.362*	*0.245*	*0.125*	*0.117*
	2	-0.0009	-0.0004	-0.0001	-0.0002	-0.0008	-0.0003
	(N=28)	*0.147*	*0.356*	*0.278*	*0.114*	*0.098*	*0.074*
	3	-0.00012	-0.00009	-0.00008	-0.00004	-0.00002	-0.00005
	(N=124)	*0.314*	*0.128*	*0.295*	*0.075*	*0.084*	*0.206*
Aristolochic acid	1	-0.019	-0.0013	0.021	0.031	-0.0004	-0.0007
	(N=114)	*0.112*	*0.165*	*0.254*	*0.321*	*0.195*	*0.418*
	2	-0.0011	-0.0007	-0.0002	-0.0004	-0.0001	-0.0008
	(N=28)	*0.097*	*0.058*	*0.074*	*0.201*	*0.119*	*0.213*
	3	-0.00004	-0.00009	-0.0001	-0.00007	-0.00006	-0.00003
	(N=124)	*0.213*	*0.147*	*0.068*	*0.201*	*0.097*	*0.114*

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; EDI-estimated daily intake; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. 'N' in each study region depicts the number of subjects in that study region or group from which the food samples were procured and the same subjects were analysed for nephropathy specific selectivity indices in the urine. Values are represented as Pearson's correlation coefficients (r) with their respective p-values 'italicized' for identification of statistically significant associations(if any) between any given two parameters [i.e. daily intake of the nephrotoxins and the variations(increase or decrease) in the tubular or glomerular nephropathy specific biochemical markers/selectivity indices]. Differences at $p<0.05$ were considered to be statistically significant correlations. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. However in the current study, negligible and statistically insignificant($p>0.05$) associations were noted between each individual nephrotoxin dietary exposure and the tubular nephropathy selectivity indices(i.e.uBCR and uNCR) of the study group 1(comprising of CKDu endemic cases) indicating no contribution of all the nephrotoxin at such levels of exposure via contaminated food consumption in the induction of tubular pathology, characteristic of the CKDu disease in the Canacona taluka. Moreover, negligible, inverse and insignificant ($p>0.05$) correlations were noted between each individual nephrotoxin intake levels and the glomerular nephropathy specific indices(i.e. uPCR,uACR,uAPR and uAlb/b2M ratio) at a comparable extent in all of the three study groups suggesting no contribution of these food based nephrotoxin exposure in triggering the glomerular pathology witnessed in the study group 2 as well. For values and units of the estimated daily intake levels of the nephrotoxin and the nephropathy specific selectivity indices, kindly refer Table 3.2.9 of the current section and Table 2.8 of chapter 2 respectively.

As indicated in **Table 3.2.11**, very weak or negligible and insignificant ($p>0.05$) correlations were noted between dietary intake of each toxins (i.e. Pb, Cd, As, Hg, OTA, AA) analysed in food sources consumed by group 1 subjects with tubular damage specific urinary indices i.e. uBCR and uNCR of same subjects consuming these food sources. This was attributed to inherent potencies of these nephrotoxins to induce tubular nephropathy viz. CTN only when present at high concentrations thus explaining insignificant associations noted with

predominant tubular injury markers in these CKDu subjects due to intake of these nephrotoxins via food at JCEFA recommended safe levels (Chandrajith et al., 2011; Jelakovic et al., 2015; Levine et al., 2016; Siriwardhana et al., 2014).Thus these results confirms no role of dietary exposure to any of these nephrotoxins (i.e. heavy metals or OTA or AA) in CKDu development in Canacona owing to presence of non-toxic or JECFA established safe levels of these nephotoxins in foods regularly consumed by this group. Moreover, negligibly inverse and insignificant ($p>0.05$) correlations were noted between EDI of nephrotoxins and glomerular injury markers i.e.uPCR, uACR, uAPR, uAlb/b2M ratio of group 1 subjects indicating complete lack of contribution of these nephrotoxins in developing diabetes or hypertensive associated CKD based glomerular nephropathy, which is consistent with tubular damaging potency of these nephrotoxins (Rajapakse et al., 2016; Ratnayake et al., 2017). On the other hand, negligible & inversely insignificant ($p>0.05$) correlations were noted between EDI's of nephrotoxins in food & glomerular injury markers i.e. uPCR, uACR, uAPR of group 2 subjects ingesting these foods.Thus indicating no contribution of these dietary nephrotoxins in triggering glomerular pathology, typical of group 2 subjects, confirming diabetes or hypertension to be disease's major causes (Ratnayake et al.,2017; Smith et al., 2011). Overall, our findings were in agreement with values obtained in a similar study of CKDu cases in Sri Lanka wherein no contribution of these analogously analysed nephrotoxins through dietary exposure route in CKDu development was noted, thus signifying lack of role of these analysed nephrotoxins in CKDu induction in Canacona, when exposed through food (dietary) route due to JECFA set safe levels of nephrotoxins in these foods (Chandrajith et al.,2011; Jelaković et al., 2015; Levine et al., 2016; Siriwardhana et al., 2014).

3.2.4 Conclusion

This study aimed at assessing the role of various nephotoxins present in the food arising through either natural contamination (by living plant or fungal organisms) or through the anthropogenic influence on the food biota (i.e. plant and animal sources) in the development of Chronic Kidney Disease of unknown etiology (CKDu) in the Canacona taluka. For this, the levels of various naturally arising nephrotoxins (i.e. ochratoxin and aristolochic acid) and anthropogenically emerging nephrotoxins (i.e. heavy metals-lead, cadmium, arsenic and mercury) in the majorly consumed food samples (i.e. cereals, pulses, vegetable and fish) in the three study groups of Canacona taluka (i.e CKDu affected group 1, diabetes & hypertension induced CKD group 2 & healthy control group 3) were analysed. Moreover the daily intakes of these nephrotoxins from the consumption of these foods were estimated for

assessment of the suitable nephrotoxicity risk. As evident from the results, the concentrations of all the nephrotoxins (i.e. Pb, Cd, As, Hg, ochratoxin and aristolochic acid) and the daily intake of these nephrotoxins through the food consumption was observed to be comparable in all of the three study groups and were well below the JECFA established reference limit and their recommended tolerable daily allowances for these nephrotoxins. The comparable levels of these nephrotoxins in the three study groups are attributed to a common source of origin of these food sources i.e. all the foodstuffs (i.e. cereals, vegetable and fish) are produced and caught by this study's healthy control villages- Molorem and Endrem due to the predominance of the farming and fishing occupations in this region, which is then further distributed to the rest of the taluka, signifying common food habits among the three study groups. These similar JECFA established safe and non-toxic concentrations of the nephrotoxins and their significantly below the tolerable threshold daily intake values arising through the consumption of these foodstuff in all the three study regions provides confirmatory evidence that these nephrotoxins on exposure via the dietary route do not contribute to the development of CKDu in Canacona due to minimal food contamination with these nephrotoxins. This was further supported by comparable nephrotoxin and daily intake values noted in foods consumed by CKDu hit and healthy groups indicating no contribution towards renal toxicity in CKDu endemic region from these food based nephrotoxins.

Furthermore from the nephrotoxicity risk point of view, the THQ values of each of these nephrotoxins and the cumulative THQ values from all the nephrotoxins combined were significantly below the JECFA established safe threshold of 1, indicating no possible risk of developing nephrotoxicity over a lifetime in Canacona on daily ingestion of these foods contaminated with the abovesaid levels of either individual or cumulative nephrotoxins.

The lack of contribution of these food based nephrotoxins in the development of CKDu in the Canacona taluka was further supported by the absence of significant associations (via correlation assessments) of the daily intake of these nephrotoxins in the induction of the elevation of tubular nephropathy specific selectivity indices of the CKDu affected subjects.

Thus highlighting no involvement of these nephrotoxins on exposure via food at such non-toxic levels in stimulating tubular pathological manifestation of CKDu in Canacona.

In summary, food /dietary based exposure to these nephrotoxins do not contribute significantly to the etiological development of CKDu in the Canacona taluka as the levels of nephrotoxin contamination and their daily intake from the commonly consumed food sources in Canacona are well within the JECFA established safe limits and moreover were comparable between the foods consumed by the CKDu affected and healthy regions.

Section 3.3

Biomonitoring of environmental nephrotoxins in the biological matrix i.e. 'blood' of the study population and evaluation of its contribution in CKDu development

3.3.1 Introduction

Our previous study stated chronic exposure to high silica and borderline lead nephrotoxin levels via contaminated groundwater consumption to be potentially responsible for CKDu causation in Canacona. Thus in order to verify, role of these nephrotoxins in CKDu etiology, it is mandatory to monitor presence of these nephrotoxins in biological matrices like blood, urine,etc. and relate their levels to disease pathology (biomonitoring) (Wimalawansa,2016).

Biomonitoring is defined as"repeated, controlled measurements of chemical/biological markers in fluids, tissues or other accessible samples from subjects previously, currently or will be exposed to chemical, physical, or biological risk factors in workplace or environment." (Manno et al.,2010). Biomonitoring (Biological monitoring) of various nephrotoxins is critical for analysis of nephrotoxin exposure levels that confers risk of developing renal-toxicity in prone populations. Ideally kidney biopsy analysis would be an ideal indicator of nephrotoxin exposure but surrogate biological matrices (i.e. blood/urine) are generally chosen for measurement of nephrotoxin doses or effects at target organ owing to latter being accessible in sufficient amounts under routine conditions without discomfort or health risks (such as biopsy linked organ (i.e. kidney) damage for the individual. Moreover, the nephrotoxins on dietary exposure are generally directly absorbed from the gastrointestinal tract into blood circulation. For these reasons blood is preferred for nephrotoxin exposure analysis as they provide surrogate endpoints of effects on target internal organs which are inaccessible for human clinical or experimental evaluations being reliable indicators of body burden of nephrotoxin exposure (Jayasumana et al., 2016; Rango et al., 2015; Sellamuthu et al., 2011a, b). Hence, blood was used in this study for nephrotoxin biomonitoring.

The ultimate outcome of prolonged exposure to high nephrotoxins levels is induction of renal tubular damage that results in CTN, pathological manifestation of CKDu. Thus in order to understand dose-response effect of these nephrotoxins i.e. nephrotoxin exposure levels that will induce tubular injury, it is mandatory to analyse for biomarkers of effect(tubular damage) and exposure(nephrotoxin)in biological matrices(Lusco et al.,2017;Sayanthooran et al.,2017).

Biomarker is described as a specific chemical detectable at low (trace-levels) that is available using minimum invasion and inexpensive to analyse which quantitatively relates to prior exposures. Biomarkers are of 2 types: biomarkers of effect and exposure (Rango et al., 2015). 'Biomarkers of effect' include measurement of changes in biological systems at cellular or molecular level like altered cellular specific metabolic enzymes/proteins levels (Wijetunge et al.,2015; Wijkström et al.,2018). As stated earlier, in this study, major implicative effect of

environmental nephrotoxin exposure is renal tubular dysfunction that is biochemically characterised by increased urinary excretion of tubular specific LMV protein (i.e.b2M,NAG). Hence by measurement of these tubular injury markers (as analysed in Chapter 2) provides an extent of tubular injury inflicted by nephrotoxins, thus results of the same will be used for analysis of the link between exposure levels of stated nephrotoxins in induction of tubular damage (Gifford et al.,2017). The extent of tubular damage effect inflicted is highly dependent on level of nephrotoxin exposure in blood (internal dose) which in turn is influenced by external environmental factors like nephrotoxin concentration in source (water/ food), daily dietary intake (from water, food) etc. Thus in order to gauge the level of nephrotoxin exposure (internal dose), it is mandatory to measure 'biomarkers of exposure' which is the level of parent nephrotoxins itself in the biological matrix (blood) (Gifford et al., 2017; Jelakovic et al., 2015). Unlike 'biomarkers of effect' that are not specific for individual nephrotoxins but common to all, 'biomarkers of exposure' are specific and restricted to direct measurements of individual nephrotoxins in blood owing to long biological half-lives and bioaccumulative tendencies of these toxins (Abiola, 2017, Cabral et al., 2015; Moody et al., 2018). Thus, both levels of biomarkers of exposure (nephrotoxin) and effect (tubular injury markers) and their association needs to be analysed to explore role of potential nephrotoxins in CKDu causation (Gifford et al.,2017;Wijkström et al., 2018).

Therefore the current study aimed at providing confirmatory evidence to the etiological contribution of chronic exposure to high silica and borderline lead nephrotoxins (via contaminated groundwater intake) in CKDu development in Canacona. This was achieved by analysis of the levels of biomarker of nephrotoxin exposure i.e measurements of these nephrotoxin levels in the blood (that reflects the toxin's internal dose), and correlation of these exposure levels with induction of tubular dysfunction represented by increased urinary elimination of tubular injury specific markers viz. b2M, NAG (biomarkers of effect) in CKDu affected subjects to explore the dose response relationship of these potential causal nephrotoxins (silica and lead) in triggering CKDu at the prevalent internal exposure levels. These exposure levels were compared with healthy unaffected controls to rule out any existing and future risk of CKDu development in these subjects (Rajapakse et al., 2016). Moreover levels of internal exposure or presence of other nephrotoxins (i.e. Cd, As, Hg, OTA, AA) in blood and correlations with tubular injury markers in CKDu affected subjects and healthy controls were analysed to rule out causative contribution (if any) of these subsidiary nephrotoxins in taluka's CKDu manifestation (Gifford et al., 2017; Jelakovic et al., 2015; Wijkström et al., 2018).

3.3.2 Materials and Methods

3.3.2.1 Ethical approval and consideration

The entire study was approved by the Government of Goa by obtaining the necessary permission from the primary government aided health safety regulatory body i.e. The Directorate of Health-Services, Panaji, Goa (Reference No:-DHS/Sp.Cell/Sect/8/1119). It was conducted in agreement with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Furthermore ethical approval for this study was granted by 2 Institutional Ethics regulatory bodies of host research-institute and Goa state's head government hospital viz.Institutional Human-Ethics Committee of BITS-Pilani, India (Reference No:-IHEC/11M/2) and Institutional Ethics Committee of Goa Medical Hospital (Dated 20th July 2015) respectively.

3.3.2.2 Study design and selection of the study population

The similar study design and stratification of the study population into three study groups as utilized in the demographic and biochemical analysis of the CKDu cases in the Canacona taluka (Chapter 2) was incorporated in the current study as well. A detailed-list of Canacona sub-district's CKD-affected patients were obtained from two main hospitals in Goa that conduct dialysis for these patients -Apollo Victor hospital and Canacona Health Centre. On analysis it was found that 142 from a combined total of 180 CKD affected patients were hailing from the Canacona taluka. From this 80% of the CKD affected patients (n=114) possessed no defined etiology hence the CKD among these subjects was recategorized as Chronic kidney disease of unknown etiology (CKDu). These CKDu affected subjects were residents of two villages namely Ponsulem and Chaudi, that were located in the vicinity of a non-operational granite mine. Hence for convenience the residing subjects in these two CKDu endemic villages were cumulatively grouped under one broad category i.e. study-group 1 and their area of residence was clubbed as study-area 1.The remaining 28 patients from the grand total of 142 patients possessed known etiology of CKD related to traditional causals of diabetes and hypertension. These CKD affected subjects were hailing from scattered villages of the taluka namely Cola, Poinguinim and Anvali and were collectively grouped under the non-endemic study-group 2 and their area of residence categorized as study-area 2. For selection of true-controls, a total of 124 volunteers with a 1: 1 sex ratio from two healthy villages of the taluka namely Molorem and Endrem (showing no prevalence of CKD whatsoever) were randomly chosen to match the age and sex distribution of the

CKDu cases. These healthy volunteers from both the non-CKD prevalent villages were clubbed together under study-group 3 with their area of residence grouped as study-area 3. The details of the three study-groups are presented in Table 2.1 of Chapter 2 (demographic and biochemical study of the CKDu cases) of this thesis.

3.3.2.3 Sample collection and preparation/processing/extraction of the nephrotoxins

Blood samples were collected under trained medical supervision by the hospital staff following attainment of written informed consent from the study subjects. The subjects provided the consent after we provided satisfactory enlightening on the study objectives, protocols and implications to the participants. Whole blood samples (5 ml) were collected from the peripheral vein of these study subjects into heparin-EDTA coated blood vacutainer tubes and preserved at -20˚C until analysis (Vlahos et al., 2018; Wanigasuriya et al., 2017)

3.3.2.4 Analytical methods

3.3.2.4.1 Reagents/chemicals

Multi-element (1000 mg/L) and Rhodium (1000 mg/L) and solution were obtained from PerkinElmer (Waltham, MA, USA). Certified Silicon reference standard solution (for estimation of silica since silicon is the major constituting element of silica) of concentration 1000 mg/L was procured from Sigma-Aldrich (MA, USA). All containers and glassware were cleaned by soaking into 20 percent nitric acid for at least 24 h and rinsed three times with deionized water prior to use. Deionized water (18 MΩ/cm resistivity) prepared from Merck Millipore system (MilliQ IQ/7003 ultrapure and pure water system, MA, USA) was used for preparation of all working solutions and utilized for all spectrophotometric analysis. All the reagents used were of analytical grade and all solutions

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3.3.2.4.2 Glassware and instruments

All the plastic ware (i.e. polyethylene bottles and tubes) used for analysis were carefully cleaned and rinsed with HNO_3 to avoid essential element, heavy metal, silica, ochratoxin and aristolochic acid contamination. This was achieved by first washing the apparatus thoroughly with water and detergent followed by rinsing with tap water and deionized water. The cleaning was eventually terminated by the final soaking in dilute acid (i.e. 500 ml conc.HNO_3 + 4500 mL deionized water) for 2 hours followed by rinsing with the same dilute acid and 5 extensive washings in deionized water and ultimately drying in a class-100 laminar flow

hood before use. A clean laboratory and laminar-flow hood capable of producing class 100 (Thomas Scientific, NJ, USA) were used for preparing solutions (Al-Saleh et al., 2017)**.** Inductively coupled plasma mass spectrometer (NexION 350 ICP-MS, PerkinElmer, Massachusetts, USA was used for the estimation of essential elements and heavy metals (except for Hg). The AAS spectra for Hg were determined using a Shimadzu AA-7000 atomic absorption spectrophotometer equipped with a cold vapor generator (CVG-1 system, Shimadzu, Tokyo Japan). Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) (iCAP6300 model, Thermo Scientific, Waltham, MA, USA) was used for estimation of silicon (the major constituting element of silica). Absorbance spectra for ochratoxin and aristolohic acid was measured on a ELISA plate reader (MultiSkanFC, Thermo-Fisher, MA, USA). Microwave oven (Multiwave 3000, Anton Paar, Bangalore, India) was used for sample digestion.

3.3.2.4.3 Sample processing and extraction of different essential elements and nephrotoxins in solution from the blood collected from the study population

3.3.2.4.3.1 Extraction of essential elements (i.e. Cu, Zn, Fe, Se and Mn), trace geogenic nephrotoxic element (viz. silica) and heavy metals (i.e. Pb, Cd, Hg and As) and into the sample solution

Essential elements like Cu, Zn, Fe, Se and Mn are required for basic metabolic activities like bone-mineral homeostasis and conferring anti-oxidative protection by constituting the structure of these anti-oxidative enzymes (Jan et al., 2015; Nordberg and Nordberg, 2016), therefore are constantly in circulation and desired to be maintained at optimal levels. Owing to their crucial functions in homeostatic maintenance of basic metabolism, they have to be frequently transported and distributed to various tissues of the body through the major transporting medium i.e. blood. These essential element lack the ability to bind to erythrocytes (RBC's) in the blood and hence their transport is regulated via binding of these essential metals to certain metal binding proteins/transporters (such as metallothioneins, metal transport proteins etc) that are solely enriched and localized in the serum fraction of the blood. Hence the levels of essential elements in the human body can be accurately estimated by measurements of the same in the serum fraction of the blood (Kowalska et al., 2015).

This estimation of the levels of these essential elements in the whole blood (specifically the serum fraction) provides an accurate indicator of the induction of metabolic and cellular stress by various toxicants like nephrotoxins in this case, wherein the concentrations of these

elements are severely reduced below optimal levels, thereby disturbing metabolic homeostasis. Nephrotoxins like heavy metals and the trace geogenic element/metalloid viz. silica are freely circulating in the blood owing to their long biological half lives in the range of 20-50 days, which makes the estimation of these elements in the whole blood as the most reliable indicator of exposure (Goyer, 2016).

Majority of the essential elements and the heavy metals (except for Hg) can be rapidly and efficiently estimated in the serum and whole blood respectively by utilization of the ICP-MS. protocol (Gajek et al., 2013). For estimation of the nephotoxins in the blood, the whole blood can be directly used for the sample extraction without any pretreatment (Mozrzymas, 2018). However, in order to extract the essential metals from the blood, the blood needs to be pretreated, that is the serum fraction of the blood has to be preliminarily isolated. This was achieved by allowing the whole blood to clot followed by centrifugation at 1000g for 10 min, after which the supernatant was isolated to give the serum portion of the blood. Since both the essential elements (i.e.Ca, Mn, Fe, Zn, Cu and Se) & nephrotoxins like heavy metals (i.e. Pb, Cd and As) are generally estimated in the various components of the same biological medium i.e. blood (with the former being estimated in the serum and the latter in the whole blood viz. the erythrocytes), both these categories of elements [i.e. essential and heavy metals except (Hg and silica)] can be extracted from the serum and blood by a common extraction procedure (Ivanenko et al., 2013). In the current study, the extraction procedure reported by Ivanenko et al., 2013 was adopted with a few modifications. For this 200 µL of the blood or serum samples, were pipetted into 15 ml conical tubes. To this, 500 µL of TMAH solution (10% v/v) was added and allowed to incubate at room temperature for 10 min, whose volume was then made up to 10 ml with a solution containing 0.05% w/v EDTA + 0.005% v/v Triton X-100. Moreover, rhodium was added as internal standard for matrix matching calibration to achieve a final concentration 10 µg/L in the sample solution which was then directly analyzed by ICP-MS for the studied heavy metals (except mercury) and the essential elements.

The presence of recently emerging trace geogenic nephrotoxin viz. silica is ideally estimated in the whole blood by measuring the presence of its constituting element viz. silicon in the blood that directly depicts the extent of silica exposure. ICP-AES is a well reported and preferred technique for the estimation of silica (specifically silicon) owing to the high specificity, large sensitivity and rapid detection times conferred by this technique (Bercowy et al., 2016). On the other hand, another nephrotoxic heavy metal (i.e. mercury) is ideally detected by a technique homologous to the ICP-AES i.e. cold vapor AAS (CV-AAS) due to the carcinogenicity of the mercury which inevitably requires the conversion of the Hg to their

less toxic and stable forms (i.e. its elemental form) that simplifies the accuracy in detection and the easy eradication of the matrix effects achieved in this technique (Gundacker et al., 2010; Shirkhanloo et al., 2015). In brief for the microwave digestion process, 0.75 ml of the blood sample was transferred into a Teflon PFA digestion vessel, to which the digestion mixture (comprising of 2ml of suprapur grade HNO_3+ 2 ml of conc. H_2O_2+ 8 ml of 18MΩ deionized water) was added and allowed to incubate for 10 min. Following which the vessels were sealed and placed in a microwave which was then heated by following a one-step digestion programme (i.e. 250W for 3-4 min). After digestion, the samples were allowed to cool at room temperature (25 ˚C) and the resultant solution was filtered through Whatman No. 42 filter paper into a volumetric flask (50 ml) which was made up to the mark with 0.2 M HNO_3 (Shirkhanloo et al., 2015). This filtered digest solution was further utilized for analysis of silica (i.e. silicon) and Hg by ICP-AES and CV-AAS respectively.

In all the sample extraction procedures for ICP-MS, ICP-AES and CV-AAS analysis, a reagent blank (i.e. devoid of sample) was also prepared in the similar manner to rule out any contamination from the chemicals used in the digestion process (Bercowy et al., 2016; Ivanenko et al., 2013; Shirkhanloo et al., 2015).

3.3.2.4.3.2 Extraction of ochratoxin and aristolochic acid into the sample solution from the blood collected from the study population

Ochratoxin and aristolochic acid are mainly estimated in the serum fraction of the blood owing to its lack of the ability to bind to the erythrocytes in the blood that allows them to freely circulate in the fluid portion (i.e. serum) of the blood. Being anionic it cannot bind to the negatively charged surface of the RBC's due to repulsion, hence are transported and taken up into circulation by binding to certain organic anionic transporters/proteins that are highly present in the serum fraction of the blood (Burckhardt and Burckhardt, 2011; Jadot et al., 2017; Stiborová et al., 2016). Therefore for analysis of these two nephrotoxins, the serum fraction of the blood was preliminarily isolated by the procedure as described earlier. For the extraction of ochratoxin from the serum, the procedure recommended by the guidelines of the Ochratoxin ELISA kit (Helica Biosystems Inc., Fullerton, CA, USA) was followed. For this, 0.25 ml of serum was mixed vigorously with 0.75 ml extraction solvent (i.e. absolute methanol) on a rotary shaker (MaxQ 2000, ThermoFisherScientific, Waltham, MA, USA) which was then allowed to incubate for 10 min at room temperature. This was followed by clarification of the digest solution by filtration of the same through Whatmann no. 1 filter paper and the filtrate containing the extracted ochratoxin from blood was further subjected to

analysis by ELISA (Khlifa et al., 2012). For the extraction of aristolochic acid from the serum, the procedure recommended by the guidelines of the Aristolochic acid ELISA kit (Helica Biosystems Inc., Fullerton, CA, USA) was adopted. For this, 0.25 ml of serum was mixed briskly with 0.75 ml extraction solvent (i.e. acetonitrile/water, 60:40, v/v) using a homogenizer (DB5000A, ThermoFisherScientific, Waltham, MA, USA) for 15 min which was then allowed to stand at room temperature for an additional 10 min. Subsequently the digested solution was filtered through Whatmann no.1 filter-paper and the filtrate containing the extracted aristolochic acid was subjected to analysis by ELISA (Jelaković et al., 2015).

3.3.2.4.4 Estimation of various essential elements in the serum and nephrotoxins in the blood collected from the study population

Various analytical methods like atomic absorption spectrometry (AAS), Inductively coupled Plasma Mass spectrometry (ICP-MS), HPLC (high performance liquid chromatography), ICP-AES, ELISA have been widely utilized for the determination of the currently studied essential trace elements and nephrotoxins in a number of biomonitoring studies till date, with each method possessing their respective share of advantages and disadvantages. After critical evaluation of these analytical methods from detailed review of literature and appropriate weighing of the pros and cons for each of these methods, we narrowed down the analysis of essential elements and heavy metals (except Hg) in serum and blood respectively by ICP-MS, Hg by cold vapor AAS, silica by ICP-AES and ochratoxin and aristolochic acid in the serum by ELISA. These methods were shortlisted for analysis owing to the ease of sample extraction for analysis, rapid and energy saving detection process, displays significantly low limit of detections and confers high sensitivity and specificity for determinations as compared to other reported methods (Cooper et al., 2013; Gökmen et al., 2013; Nordberg et al., 2014; Vettorazzi et al., 2014). The details of each analytical method employed for determination of various essential elements and nephrotoxins (viz. heavy metals, silica, ochratoxin and aristolochic acid) in the blood are described below.

3.3.2.4.4.1 Determination of essential elements (i.e. Ca, Mn, Zn, Cu, Fe and Se) and heavy metals (i.e. Pb, Cd, As) by ICP-MS and the highly carcinogenic heavy-metal (i.e. Hg) by cold-vapor AAS

Flame Atomic absorption spectrometry (AAS), is still used for the routine biomonitoring of heavy-metal exposure and essential trace element exposure and/or deficiency (Barton, 2011; Olmedo et al., 2010; Sardan et al., 2010). However, the use of inductively coupled plasma–

mass spectrometry (ICP-MS) is recently predominating in clinical lab analysis due to its distinct advantages in comparison with the AAS techniques which includes multi-element measurement ability combined with much lower detection limits. Moreover, ICP-MS provides a linear and wider dynamic range, which allows for determination of trace essential elements and heavy-metals at the same sample injection in one go (D'Ilio et al., 2010; Gil et al., 2015; Lemos and de Carvalho, 2010). Hence the ICP-MS method was used in the current study as well for biomonitoring of essential elements and heavy-metals (except Hg) by a procedure reported by Ivanenko et al (2013) with a few modifications. For this, multi-element stock solutions containing 1 g/L of each element were obtained from Perkin-Elmer (PerkinElmer, Waltham, MA, USA). Analytical calibration standards were prepared daily in 25ml volumetric flasks over the range 0-500 μg/ L for heavy metals (i.e. Pb, Cd, As) and a range of 0-150 mg/L for essential trace elements (Ca, Mn, Zn, Cu, Fe and Se) by suitable serial dilutions of multi-element stock solution in 0.5% v/v TMAH + 0.05% w/v EDTA + 0.005% v/v Triton X-100 to prepare a calibration curve for obtaining the unknown concentrations. Rhodium was used as internal standard at the concentration 10 μg/L Rh prepared from a Rhodium stock solution (of 1g/L), that was obtained from Perkin-Elmer (Waltham, MA, USA). TMAH was purchased from Sigma (MA, USA) and its solution was prepared in 18 MΩ deionized water (25% w/v). PerkinElmer NexION 350 ICP-MS, Massachusetts, USA

For the ICP-MS analysis, a Perkin Elemer ICP-MS instrument (NexION 350 ICP-MS, Massachusetts, USA) with high-purity argon (99.99%), which utilized a Meinhard concentric nebulizer (Perkin Elemer, Waltham, MA, USA) connected to a cyclonic spray chamber was used. A radiofrequency (rf) of 1100 W power was selected in pulse mode with autolens one and sample uptake rate was set at 1.1 ml/min. Sample data were acquired by using 20 sweeps/reading, 1 reading/replicate, and a dwell time of 60 ms. Argon nebulizer gas flow, auxillary gas flow and plasma gas flow rate were optimized daily from 0.5 to 0.9 L/min, 1.5-1.85 L/min and 12-15L/min respectively. Data were acquired in counts per second (cps). The following isotopes for the essential elements and heavy metals were selected: ^{44}Ca, ^{66} Zn, ^{63}Cu, ^{55}Mn, ^{56} Fe, ^{82}Se, ^{208}Pb, ^{111}Cd and ^{75}As. For verification ,the absorbance of the reagent blank (prepared in the same manner as the digested sample to determine the contamination of the reagents used during sample extraction), working standards of each of the analysed heavy metals cum essential elements and the sample solutions were recorded at their respective metal specific wavelength, after which the corresponding heavy metals and essential element

content were calculated from their standard calibration curves by interpolation (Gajek et al., 2013; Ivanenko et al., 2013).

On the other hand, the heavy metal-Hg were determined by using a Shimadzu AA-7000 atomic absorption spectrophotometer equipped with cold vapor generator (HVG-1 system, Shimadzu, Tokyo, Japan). The measurement of Hg by cold vapor atomic absorption spectrometry (CV-AAS) was based on the reduction and volatilization of mercury from the sample digest solution to its less toxic elemental state following a chemical transformation by sodium borohydride without generating hydrides. Since most of the commercially available Hg standards are usually prepared from their respective inorganic salts (i.e. Hg(I) salts, it was mandatory for the Hg standard and sample digest solutions containing unknown concentration of these metals to be correspondingly reduced to the elemental form of Hg for accurate estimation of the same (Nordberg et al., 2014). In the current study, Hg was estimated by the method reported by Shirkhanloo et al (2015) with a few modifications. The purity of argon and acetylene gases used for flame generation was 99.999% and 99.99% respectively. Six working standard solutions over a range of 0-500µg/L were daily prepared in 25ml volumetric flasks by appropriate dilution of their respective 1000 µg/L stock standard solutions using 1%(w/w) suprapure grade nitric acid (Merck, Mumbai, India) to prepare the calibration curve for calculation of the unknown concentrations. Hollow cathode lamps for were used for the determination of Hg (253.7 nm and slit 0.5 nm) and the conditions were operated according to the guidelines recommended by the manufacturer as given in the Instruction manual accompanying the instrument for estimation of the unknown concentrations of the Hg in the test samples. For verification, the absorbance of the reagent blank (prepared in the same manner as the digested sample to determine the contamination of the reagents used during sample extraction), working standards of Hg and the sample solutions were recorded at its metal specific wavelength, after which the corresponding Hg content were calculated from their standard calibration curves by interpolation (Shirkhanloo et al., 2015). The detection limits for the heavy metals were:-Cd, 0.1 µg/L; Pb, 1 µg/L; Hg and As- 0.2 µg/L.

3.3.2.4.4.2 Determination of silica (specifically its major constituting element silicon) by ICP-AES

It has been well reported that in order to accurately estimate the silica content in clinical samples like blood, it is obligatory to measure its major constituting metallic element viz. silicon in such matrices as this element confers specificity for detection of silica and

moreover the other constituting element (i.e. oxygen) being non-metallic cannot be subjected to the atomization process commonly employed for the detection of metals in various analytical techniques (Maraschi et al., 2013). One of the traditionally used methods to measure the silica(specifically silicon) levels in the clinical samples like blood is ICP-AES which has a few advantages over the AAS technique that include ease of sample processing, rapid and energy saving detection process, high sensitivity and selectivity and lower limits of detection (Bercowy et al., 2016; Chiappini et al., 2015). Hence in the current study, the silica (especially silicon) was estimated by the ICP-AES method described by Bercowy et al.(2016) with the a few modifications. For this the inductively coupled Plasma Atomic Emission Spectrometer (iCAP6300 model, Thermo Scientific, Waltham, MA, USA) was set up as previously reported (Bercowy et al., 2016). The instrument settings for detector sensitivity (-800 V) and slit width (horizontal slit: 50 microns; vertical slit: 200 microns) were optimized in accordance with manufacturer's instructions. The silicon emission line at a wavelength of 251.611 nm was used for the analysis. For all purposes, triplicate dilutions were run for each sample. Analytical working calibration standards were prepared daily in 25ml volumetric flasks over the range of 0-500 mg/ L by suitable serial dilutions of a 1000 mg/L stock solution of a certified silicon reference standard obtained from Sigma-Aldrich (MA, USA) in 1%(w/w) suprapure grade nitric acid (Merck, Mumbai,India). Using these working standards, a six-point calibration curve was obtained from the emission intensity v/s the standard concentrations for calculation of the unknown concentrations in the test samples. Results were deemed reproducible when the standard deviation in the emission intensities was less than 5%. The silica levels were reported in mg/L in the blood samples (Bercowy et al., 2016).
The accuracy of all the aforementioned analytical methods (i.e. ICP-MS, CV-AAS and ICP-AES) were evaluated by analysing a certified reference material viz. NIST CRM specific for the human blood by the same procedure used for their respective test sample analysis for every 20 samples. If the mean recoveries of the analysed metals remarkably deviated from the certified values, the instrument would have to be recalibrated. However in the current study the mean recoveries of the analysed metals were in the range of 97.3 % to 99.1%, indicating a good agreement between certified and measured value thereby negating the requirements for frequent recalibration (Bercowy et al., 2016; Chiappini et al., 2015; Gajek et al., 2013; Ivanenko et al., 2013).

3.3.2.4.4.3 Determination of ochratoxin by ELISA

The measurement of ochratoxin levels in the digested serum samples were done by a solid-phase direct competitive enzyme immunoassay technique using MycoMonitor Ochratoxin A ELISA assay kit (Helica Biosystems Inc., Fullerton, CA, USA) according to the manufacturer's instructions. In brief, ochratoxin levels in test samples (serum samples) were estimated from the standard curve of 6 provided ochratoxin standards of increasing concentrations i.e. 0.2, 0.4, 1, 2, 4, 8 µg/L. Ochratoxin in the serum was analysed in the same manner as that followed for analysis in food grains (Please refer section no. **3.3.2.4.4.3)**. A dose-response curve of absorbance versus concentration using the provided working standards of ochratoxin in the range from 0.2-8 µg/L ppb was constructed and the unknown concentration in the test serum samples were measured by interpolation from the standard curve.

Each blood sample was analysed in triplicates and the average results were reported for better reproducibility

3.3.2.4.4.4 Determination aristolochic acid by ELISA

The levels of aristolochic acid(AA) in the serum sample digest or extract was estimated by the solid-phase sandwich enzyme immunoassay technique using PhytoMonitor Aristolochic acid ELISA assay kit (Helica Biosystems Inc., Fullerton, CA, USA) in accordance with the manufacturer's guidelines. To summmarise, levels of aristolochic acid in the test serum samples were determined from the standard curve of 6 furnished standards of increasing concentrations i.e. 0.1, 0.25, 0.50, 1, 2 and 5 µg/L. Aristolochic acd in the serum was analysed in the same manner as that followed for analysis in food grains (Please refer section no. **3.3.2.4.4.4)**. A dose-response curve of absorbance versus concentration using the provided working standards of AA in the range from 0.1-5 µg/L was constructed and the unknown concentration in the test samples were estimated by interpolation from the standard curve.

Each blood (i.e. serum) sample was evaluated in triplicates and the mean values were reported for better reproducibility.

In general, the overall prevalence and distribution in various concentration ranges for all the measured essential elements and the nephrotoxin were analysed across the three major demographic variables/risk-factors (i.e Sex, age and occupation) to assess for differences(if any) in the nephrotoxin exposure in these demographic groups in order to establish the susceptible segments of the population that are at higher risk of developing CKDu in the

Canacona taluka (Bercowy et al., 2016; Chiappini et al., 2015; Gajek et al., 2013; Ivanenko et al., 2013; Jelaković et al., 2015; Khlifa et al., 2012).

3.3.2.4.5 Statistical analysis

The analysis of the biomarkers of nephrotoxin exposure i.e. presence of the nephrotoxin in the blood sample was duplicated for better data reproducibility. All statistical analyses were performed with SPSS (Version 20.0) for Windows. The data for each continuous variable were expressed as a mean±SD with a range of minimum and maximum values provided of two independent experiments. The differences between the values of the continuous variable of the three study groups (i.e. CKDu affected groups, diabetic and hypertensive CKD affected group and healthy control groups) were analysed by one-way analysis of variance (ANOVA) test and Dunnett's test was applied post-hoc to analyse the difference in the biochemical variable distributed among various demographic variables like sex, age and occupation.

The interrelationship between the presence of nephrotoxin concentrations in the major exposure sources i.e. drinking groundwater and food and the resulting levels of the biomarkers of exposure in the blood (i.e. the levels of these nephrotoxins in the blood) were independently identified by the univariate correlation assessments by calculation of the Pearson's correlation coefficients (r) between these groups with their respective p-values. This helped to identify the accurate and significant ($p<0.05$) contributing source of the nephrotoxin absorption in the body and circulation of the same in the blood (Nanayakkara et al., 2014; Wijkström et al., 2018).

The relationship or association between various measured nephrotoxins in the blood samples were analysed via calculation of Pearson's correlation coefficients (r) with their respective p-values to identify the significantly ($p<0.05$) common source of origin (if any) of these nephrotoxins, enabling the adoption of necessary remediation measures to eradicate the source of exposure which could assist in the possible reduction of the CKDu incidence in the taluka (Jayasumana et al., 2011; Levine et al., 2016).

Moreover the association with the levels of essential elements in influencing the bioavailability of the nephrotoxins in the blood for the resulting induction of renal toxicity effects was examined by measurement of statistically significant Pearson's correlation coefficients (r) (Jayatilake et al.,2013;Levine et al.,2016).

Additionally,independent interrelationships between level of biomarkers of exposure (i.e.nephrotoxin levels in blood) with levels of the biomarkers of renal damaging effect (i.e.

tubular or glomerular nephropathy specific indices, as already determined in Chapter 2 of this thesis) was examined via calculation of Pearson's correlation coefficients (r) with their respective p-values.This was done to understand and establish the significant ($p<0.05$) dose-response relationships between nephrotoxin exposure and possible renal damage inflicted i.e. tubular dysfunction, thereby permitting us to confirm the etiological contribution of specific chronic nephrotoxin exposure in the development of tubular specific pathology of CKDu in the taluka(Fadrowski et al., 2013; Jelakovic et al., 2015; Levine et al, 2016; Lin et al., 2006; Tatapudi et al., 2018; Wijkström et al., 2018; Wesseling et al., 2013).

Differences at *p-values<0.05 were deliberated to be statistically significant in all the sets of assessments (i.e. analysis of the continuous variables between the three study groups and correlation assessments).

3.3.3 Results and discussion

In order to assess and confirm the contribution of potential causal nephrotoxins (derived from analysis of their major exposure sources i.e. water & food) in development of CKDu (i.e. renal tubular nephropathy) in Canacona, the most appropriate way will be to monitor the presence of these toxins in biological matrices (i.e.biomonitoring) so as to confirm exposure of individuals to these substances.Through biomonitoring we can assess various biochemical marker of nephrotoxin exposure (i.e. concentration of nephrotoxins in the blood) and induced renal tubular damage (biomarkers of effects in the urine) in order to establish a dose-response effect relationship of the internal level of nephrotoxin exposure (i.e. blood nephrotoxin levels) in the induction of tubular dysfunction by examining associations between blood nephrotoxins levels (biomarkers of exposure) and levels of tubular injury specific biomarkers-uBCR, uNCR (biomarkers of effect).

3.3.3.1 Levels of the biomarkers of nephrotoxin exposure (i.e. concentration of nephrotoxins) in blood of the study population

In our previous environmental monitoring study of various nephrotoxins in major exposure sources i.e. drinking groundwater & food (described in previous 2 sections of this chapter), it was strongly suggested that chronic exposure to borderline lead levels & high silica levels via long-term intake of contaminated groundwater seem to be potential causals responsible for CKDu development in Canacona. Thus in order to verify the contribution of these nephrotoxins exposure in CKDu induction, levels of these nephrotoxins were analysed in the blood and compared with respective WHO permissible levels in CKDu affected subjects & non-affected subjects (comprising of general CKD cases & healthy controls).The levels of other unsuspected nephrotoxins (i.e. Cd, As, Hg, ochratoxin, aristolochic acid) as established from the environmental monitoring study (described in previous 2 sections of this chapter) were also measured in blood to conformingly rule out the role (if any) of these toxins in CKDu induction.

The mean concentrations of all nephrotoxins analysed in the blood with a range of minimum to maximum values noted in the 3 study groups for both sexes are detailed in **Figure 3.3.1 & Table 3.3.1.**

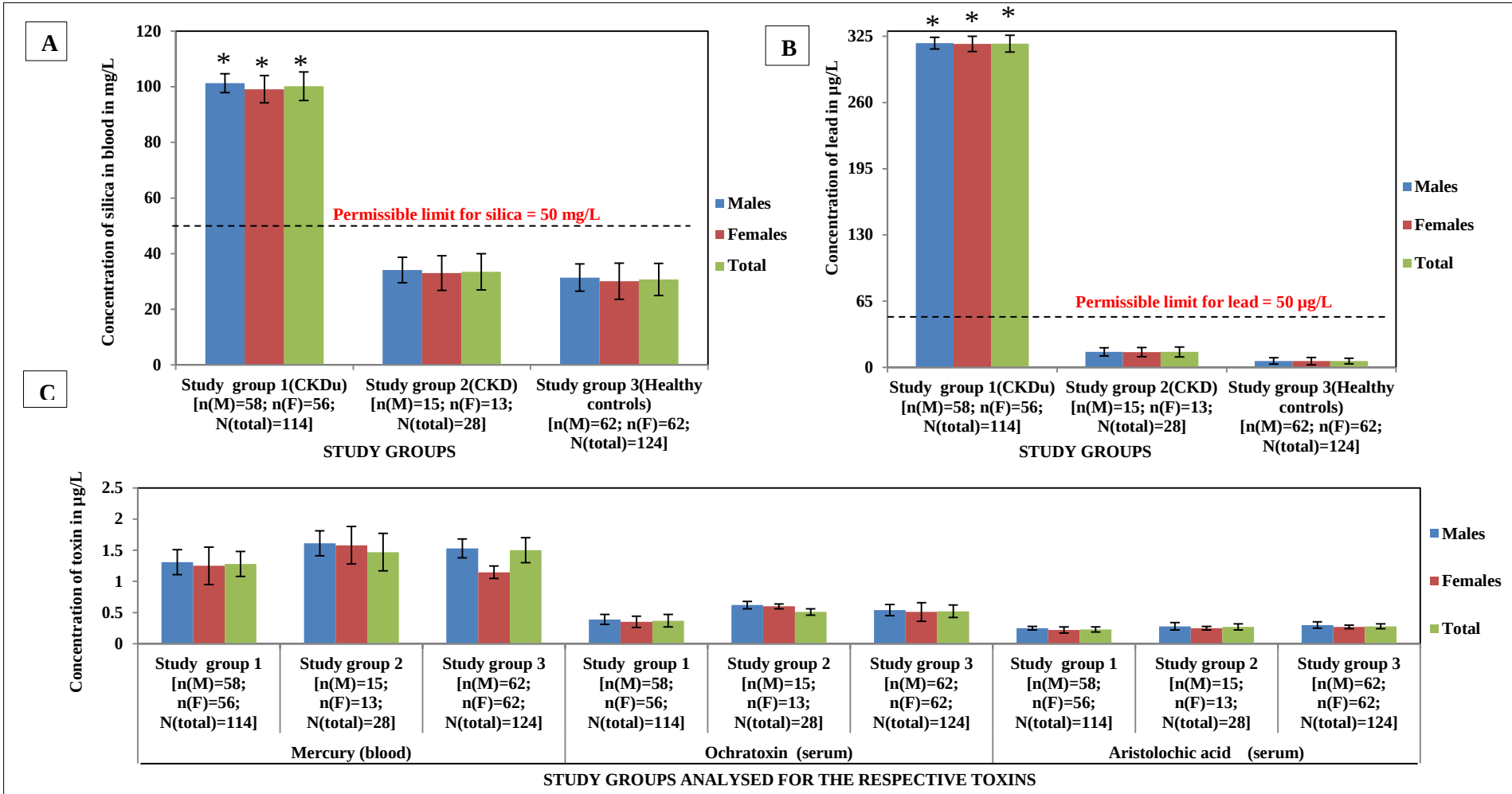


Figure 3.3.1. Mean levels of the nephrotoxins in the blood of both sexes of all three study groups viz. CKDu affected group, diabetes and hypertensive CKD affected group and healthy controls

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; M-male; F-female. Data are derived from two independent experiments with each nephrotoxin of an individual experiment measured in duplicates.Study group 1, 2 and 3 comprises of the CKDu affected subjects, diabetes or hypertension induced CKD affected subjects and healthy controls subjects respectively. n(M) and n(F) in each study group denotes the total number of males and females respectively from whom the blood samples for nephrotoxin analysis were obtained.n(M) + n(F)=N(total) of a given study group which represents the total number of subjects in that group.(A)Concentration of silica in the blood of the male subjects,female subjects and both sexes combined of all three study groups(B) Concentration of lead in the blood of the study subjects (C) Concentration of mercury in blood & ochratoxin and aristolochic acid in serum of the study subjects. Values are represented as mean±SD(standard deviation) of the nephrotoxin concentrations estimated independently in the male sex,female sex and both sexes combined for all the three study groups. All concentration values are in µg/L except for silica which is depicted in mg/L. Differences at *p<0.05 were considered to be significant. The WHO established permissible limits in blood for toxins like silica= 50mg/L; lead=50µg/L; mercury=10 µg/L and in serum for ochratoxin=5µg/L; aristolochic acid=2µg/L. Cadmium and Arsenic were present at below detectable levels in the blood of all three study groups' subjects, hence are not represented in the figure. Significantly higher levels of the nephrotoxins (viz.silica and lead) were noted in the blood of the CKDu affected subjects(study group 1) as compared to the diabetes or hypertensive CKD affected group(study group 2)and healthy control groups(study group 3) and were also found to remarkably exceed their respective WHO established permissible limits thus indicating significantly higher nephrotoxin exposure in study group 1.The blood silica & lead levels in CKDu affected subjects were noted to significantly surpass the limit of 80mg/L & 60 µg/L respectively which are reported in causing oxidative damage to proximal tubules of the renal nephron which ultimately manifests in the Chronic tubulointerstitial nephritis,the major pathological manifestation of CKDu.Hence confirming the causal role of these two toxins viz.silica & lead in CKDu development in Canacona.The details on the range of minimum to maximum values of the levels of each nephrotoxin and prevalence distribution(%) of the nephrotoxin concentration in various ranges for each sex and both sexes combined(i.e.total number of subjects) in all three study groups are listed in **Table 3.3.1.**

Table 3.3.1: Mean and range concentrations of the nephrotoxins estimated in the blood in both sexes of the entire study population and comparison of their levels with the respective WHO established normal reference ranges

CKD status		CKDu endemic cases			CKD non-endemic cases			No CKD prevalence (Healthy controls)			Annnotation for the prevalence % of the concentration ranges (in their corresponding units) of the resepctive biomarkers	WHO established permissible limits
Nephrotoxin	Gender	Study group 1 [N(males)=58; N(females)=56; N(total)=114]			Study group 2 [N(males)=15; N(females)=13; N(total)=28]			Study group 3 [N(males)=62; N(females)=62; N(total)=124]				
		Mean	Range (min-max)	Prevalence %	Mean	Range (min-max)	Prevalence %	Mean	Range (min-max)	Prevalence %		
Silica (blood)	M	**101.3± 2.4***Δ	(97.9-103.7)	E=5.2%; F=37.9%; G=2.1%; H=54.8%	34.1± 3.6	(30.6-38.8)	B=46.5%; D=53.5%	31.4± 3.9	(28.0-35.4)	A=41.9%; B=41.9%; C=16.2%	A=27-30 mg/L; B=30-33 mg/L; C=33-36 mg/L; D=36-39 mg/L; E=95-98 mg/L; F=98-100 mg/L; G=100-102 mg/L; H=102-104 mg/L	**50 mg/L**
	F	**99.1± 1.9***Δ	(95.1-102.9)	E=5.2%; F=37.9%; G=3.4%; H=53.5%	33.0±3.2	(27.2-37.9)	B=47.7%; D=52.3%	30.1± 3.5	(27.2-34.9)	A=42.9%; B=41.6%; C=15.5%		
	Total	**100.2± 2.1***Δ	(95.1-103.7)	E=5.2%; F=37.9%; G=2.8%; H=54.2%	33.5± 3.5	(27.2-38.8)	B=47.1%; D=52.9%	30.7± 3.8	(27.2-35.4)	A=42.4%; B=41.8%; C=15.8%		
Lead (blood)	M	**318.2±3.7***Δ	(315.6-322.3)	G=5.2%; H=37.9%; I= 56.9%	15.3± 1.9	(15.0-15.8)	D=46.7%; E=6.7%; F=46.7%	6.4± 0.9	(5.9-6.8)	A=21.4%; B=62.5%; C=16.1%	A=5.5-6.0 µg/L; B=6.6-6.5 µg/L; C=6.5-6.9 µg/L; D=15.0-15.1 µg/L; E=15.1-15.4 µg/L; F=15.4-15.8 µg/L; G= 315-317µg/L; H=317-319 µg/L; I=319-322 µg/L	**50 µg/L**
	F	**317.4± 2.9***Δ	(315.2-322.0)	G=5.0%; H=39.5%; I= 55.5%	15.1± 1.5	(15.0-15.6)	D=46.2%; E==7.7% F=46.2%	6.2± 0.7	(5.8-6.7)	A=20.9%; B=62.8%; C=16.3%		
	Total	**317.8± 3.3***Δ	(315.2-322.3)	G=5.1%; H=38.7%; I= 56.2%	15.2± 1.8	(15.0-15.8)	D=46.4%; E=7.2%; F=46.4%	6.3± 0.8	(5.8-6.8)	A=21.2%; B=62.6%; C=16.2%		
Cadmium (blood)	M	BDL	BDL		BDL	BDL		BDL	BDL		BDL	**2 µg/L**
	F	BDL	BDL		BDL	BDL		BDL	BDL			
	Total	BDL	BDL		BDL	BDL		BDL	BDL			
Arsenic (Blood)	M	BDL	BDL		BDL	BDL		BDL	BDL		BDL	**15µg/L**
	F	BDL	BDL		BDL	BDL		BDL	BDL			
	Total	BDL	BDL		BDL	BDL		BDL	BDL			
Mercury (blood)	M	1.31±0.2	(1.0-1.54)	A=5.17%; B=37.9%; C=56.9%	1.61±0.4	(1.53-1.71)	C=6.7%; D=40%; E=53.3%	1.53±0.5	(1.29-1.70)	B=40.9%; D=42.9%; E=16.1%	A=0.95-1.15 µg/L; B=1.25-1.35 µg/L; C=1.35-1.55 µg/L; D=1.55-1.65 µg/L; E=1.65-1.75 µg/L	**10 µg/L**
	F	1.25±0.4	(0.98-1.50)	A=6.1%; B=38.5%; C=55.4%	1.58±0.3	(1.50-1.68)	C=8.5%; D=38.4%; E=53.1%	1.47±0.3	(1.26-1.61)	B=42.9%; D=41.6%; E=15.5%		
	Total	1.28±0.3	(0.98-1.54)	A=5.6%; B=38.2%; C=56.2%	1.60±0.3	(1.50-1.71)	C=7.6%; D=39.2%; E=53.2%	1.50±0.4	(1.26-1.70)	B=41.9%; D=42.3%; E=15.8%		
Ochratoxin (serum)	M	0.39±0.18	(0.23-0.59)	A=5.4%; B=39.9%; C= 54.7%	0.62±0.06	(0.56-0.69)	C=6.7%; D=93.3%	0.54±0.17	(0.34-0.68)	B=37.9%; C=41.9%; D=20.2%	A=0.2-0.3 µg/L; B=0.3-0.4 µg/L; C=0.5-0.6 µg/L; D=0.6-0.7 µg/L	**5µg/L**
	F	0.35±0.16	(0.20-0.55)	A=5.0%; B=41.5%; C= 53.5%	0.60±0.04	(0.54-0.68)	C=7.1%; D=92.9%	0.51±0.19	(0.32-0.65)	B=36.9%; C=45.1%; D=18.0%		
	Total	0.37±0.17	(0.20-0.59)	A=5.2%; B=40.7%; C= 54.1%	0.61±0.05	(0.54-0.69)	C=6.9%; D=93.1%	0.52±0.18	(0.32-0.68)	B=37.4%; C=43.5%; D=19.1%		
Aristolochic acid (serum)	M	0.25±0.03	(0.24-0.28)	A=5.0%; B=36.7%; C= 58.3%	0.28±0.06	(0.27-0.31)	C=4.2%; D=95.8%	0.3±0.05	(0.29-0.33)	B=29.4%; C=44.9%; D=25.7%	A=0.1-0.2 µg/L; B=0.2-0.3 µg/L; C=0.4-0.5 µg/L; D=0.5-0.6 µg/L	**2µg/L**
	F	0.22±0.05	(0.20-0.23)	A=4.5%; B=38.6%; C= 56.9%	0.25±0.03	(0.24-0.28)	C=6.6%; D=93.4%	0.27±0.03	(0.26-0.29)	B=35.5%; C=42.2%; D=22.3%		
	Total	0.23±0.04	(0.20-0.28)	A=4.8%; B=37.7%; C= 57.5%	0.27±0.05	(0.24-0.31)	C=5.4%; D=94.6%	0.28±0.04	(0.29-0.33)	B=32.5%; C=43.5%; D=24.0%		

Abbreviations: BDL-below detectable levels; CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; M-male; F-female; WHO-world health organization. Data are derived from two independent experiments with each nephrotoxin of an individual experiment measured in duplicates. 'N' in each study group depicts the number of subjects in that group from whom the blood samples for nephrotoxin analysis were procured.Values are represented as mean±SD with the range of minimum to maximum concentrations of each nephrotoxin depicted as well. All concentration values are in µg/L except for silica which is depicted in mg/L. The prevalence of the

nephrotoxin concentration in various ranges for each sex category of the three study groups are represented by prevalence % and annotated by uppercase letters (i.e. A to I) for convenience. The annotations for each estimated nephrotoxin varies and have been independently described. The prevalence % denotes the proportion of subjects from the total number of a particular study group in their respective sex categories (N for males and N for females being different in all the study groups) bearing the nephrotoxin at a given concentration range. The total number of males(M)/females(F) in study group one, two and three are 58(M)/56(F); 15(M)/13(F) and 62(M)/62(F) respectively.
For example, the prevalence % annotation of 'A' in the case of blood silica levels denotes the percentage of the total population of a particular study group in a particular sex category possessing concentrations at the range of 27-30 mg/L. Differences at *$p<0.05$ were considered to be statistically significant as compared to the true controls (study group 3). Differences at $^{\Delta}$ $p<0.05$ were considered to be statistically significant as compared to the non-endemic CKD cases(study group 2). The values highlighted in bold denote the levels of the nephrotoxins (viz.silica and lead) in the blood that were significantly exceeding the WHO established normal permissible range and were higher only in the CKDu endemic group(study group 1) depicting higher nephrotoxin exposure in this group.

3.3.3.1.1 Level of lead (Pb) exposure in the blood of the study population

Lead in whole blood is a useful indicator of human exposure to lead.Blood Pb is derived from several body compartments, & is related mainly to recent exposure (Jones et al.,2017).On absorption it freely circulates in the blood stream and majorly accumulates in the kidney and bone. In the bone it competes with calcium (Ca) binding sites and replaces Ca due to similar in valencies and structure of these 2 metals causing mineral Ca loss and Ca homeostasis disturbance that can result in skeletal disorders as well apart from inducing nephrotoxicity (Assi et al.,2016; Caito et al.,2017; de Souza et al.,2018; Gil et al.,2015; Tomaszewska et al., 2016). This Ca inhibition by lead explains below normal reference levels of Ca noted in CKDu affected subjects blood and prevalence of skeletal pains among them as well (as noted in demographic survey). Due to rapid & specific accumulation in these 2 reserves/target organs of body (i.e. kidney and bone), it can easily be remobilized from these reserves into free circulation in blood due to inherent reabsorptive tendencies of these organs that explains elevated levels noted in blood even after cessation of exposure. Thus, blood Pb concentration is a preferred indicator of human exposure as it accurately reflects the body and renal burden of Pb (García-Esquinas et al.,2015; Kim et al.,2015; Klotz and Goen,2017; Wu et al., 2016).

The kinetics of blood Pb has been well described as a two-compartment model with a fast phase with half-life of 100-150 days and a slow phase with half-life of 25-30yrs. Therefore indicating that blood Pb accurately reflects lead's body burden especially on prolonged and high level exposure and after exposure stops (Buser et al.,2016; Klotz and Goen,2017; Morrissey et al.,2017; Tchounwou et al. 2012). Hence was determined in this study as well. As indicated in **Figure 3.3.1B & Table 3.3.1**, blood Pb levels varied significantly ($p<0.05$) between the 3 study groups with no significant difference noted between both genders for all groups indicating no gender bias in Pb accumulation suggesting a common source of Pb exposure for both sexes. Highest blood Pb level (mean=317.8±3.3 µg/L, min-max=315.2-322.3 µg/L) were noted in CKDu affected subjects (group 1) that significantly ($p<0.05$) surpassed WHO permissible limit of 50 µg/L with lower levels (within permissible limits) noted in general diabetes & hypertensive CKD hit subjects (group 2) [mean=15.2± 1.8 µg/L,

min-max=15.0-15.8 µg/L] & healthy control (group 3) [mean= 6.3±1.0 µg/L, min-max=5.8-6.8 µg/L].

Moreover, blood Pb levels of 94.9% of CKDu affected subjects of Canacona were prevalent in the highest Pb concentration range (i.e.317-322 µg/L) **(Table 3.3.1).** The significantly higher blood Pb levels noted in CKDu affected subjects were in agreement with blood Pb levels noted in CKDu cases of Sri Lanka and Central America wherein such high levels were stated to be indicative of long-term and higher exposure and Pb accumulation in this population, which stemmed from chronic exposure of patients to Pb through diet for approx. 38-40 yr. This accumulated Pb manifested in prolonged induction of tubular injuries (CKDu). Thus highlighting that CKDu hit subjects of this study were also chronically exposed to Pb which resulted in enhanced Pb accumulation and associated induction of tubular damage that manifested in CKDu (Laws, 2015; Levine et al.,2016; Nanayakkara et al.,2014).

Many studies have well-reported a dose response relationship between Pb exposure levels (i.e. blood Pb levels) and degree of renal tubular damage inflicted in analysis of CKDu cases in Sri Lanka and Central America. In these studies, a blood Pb level of > 55 µg/L (range of 50-90 µg/L) over chronic exposure is well reported in causing oxidative injury to proximal tubular structural and functional architecture (Abiola, 2017; Brooks, 2009; Gunatilake et al., 2015; Jayasumana et al., 2014; Jayasumana et al., 2015a; Laws, 2015; Laws et al., 2015; Levine et al., 2016; Lusco et al., 2017; Nanayakkara et al; 2014; Sanoff et al., 2010; Weaver and Jaar, 2015; Wimalawansa, 2016). At such blood levels, Pb forms deposits in proximal tubular cells due to their bioaccumulative tendencies which causes swelling of the tubular mitochondria that disrupts cellular respiration resulting in excessive ROS generation by depleting antioxidant defenses manifesting in oxidative stress. The excess ROS consequently induces DNA damage that ultimately triggers the apoptotic pathway of tubular cell death via activation of p53 apoptotic gene. Simultaneously, the generated ROS signals activation of the inflammatory cascade mediator i.e. NF-kβ which attracts macrophages that triggers inflammation in the interstitium surrounding proximal tubules (Jan et al.,2015; Lentini et al., 2017; Orr and Bridges, 2017; Xu et al., 2018). Therefore this Pb induced prolonged and amplified inflammation and apoptosis in the interstitial and proximal tubular cells at such blood levels ultimately results in interstitial fibrosis and tubular atrophy that manifests in development of chronic tubulointerstitial nephritis (CTN), typical of CKDu which advances in renal function degeneration resulting in renal failure (Gunatilake et al., 2015; Jayasumana et al., 2015 a & b; Levine et al., 2016; Lusco et al, 2017; Nanayakkara et al., 2014;Sanoff et al., 2010; Weaver & Jaar, 2015; Wimalawansa,2016).

Similarly, in the current study as well, the mean blood lead levels (i.e. biomarker of lead exposure) of CKDu affected subjects were observed to be significantly ($p<0.05$) exceeding the levels demonstrated in aforementioned studies (i.e. 50-90 µg/L) and WHO permissible levels (50 µg/L) known to cause oxidative damage to the kidney (specifically proximal tubules) resulting in induction of CTN (typical of CKDu).Thus highlighting and confirming significant etiological contribution (as established from the environmental monitoring study described in this chapter's previous sections) of prolonged exposure to high Pb levels in CKDu development in Canacona.This was further supported by observance of below WHO permissible and non-toxic levels of Pb noted in blood of diabetic and hypertensive CKD subjects viz. study group 2 and healthy controls viz. study group 3 of the taluka thus negating the contribution of Pb nephrotoxin in CKD causation of group 2.

3.3.3.1.2 Level of cadmium (Cd) exposure in the blood of the study population

No such trend as noted in levels of Pb exposure in the blood were witnessed for the other 3 heavy metals (i.e. Cd, As, Hg) in all 3study groups. For cadmium, blood levels of Cd are a useful biomarker of Cd exposure. The blood Cd levels are highly prominent during ongoing exposure and persist even after exposure stops like Pb (Friberg, 2017). This is mainly attributed to the kinetics of Cd which has also been described as a 2 phase model with a rapid phase possessing a half-life of 75-130 days and a slow-phase possessing a half-life of 7.4-16 yrs. Due to its 2 phase kinetics it persistently circulates in the blood and simultaneously accumulates in its main target organ i.e. kidneys (mainly proximal tubules, PT) owing to the inherent potency of the PT to reconcentrate these divalent heavy metals. This accumulation in PT of kidney allows remobilization of Cd from reserves in the kidney into blood due to its reabsorptive properties, thus explaining ability of blood Cd to represent renal & body burden of Cd on both prolonged & terminated exposure (Amzal et al.,2009; Byber et al., 2016; Ishizaki et al.,2015; Rinaldi et al.,2017; Sankhla et al., 2016; Saravanabhavan et al.,2017).

As indicated in **Table 3.3.1**, mean blood Cd levels in all 3 study groups were comparable and below detectable levels (BDL) in both genders indicating no gender bias for cadmium accumulation. Previous studies of CKDu cases in Sri Lanka and Central America have depicted a good dose-effect relationship between blood Cd levels and renal tubular dysfunction (CKDu), thus highlighted Cd's role in CKDu causation. Herein, blood Cd at levels greater than 10µg/L were reported to induce oxidative damage to the proximal tubules that ultimately manifested in tubular atrophy and fibrosis, characteristic of CTN which is typical of CKDu pathology. This Cd induced nephrotoxicity mechanism is detailed in

Chapter1 (Navas-Acien et al.,2009; Bandara et al.,2008; Bandara et al.,2010a; Jayasumana et al.,2016; Price, 2017; Wanigasuriya et al.,2011). However, in the current study, blood Cd levels noted in all 3 study cohorts were BDL being nowhere close to levels known to cause oxidative injury in proximal tubules and were below WHO permissible levels of 5µg/L. Thus signifying the lack of contribution of Cd in CKDu development in the taluka. These results were in agreement with findings of a similar study of CKDu cases in Central America which witnessed negligible levels of Cd exposure in blood from all dietary sources (food, water) thus ruled out Cd as a causal for CKDu in that region at such levels of exposure (Almaguer et al.,2014; Abiola,2017; Correa-Rotter et al.,2014; Correa-Rotter,2017; McClean et al., 2012).

3.3.3.1.3 Level of arsenic (As) exposure in the blood of the study population

As depicted in **Table 3.3.1**, mean blood As concentrations in all 3 study cohorts were similar and BDL in both sexes, again indicating no gender bias for arsenic exposure. Few studies of CKDu cases in Sri Lanka have portrayed a significant dose-response relationship between levels of As exposure in blood and prevalence of renal tubular nephropathy. Thus supported role of As in CKDu induction. These reports stated that, blood As at levels greater than 22µg/L induces proximal tubular nephrotoxicity mediated by oxidative injuries to tubules that manifested in tubular atrophy and fibrosis, characteristic of CTN which is typical of CKDu pathology. This As elicited nephrotoxicity mechanism is detailed in Chapter1 (Athuraliya et al.,2011;Chang and Singh,2018; Levine et al.,2016; Jayasumana et al.,2013; Jayasumana et al.,2014; Jayasumana et al.,2015c; Wanigasuriya et al.,2012). Contrarily, in the present study, levels of biomarkers of As exposure (i.e. As levels in blood) in all 3study groups were noted at BDL being completely divergent from the level known to cause renal tubular dysfunction & WHO established permissible levels of 15 µg/L. Thus highlighting, lack of role of As exposure in CKDu manifestation in Canacona. These findings were consistent with results from analysis of CKDu cases in Central America which had negated As as a causal factor for CKDu in that region owing to observance of negligible levels of As exposure in blood from all dietary sources (food, water) (Almaguer et al., 2014; Abiola, 2017; Correa-Rotter et al.,2014; García-Trabanino et al.,2015; McClean et al., 2012).

3.3.3.1.4 Level of mercury (Hg) exposure in the blood of the study population

As indicated in **Figure 3.3.1C & Table 3.3.1**, mean blood Hg levels in all 3 groups were homologous and well below WHO permissible levels (10µg/L) in both genders, depicting no gender bias for Hg accumulation. Previous analysis of CKDu cases in Central America and

Sri Lanka have documented a significant dose-effect association between blood Hg exposure levels and renal tubular injuries, thus advocated role in CKDu causation (Akerstrom et al., 2017; Levine et al., 2016). In these reports, blood Hg at levels greater than 12µg/L triggered incessant oxidative injury to proximal tubules that disrupted tubular structural and functional integrity which ultimately resulted in tubular proteinuria development (characterized by minimal albumin and high b2M levels in the urine) that is unique to CTN (typical of CKDu pathology) (Bridges and Zalups,2017; Orr and Bridges, 2017). However in the current study, blood Hg levels in all 3 study groups were largely below levels noted to cause oxidative injury to proximal tubules and were well within the WHO set safe and non-toxic levels with 43.1 and 56.9% of CKDu affected subjects being prevalent in the lower two blood Hg concentration range of 0.95-1.35 and 1.35-1.55µg/L respectively **(Table 3.3.1).** Our findings were in accordance with prevalence of blood values of Hg exposure in the lower range noted in the analysis of CKDu cases in Central America which had potentially ruled out the role of Hg in CKDu development of that area at the prevalent exposure levels (Almaguer et al.,2014; Abiola, 2017; Correa-Rotter et al.,2014;García-Trabanino et al.,2015;McClean et al., 2012). Thus signifying no etiological contribution of Hg exposure in CKDu induction in the taluka.

3.3.3.1.5 Level of silica exposure in the blood of the study population

Level of silica exposure in blood can be suitably quantified by estimation of its major constituting element i.e. silicon, as silicon being specific to silica composition confers selectivity for silica detection, unlike non-metallic constituent of silica i.e. oxygen which is ubiquitously distributed in various compounds (Bercowy et al.,2016;Chiappini et al., 2015).

It is well reported that silicon in the blood is an accurate indicator of human exposure to silica (Maraschi et al.,2013; Khandare et al.,2015). Silica (specifically silicon) can persist in blood circulation owing to its bioacccumulative tendencies. This perseverance in circulation is explained by the kinetics of silica which has been described as a 2 phase model with a fast phase of silica occurrence depicting a half-life of 105-150 days and a slow phase depicting a half-life of 32-36 yrs. Due to this kinetics, it is rapidly absorbed into circulation from gastrointestinal tract wherein it largely circulates in the blood & concurrently deposits in its target organ i.e.kidney mainly proximal tubule (PT) accounted to PT's intrinsic concentrating capacity to accumulate metals. Furthermore, due to PT's reabsorptive tendencies, silica is readily remobilized from deposits in the kidney into blood circulation, thereby justifying silica levels in the blood to be indicative of both recent on-going & terminated exposure. Thus silica levels (specifically silicon) in the blood is an optimal indicator of renal & body

load of silica (Ghahramani,2010; Mohner et al.,2017; Sergent et al.,2017; Sponholtz et al, 2016; Tsuchiya et al.,2017). Hence was determined in the current study as well.

As indicated in **Figure 3.3.1A & Table 3.3.1**, the blood silica concentrations (specifically silicon) were significantly ($p<0.05$) different between all 3 study cohorts with no significant variations noted in either sexes in all cohorts depicting absence of a gender predisposition in silica accumulation, thus highlighting a common silica exposure source in both genders. Significantly elevated blood silica levels [mean=100.2±2.1mg/L, min-max=95.1-103.7mg/L] were noted in CKDu affected subjects of Canacona that exceedingly surpassed the previously reported threshold of 90 mg/L (which was well documented in causing significant tubular nephrotoxic effects in animal models and a few human epidemiological studies) (Dobbie & Smith,1982; Newberne & Wilson,1970; Markovic & Lebedev,1965; Radovanovic et al., 1991). Contrarily, blood silica levels in general diabetes & hypertensive CKD subjects (study group 2) [mean=33.5±3.5 mg/L, min-max=27.2-38.8 mg/L] & healthy controls (study group 3) [mean=30.7±3.8 mg/L, min-max=27.2-35.4 mg/L] were significantly lower than the set threshold of 90 mg/L and below levels noted in the CKDu group. Moreover 95% of CKDu subjects were predominant in the highest blood silica concentration range (i.e. 98-104 mg/L). The presence of such significantly elevated blood silica levels in CKDu affected subjects were in accordance with and exceeded the blood concentrations noted in CKDu patients(89-95 mg/L approx.) of the Uddanam region of Andhra Pradesh and Balkan region of Europe; wherein such levels were implicated in CKDu causation in those regions.These studies also stated that such high blood silica levels were symbolic of silica accumulation and prolonged exposure of the population to high silica levels in its environmental exposure source, which in those studies emerged from chronic exposure of patients to silica through the diet (i.e. from water) for approx.35-42 yrs. This accumulated silica resulted in continual induction of tubular injuries typical of CKDu. Thus similarly signifying that CKDu subjects of this study were also chronically exposed to silica (via groundwater intake) which resulted in enhanced silica accumulation and associated induction of incessant tubular damage that manifested in CKDu (Khandare et al.,2015; Radovanovic et al.,1991; Stiborova et al.,2016;Tatapudi et al.,2018).

Few animal studies have well-documented a strong dose-effect relationship between silica exposure levels (i.e. blood silica levels) & degree of renal tubular injuries inflicted on prolonged silica exposure. In these studies, blood silica levels of more than 55mg/L over a long-term exposure via chronic intake of contaminated drinking water caused severe renal histopathological alterations comprising of tubular atrophy, mononuclear cell infiltration & interstitial fibrosis which are characteristic of CTN manifestation (typical of CKDu).

Moreover, a significant elevation in tubular proteinuria levels (indicated by increased urinary excretion of LMV protein-b2M, NAG) were noted with increasing exposure to high blood silica levels in these animals,indicative of silica's role in tubular injury manifestation (Dobbie and Smith,1982; Kawanabe et al.,1992; Newberne and Wilson,1970). This was further backed by observance of a similar urinary increase in tubular injury specific markers (i.e. b2M, NAG) at blood silica levels of greater than 89-95 mg/L noted in human epidemiological studies of CKDu cases of Andhra Pradesh and Balkan (Europe), wherein those patients were chronically exposed to elevated silica for over 35 years via consumption of untreated silica contaminated groundwater. Overall these findings (animal and human studies) strongly highlighted the nephrotoxic potency of silica in induction of tubular dysfunction at blood exposure levels (of > 89-95 mg/L) over a long-term that ultimately progresses to renal failure due to continually induced compromise in renal tubular functional and structural integrity (Goldsmith and Goldsmith,1993; Hotz et al.,1995; Khandare et al., 2015; Markovic and Lebedev,1965; Markovic,1968 and 1974; Markovic and Arambasic,1971; Ng et al.,1993; Radovanovic et al.,1991; Stiborova et al.,2016; Tatapudi, et al.,2018).

In unity with these previous findings of silica dose-response animal models and human epidemiological studies, the current study also reported mean blood silica levels (i.e. the primary biomarker of silica exposure) of CKDu affected subjects to significantly exceed ($p<0.05$) the levels demonstrated in aforementioned studies (i.e. 55-95 mg/L) known to cause renal tubular injuries and associated induction of CTN (typical of CKDu pathology).Thus highlighting and confirming the significant etiological contribution (as established from this thesis'environmental monitoring study) of prolonged exposure to high silica levels in CKDu development in the Canacona taluka.This was further backed by observance of safe, below the threshold and non-toxic levels of silica noted in blood of diabetic and hypertensive CKD subjects viz. study group 2 and healthy controls viz. study group 3 of the taluka signifying absence of a prolonged and a higher rate of silica exposure in these cohorts and successfully negating the role of silica in causality of the diabetic and hypertensive CKD noted in group 2.

3.3.3.1.6 Level of ochratoxin (OTA) exposure in the blood of the study population

No such trend as noted in levels of silica exposure in blood of all 3 study groups were noted for food based nephrotoxins (i.e. ochratoxin & aristolochic acid) analysed in this population.

As indicated in **Figure 3.3.1C & Table 3.3.1**, mean blood ochratoxin levels in all 3 study cohorts were similar in both sexes indicating no gender bias for OTA accumulation. Few reports of CKDu cases in Sri Lanka, Tunisia and Balkan region (Europe) have depicted a

good dose-response relationship between blood ochratoxin levels and renal tubular damage induction, thus supported OTA's role in CKDu causation studies reported that blood ochratoxin at levels greater than 8 µg/L triggered continual oxidative injuries to proximal tubules that disrupted tubular structural and functional integrity which ultimately manifested in tubular atrophy and fibrosis that are characteristic of CTN manifestation, which encompasses the CKDu pathology. This OTA induced renal-toxicity mechanism is detailed in Chapter1 (Abid et al.,2003; Badurdeen et al., 2016; Bui-Klimke and Wu,2015; Desalegn et al.,2011; Gifford et al.,2017; Gruia et al.,2018; Hassen et al.,2004; Khlifa et al.,2012; Pavlovic, 2014; Tao et al., 2018). However, in the current study, the blood OTA in all 3 study cohorts were nowhere close to levels known to cause oxidative damage in the renal proximal tubule and were well-below WHO permissible levels of 5 µg/L with 45.9 and 54.1% of CKDu affected subjects being prevalent in the lower two blood OTA concentration ranges of 0.2-0.4 and 0.5-0.6 µg/L respectively **(Table 3.3.1)**. Our results were in agreement with the prevalence of blood OTA exposure at lower, non-toxic and safe concentration ranges in CKDu patients of Sri Lanka and Central America which had strongly negated the role of OTA as a causal factor for CKDu induction in those areas at the prevalent exposure levels (Almaguer et al.,2014; Abiola,2017; Correa-Rotter et al.,2014; García-Trabanino et al,2015; Levine et al.,2016; McClean et al,2012; Wanigasuriya et al.,2008; Wesseling et al.,2013). Thus, signifying lack of OTA role in CKDu development in Canacona.

3.3.3.1.7 Level of aristolochic acid (AA) exposure in the blood of the study population

As depicted in **Figure 3.3.1C & Table 3.3.1**, mean blood aristolochic acid levels in all 3 study groups were homologous and well below WHO permissible level in both genders,indicating no gender bias for AA accumulation & suggesting a common exposure source in both sexes. Previous analysis of CKDu cases in Sri Lanka and India has documented a significant dose-effect association between blood AA exposure levels and renal tubular dysfunction. Thus advocating role of AA in CKDu manifestation Herein, blood AA at levels greater than 4µg/L inflicted oxidative damage to proximal tubules that manifested in CTN specific renal histological alterations of tubular atrophy and fibrosis, which is typical of CKDu pathology. AA triggered nephrotoxicity mechanism is detailed in Chap.2 (Gökmen et al.,2013; Grollman,2013; Jadot et al.,2017; Jha and Chugh, 2003; Jha2010;Rajapakse et al.,2016; Stanifer et al.,2016;Vanherweghem,1997; Wanigasuriya et al.,2011). However in this study, blood AA levels in all 3 study groups were critically well below concentrations known to induce oxidative proximal tubular injuries and malignancies

and moreover were well within WHO established non-toxic levels (safe) with 42.5 & 57.5 % of CKDu subjects being prevalent in the lower 2 blood AA concentration range categories of 0.1-0.3 and 0.4-0.5µg/L respectively **(Table 3.3.1).** These findings were consistent with the values of blood AA exposure found to be prevalent in lower concentration ranges in CKDu cases in Central America, which had ruled out the role of AA in CKDu development in those area at such levels of exposure (Almaguer et al.,2014; Abiola, 2017;Correa-Rotter et al.,2014; García-Trabanino et al.,2015; McClean et al.,2012; Wesseling et al.,2013). Thus signifying no role of AA exposure in etiology of CKDu in Canacona.

Overall, from the analysis of various biomarkers of nephrotoxin exposure in the blood of the study population, it was evident that silica and Pb seem to be 2 nephrotoxins that majorly contributed to the etiological development of CKDu in Canacona attributed from the significantly ($p<0.05$) higher levels(beyond WHO permissible range) of these nephrotoxins witnessed in the blood of CKDu affected subjects indicative of a prolonged and higher rate of exposure of CKDu patients to these causative nephrotoxins.This was further supported by the observance of significantly lower, safe and non-toxic exposure levels (set by WHO) of these nephrotoxins in the blood of diabetes and hypertensive CKD subjects (study group 2) and healthy controls (group3) depicting no causal role of these nephrotoxins in general CKD development witnessed in Canacona's non-endemic region. Moreover, other nephrotoxins (i.e. Cd, Hg, As, OTA, AA) were present at below their respective WHO permissible range & at a comparable extent in all 3 study groups depicting no involvement of these nephrotoxins in causation of either CKDu or CKD in the endemic and non-region of Canacona taluka.

3.3.3.2 Age, sex and occupation stratified distribution and prevalence of biomarkers of nephrotoxin exposure (i.e. toxins levels in blood) in the study population for prediction of susceptible population groups to nephrotoxin exposure and CKDu development

A critical step in screening the levels of nephrotoxin exposure in the suspected exposed populations for analysis of their involvement in CKDu causation is the assessment of levels of various biomarkers of exposure in the demographically stratified risk groups that are highly susceptible towards development of CKDu in order to check the role of these demographic variables in influencing rate of nephrotoxin exposure. In order to achieve this purpose, an analysis of the demographic distribution of biomarkers of nephrotoxin exposure (i.e. blood nephrotoxin levels) of the study population stratified by three variables i.e. age, sex and occupation is mandatory (Lunyera et al.,2016).Hence this was undertaken in this study.

Previous epidemiological studies of CKDu in Sri Lanka and Central America have suggested CKDu to mainly affects higher age groups and males (Abiola,2017;Lebov et al.,2015;Orantes et al.,2014; Orantes-Navarro et al.,2016; Raines et al,2014; Wanigasuriya,2012). Hence the current study desired to validate these findings by conducting a preliminary analysis of the differences (if any) in presentation and prevalence of these biomarkers (blood nephrotoxin levels) in different ranges across various age groups, both sexes and type of occupation prevalent in the study population. We also investigated the extent to which these typical CKDu risk factors may affect prevalence of biomarkers of nephrotoxin exposure(blood nephrotoxin levels)in the abnormally high ranges and tried to verify that male sex is not a typical risk factor in our study (Rango et al.,2015). Firstly, prevalence distribution of estimated clinical biomarkers of nephrotoxin exposure (i.e. levels of nephrotoxins in blood)in various concentration ranges were analysed across 2 major demographic variable/risk-factor (i.e. age, sex) in the study population, which are presented in **Figure 3.3.2 & Table 3.3.2.** As shown in **Figure 3.3.2 & Table 3.3.2,** mean values of all the biomarkers of nephrotoxin exposure (blood neprhrotoxin levels) did not vary significantly with sex but showed considerable variation with age in all 3 study groups.Mean values of biomarkers of Pb heavy metal exposure and silica exposure (i.e. blood nephrotoxin levels) in CKDu affected subjects (i.e. study group 1) were found to be abnormally and significantly ($p<0.05$) well beyond WHO reference limits and were prevalent at similar ranges of their levels in both males and females of various age groups. Moreover, the levels of these nephrotoxins were comparable in diabetes and hypertensive CKD affected subjects and healthy controls and were well below WHO permissible limits with homologous prevalence of both the groups in the lower nephrotoxin concentration ranges for both genders in each of the age categories. Additionally,other analysed nephrotoxins (i.e. Hg, OTA, AA) were prevalent at comparable levels in their respective lower ranges in all 3 study groups without exceeding WHO permissible limits to a uniform extent in the both sexes as well. Cd, As were excluded from this analysis due to BDL of the same. Thus this similarity in blood nephrotoxins levels noted in both genders in all 3 study groups are indicative of the absence of gender bias for accumulation of any of the nephrotoxins, further suggesting a common source of nephrotoxin exposure in both sexes. Thus further supporting lack of a gender predisposition in CKDu development, confirming sex to be not a risk factor in this disease manifestation of Canacona which was consistent with the results of CKDu analysis of Sri Lanka (Badurdeen et al., 2016; Rajapakse et al., 2016; Wanigasuriya et al., 2011) & Andhra Pradesh (Ganguli, 2016).

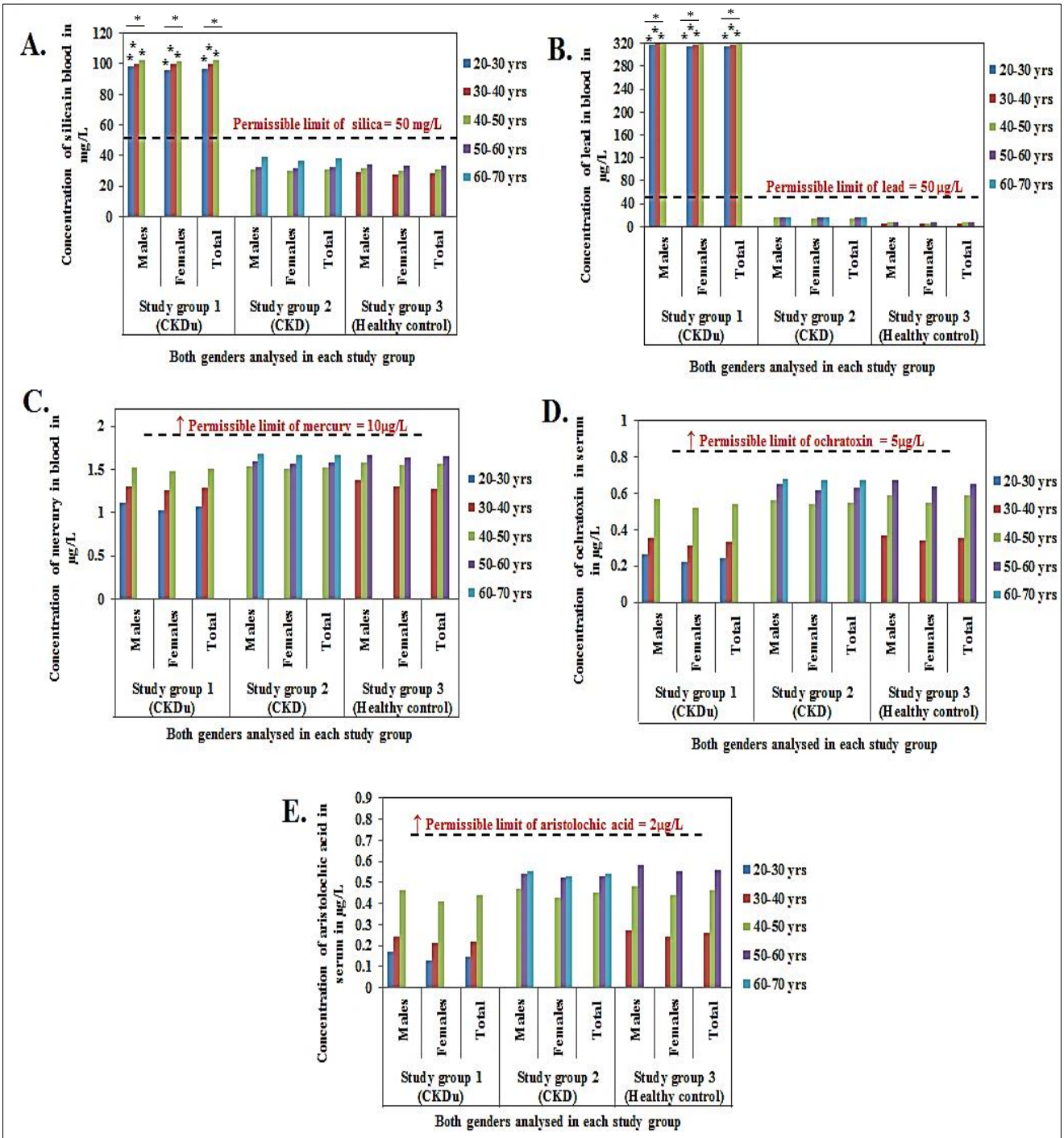


Figure 3.3.2. Age and sex based stratification of the levels of analysed nephrotoxins in the blood of the entire study population for identification of the risk-groups of the population that are prone to higher nephrotoxin exposure and related CKDu development.

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology.Study group(cohort) 1, 2 & 3 consists of CKDu affected,diabetes/hypertension induced CKD affected & healthy controls subjects respectively. The number of males (M) / females (F)/total number of subjects(total) in study group one, two and three are 58(M)/56(F)/114(total); 15(M)/13(F)/28(total) and 62(M)/62(F)/124(total) respectively.The age based distribution(i.e. the number of subjects under each age category) among males,females and total number of subjects (combination of both sexes) in all three study groups are detailed in the affixed table(**Table 3.3.2)** of this figure. (A)Concentration of silica in the blood of various age groups among male sex,female sex and both sexes combined in all three study groups(B) Concentration of lead in the blood of age and gender stratified sub-groups of the population of the three study cohorts (C) Concentration of mercury in the blood of age and gender stratified sub-groups of the three study cohorts' population (D) Concentration of ochratoxin in the serum of age and gender stratified sub-groups of the three study cohorts' population (E) Concentration of aristolochic acid in the serum of age and gender stratified sub-groups of the three study cohorts' population.Values are represented as mean concentrations of the nephrotoxin in age based stratified sub-groups of the population i.e. subjects in the age group of 20-30yrs,30-40yrs,40-50yrs,50-60yrs and 60-70yrs under each of the broad population segments stratified by gender i.e.males,females and total number of subjects (i.e.both sexes combined) in all three study groups. All concentration values are in µg/L except for silica which is depicted in mg/L. Differences at *$p<0.05$ were considered to be statistically significant as compared to the healthy controls(group 3) & CKD affected group(group 2) and also relative to the lower age categories (in a given study group).The WHO established permissible limits in blood for toxins like silica= 50mg/L; lead=50µg/L; mercury=10 µg/L and in serum for ochratoxin=5µg/L; aristolochic acid=2µg/L. Cadmium and Arsenic were present at below detectable levels in the blood of all three study groups' subjects, hence are not represented in the figure.The absence of a bar representation of the levels of a given nephrotoxin for a particular age category among males,females and total number of subjects in each of the study groups indicated lack of subjects in that particular age category. No gender bias in nephrotoxin exposure was noted which was evident from similar mean levels of a given nephrotoxin being reported in both genders of all three study groups.The nephrotoxin levels were found to rise with increasing age but the higher age groups (i.e.40-50 yrs in case of study group 1 and 60-70 yrs in study group 2) did not exhibit statistically significant ($p>0.05$) differences in the nephrotoxin levels as compared to their respective lower age groups. However, silica and lead levels in the CKDu affected subjects(study group 1) were found to remarkably exceed their respective WHO set permissible limits in all age groups with significantly($p<0.05$) elevated levels being noted in the higher age group of 40-50 yrs as compared to lower age groups, depicting enhanced nephrotoxin accumulation with increased exposure(i.e. age) due to their bioaccumulative potencies. The details on the range of minimum to maximum values of the concentration of each nephrotoxin & prevalence distribution(%) of the nephrotoxin concentration in various ranges for different age groups of each sex and both sexes combined(i.e. total number of subjects) in all three study groups are listed in **Table 3.3.2**. For more details of the levels of each nephrotoxin, kindly refer Figure3.3.1and Table 3.3.1.

Table 3.3.2: Age and sex dependent stratification of the prevalence of the levels of various nephrotoxins in the blood of the entire study population for identification of the demographic risk-groups susceptible to higher nephrotoxin exposure and associated CKDu development.

Age in years	Gender	Study group	Number of subjects in each category	Nephrotoxin analysed														
				Blood lead			Blood mercury			Blood silica			Serum ochratoxin			Serum aristolochic acid		
				mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)	mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)	mean in mg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/L)	mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)	mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)
20-30 yrs	M	1	3	316.0 (315.6-316.5)	G=5.17%	A=5.5-6.0	1.11 (1.0-1.15)	A=5.17%	A=0.95-1.15	98.0 (97.9-98.3)	E=5.17%	A=27-30	0.26 (0.23-0.29)	A=5.17%	A=0.2-0.3	0.17 (0.13-0.18)	A=5.17%	A=0.1-0.2
		2	-	-	-	B=6.0-6.5	-	-	B=1.25-1.35			B=30-33	-	-	B=0.3-0.4	-	-	B=0.2-0.3
		3	-	-	-	C=6.5-6.9	-	-	C=1.35-1.55			C=33-36	-	-	C=0.5-0.6	-	-	C=0.4-0.5
	F	1	3	315.5 (315.2-316.1)	G=5.17%	D=15.0-15.1	1.02 (0.98-1.10)	A=5.17%	D=1.55-1.65	95.5 (95.1-96.5)	E=5.17%	D=36-39	0.22 (0.20-0.26)	A=5.17%	D=0.6-0.7	0.13 (0.10-0.16)	A=5.17%	D=0.5-0.6
		2	-	-	-	E=15.1-15.4	-	-	E=1.65-1.75			E=95-98	-	-		-	-	
		3	-	-	-	F=15.4-15.8	-	-				F=98-100	-	-		-	-	
	Total	1	6	315 (315.2-316.5)	G=5.17%	G=315-317	1.07 (0.98-1.15)	A=5.17%		96.7 (95.1-98.9)	E=5.17%	G=100-102	0.24 (0.20-0.29)	A=5.17%		0.15 (0.10-0.18)	A=5.17%	
		2	-	-	-	H=317-319	-	-				H=102-104	-	-		-	-	
		3	-	-	-	I=319-322	-	-					-	-		-	-	
30-40 yrs	M	1	22	318.3 (317.5-319.8)	H=37.93%		1.3 (1.25-1.33)	B=37.93%		100.2 (99.5-100.8)	F=37.93%		0.35 (0.32-0.39)	B=37.93%		0.24 (0.22-0.28)	B=37.93%	
		2	-	-	-		-	-					-	-		-	-	
		3	26	6.1 (5.9-6.2)	A=19.35%; B=22.58%		1.37 (1.29-1.35)	B=41.93%		28.8 (28.0-29.9)	A=41.93%		0.37 (0.34-0.39)	B=41.93%		0.27 (0.25-0.29)	B=41.93%	
	F	1	22	317.9 (317.1-319.5)	H=37.93%		1.26 (1.24-1.30)	A=0.9%; B=37.0%		99.9 (98.5-100.1)	F=37.93%		0.31 (0.30-0.37)	B=37.93%		0.21 (0.20-0.26)	B=37.93%	
		2	-	-	-		-	-					-	-		-	-	
		3	26	5.9 (5.8-6.1)	A=20.9%; B=20.9%		1.30 (1.26-1.32)	B=41.93%		27.2 (27.0-29.2)	A=41.93%		0.34 (0.32-0.35)	B=41.93%		0.24 (0.22-0.26)	B=41.93%	
	Total	1	44	318.1 (317.1-319.8)	H=37.93%		1.28 (1.24-1.33)	A=0.9%; B=37.45%		100.0 (98.5-100.8)	F=37.93%		0.33 (0.30-0.37)	B=37.93%		0.22 (0.20-0.28)	B=37.93%	
		2	-	-	-		-	-					-	-		-	-	
		3	52	6.0 (5.8-6.2)	A-=20.13%; B=21.74%		1.27 (1.26-1.35)	B=41.93%		28.0 (27.0-29.9)	A=41.93%		0.35 (0.32-0.39)	B=41.93%		0.26 (0.22-0.29)	B=41.93%	
40-50 yrs	M	1	33	320.3 (319.4-322.3)*	I=56.89%		1.52 (1.50-1.54)	C=56.89%		102.5 (100.9-103.7)*	G=2.1%; H=54.88%		0.57 (0.54-0.59)	C=56.89%		0.46 (0.44-0.48)	C=56.89%	
		2	1	15.1	D=6.6%		1.53	C=6.6%		30.6	B=6.67%		0.56	C=6.6%		0.47	C=6.6%	
		3	26	6.4 (6.2-6.5)	B=41.93%		1.58 (1.55-1.61)	D=41.9%		31.6 (30.9-32.4)	B=41.9%		0.59 (0.57-0.60)	C=41.9%		0.48 (0.45-0.50)	C=41.9%	
	F	1	31	320.1 (319.0-322.0)*	I=55.36%		1.48 (1.44-1.50)	C=55.36%		102.0 (100.3-102.9)*	G=1.8%; H=53.5%		0.52 (0.50-0.55)	C=55.36%		0.41 (0.40-0.43)	C=55.36%	
		2	1	15	D=6.6%		1.5	C=6.6%		30.2	B=6.67%		0.54	C=6.6%		0.43	C=6.6%	
		3	26	6.2 (6.1-6.4)	B=41.93%		1.55 (1.54-1.59)	D=41.9%		30.3 (30.0-31.3)	B=41.9%		0.55 (0.53-0.58)	C=41.9%		0.44 (0.42-0.47)	C=41.9%	
	Total	1	61	320.2 (319.0-322.3)*	I=56.14%		1.50 (1.44-1.54)	C=56.14%		102.2 (100.3-103.7)*	G=2.0%; H=54.1%		0.54 (0.50-0.59)	C=56.14%		0.44 (0.40-0.48)	C=56.14%	
		2	2	15	D=6.6%		1.52	C=6.6%		30.4	B=6.67%		0.55	C=6.6%		0.45	C=6.6%	
		3	52	6.3 (6.2-6.5)	B=41.93%		1.57 (1.54-1.61)	D=41.9%		30.9 (30.0-32.4)	B=41.9%		0.59 (0.53-0.60)	C=41.9%		0.46 (0.42-0.50)	C=41.9%	
50-60 yrs	M	1	-	-	-		-	-					-	-		-	-	
		2	6	15.3 (15.2-15.4)	D=40%		1.59 (1.58-1.65)	D=40%		32.5 (31.6-33.0)	B=40%		0.65 (0.63-0.67)	D=40%		0.54 (0.52-0.56)	D=40%	
		3	10	6.7 (6.6-6.8)	C=16.13%		1.66 (1.65-1.70)	E=16.13%		33.8 (33.4-35.4)	C=16.13%		0.67 (0.64-0.68)	D=16.13%		0.58 (0.54-0.59)	D=16.13%	
	F	1	-	-	-		-	-						-		-	-	
		2	5	15.2 (15.1-15.4)	D=38.4%		1.57 (1.56-1.61)	D=38.4%		31.9 (30.7-32.5)	B=38.4%		0.62 (0.60-0.65)	D=38.4%		0.52 (0.50-0.55)	D=38.4%	
		3	10	6.6 (6.5-6.7)	C=16.3%		1.64 (1.65-1.68)	E=16.13%		33.2 (33.0-34.9)	C=16.29%		0.64 (0.62-0.65)	D=16.13%		0.55 (0.53-0.56)	D=16.13%	
	Total	1	-	-	-		-	-					-	-		-	-	
		2	11	15.2 (15.0-15.4)	D=39.2%		1.58 (1.56-1.65)	D=39.2%		32.2 (30.7-33.0)	B=39.2%		0.63 (0.60-067)	D=39.2%		0.53 (0.50-0.56)	D=39.2%	
		3	20	6.6 (6.5-6.8)	C=16.2%		1.66 (1.65-1.70)	E=16.13%		33.5 (33.0-35.4)	C=16.29%		0.65 (0.62-0.69)	D=16.13%		0.56 (0.53-0.59)	D=16.13%	
60-70 yrs	M	1	-	-	-		-	-					-	-		-	-	
		2	8	15.5 (15.3-15.8)	E=6.67% F=46.67%		1.68 (1.66-1.71)	E=53.3%		39.0 (37.9-38.8)	D=53.3%		0.66 (0.62-0.69)	D=53.33%		0.55 (0.53-0.60)	D=53.33%	
		3	-	-	-		-	-					-	-		-	-	
	F	1	-	-	-		-	-					-	-		-	-	
		2	7	15.3 (15.2-15.6)	E==7.69% F=46.15%		1.66 (1.65-1.68)	E=53.1%		36.9 (36.1-37.9)	D=53.1%		0.65 (0.61-0.68)	D=53.18%		0.53 (0.51-0.57)	D=53.18%	
		3	-	-	-		-	-					-	-		-	-	
	Total	1	-	-	-		-	-					-	-		-	-	
		2	15	15.3 (15.2-15.8)	E=7.18%; F=46.41%		1.67 (1.65-1.71)	E=53.2%		37.9 (36.1-38.8)	D=53.23%		0.65 (0.61-0.69)	D=53.25%		0.54 (0.51-0.60)	D=53.25%	
		3	-	-	-		-	-					-	-		-	-	

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; M-male; F-female.Values are represented as a mean with the range of minimum to maximum concentrations of each nephrotoxin depicted as well. All concentration values are in µg/L except for silica which is depicted in mg/L. The prevalence of the nephrotoxin concentration in various ranges for each age and sex category of the three study groups are represented by prevalence % and annotated by uppercase letters (i.e. A to I) for convenience. The annotations for each estimated nephrotoxin varies and have been independently described. The prevalence % of a particular study group

denotes the proportion of subjects from its total number in their respective sex categories of a particular age group bearing the nephrotoxin at a given concentration range. The total number of males (M) / females (F) in study group one, two and three are 58(M)/56(F); 15(M)/13(F) and 62(M)/62(F) respectively. For example, the prevalence % annotation of 'A' in the case of blood lead levels denotes the percentage of the total population of a particular study group in a particular age and sex category possessing concentrations at the range of 5.5-6.0 µg/L. . Differences at *$p<0.05$ were considered to be statistically significant as compared to the healthy controls(group 3) & CKD affected group(group 2) and also relative to the lower age categories (in a given study group). No gender bias in the nephrotoxin exposure was noted as represented from the similar levels of toxins being reported in the blood of both sexes in all of the three study groups. Moreover, all of the nephrotoxin concentrations in the subjects of the three study groups were prevalent at higher ranges of their levels with increasing age but the differences in the higher age groups (i.e.40-50yrs in case of study group 1 and 60-70 yrs in study group 2) were not statistically significant ($p>0.05$) as compared to their respective lower age groups. However, silica and lead levels among the CKDu affected subjects (study group 1) were largely surpassing their respective WHO established permissible levels in all age groups with their significantly($p<0.05$) highest concentration range noted in the older individuals in the age group of 40-50 years as compared to lower age groups, demonstrating nephrotoxin accumulation with increased exposure(i.e. age) due to their bioaccumulative potencies. For details of each nephrotoxin, kindly refer Table 3.3.1.

These findings were contradictory to previous studies of CKDu in Central America and Sri Lanka which had established male sex to be the major risk group prone to CKDu development (Orantes et al.,2017; Vela Parada et al.,2014). Since no significant gender bias for nephrotoxin exposure & CKDu induction was noted, levels of various biomarkers of nephrotoxin exposure (blood nephrotoxin levels) were further stratified by age (**Table3.3.2)**.

As indicated in **Figure 3.3.2 & Table 3.3.2,** the levels of blood nephrotoxins in all the three study groups proportionately increased with increasing age groups to a uniform extent in both sexes. This is attributed to the longer half-lives and bioaccumulative tendencies of these nephrotoxins that causes an increase in the body burden with simultaneous elevation in the blood with increasing duration of exposure (i.e.rising age) (Abiola,2017;Gamboa et al. ,2016; Gil and Hernández,2015; Sabath and Robles-Osorio,2012).Thus this explains the prevalence of the nephrotoxins in their respective higher concentration ranges at 37.9% and 56.2% in the age category of 30-40 yrs. and 40-50 yrs. respectively for both genders in study group 1.

Whereas in study group 2, higher levels of the nephrotoxins were prevalent at roughly 35% and 53.3% in the age groups of 50-60 yrs. and 60-70 yrs. respectively for both sexes. However the levels of nephrotoxins-Hg, ochratoxin and aristolochic acid although prevalent at a comparable extent in their higher ranges with rising age, but the differences with their respective lower age groups were not statistically significant ($p>0.05$) and moreover were established to be well below their respective WHO permissible level even in the highest age groups (i.e. 40-50 yrs. in study group 1 and 60-70 yrs. in study groups 2) signifying no contribution of these nephrotoxins in CKDu development of the Canacona taluka.

On the contrary the levels of silica and lead were found to be remarkably surpassing their respective WHO established permissible levels in the all the age groups with the significantly ($p<0.05$) highest range of their levels noted in the oldest age group of the CKDu affected subjects i.e. 40-50 yrs depicting prolonged and higher rate of these nephrotoxin exposure coupled with increased accumulation of silica and lead in these CKDu affected subjects. These findings were in agreement with a similar trend of higher exposure of the causative

nephrotoxins i.e. silica and lead noted at higher age groups of the CKDu affected subjects analysed in another Indian state and Sri Lanka respectively (Ekong et al., 2006; Khandare et al., 2015; McClean et al., 2012; Nanayakkara et al., 2014; Rango et al., 2015; Ganguli, 2016; Vlahos et al., 2018; Weaver and Jaar,2015). Therefore further justifying the causative role of prolonged and higher rate of silica and Pb exposure in CKDu development in Canacona.

These findings further supported the previously reported observation that a nephrotoxin exposure beyond 25 yrs is mandatory to induce considerable amount of tubular dysfunction that manifests in renal failure owing to the inherent propensity of the triggered nephrotoxic effects to inflict severe renal damage only after sufficient aggravation and accumulation with time. This explains the higher nephrotoxin (viz. silica and lead) exposure levels and induced renal damaging effects noted at higher age groups among the CKDu affected subjects of the Canacona taluka.Thus further supporting & explaining the earlier induction(at the 3rd decade) of CKDu in this taluka (Lebov et al.,2015; Orantes et al.,2017; Vela Parada et al., 2014; Raines et al.,2014; Wanigasuriya,2012) in comparison to the typical diabetes & hypertensive induced CKD cases of study group2 that stereotypically manifests at an advanced age due to the inevitable compromise in renal functional integrity with age (Defina et al., 2016). Following age and sex based stratification of biomarkers of nephrotoxin exposure, the prevalence and range distribution in levels of these markers in different age groups distinguished by occupation were analysed **(Figure 3.3.3 & Table 3.3.3).** This was done to check for predisposition of any occupation type to be prevalent at highest range of values of each biomarker for assessment of occupational risk groups for nephrotoxin exposure and associated CKDu development (Lunyera et al.,2016). As indicated , each of the biomarkers of nephrotoxin exposure were prevalent at elevated ranges of their concentration to a comparable extent in all occupation types (i.e. mining, fishing, farming) for both sexes and various age categories in all 3 study groups. Here also the levels of nephrotoxins increased with rising age to a similar extent in all occupation types with their highest concentration ranges being prevalent at the highest age category in all occupational categories of all study cohorts (Ekong et al.,2006; Khandare et al.,2015; McClean et al.,2012; Levine et al., 2016; Nanayakkara et al.,2014; Rango et al.,2015; Ganguli,2016; Tatapudi et al.,2018;Vlahos et al., 2018; Weaver and Jaar,2015). The levels of nephrotoxins were prevalent in their higher conc.ranges at approximately 38.9 & 56.3% in the age category of 30-40 & 40-50 yrs respectively to a similar level in all occupational groups of group1. Whereas in group 2, higher levels of nephrotoxins were prevalent at roughly 37.1 & 54.1% in age groups of 50-60 & 60-70 yrs.respectively to a uniform extent in all occupations (**Table 3.3.3).**

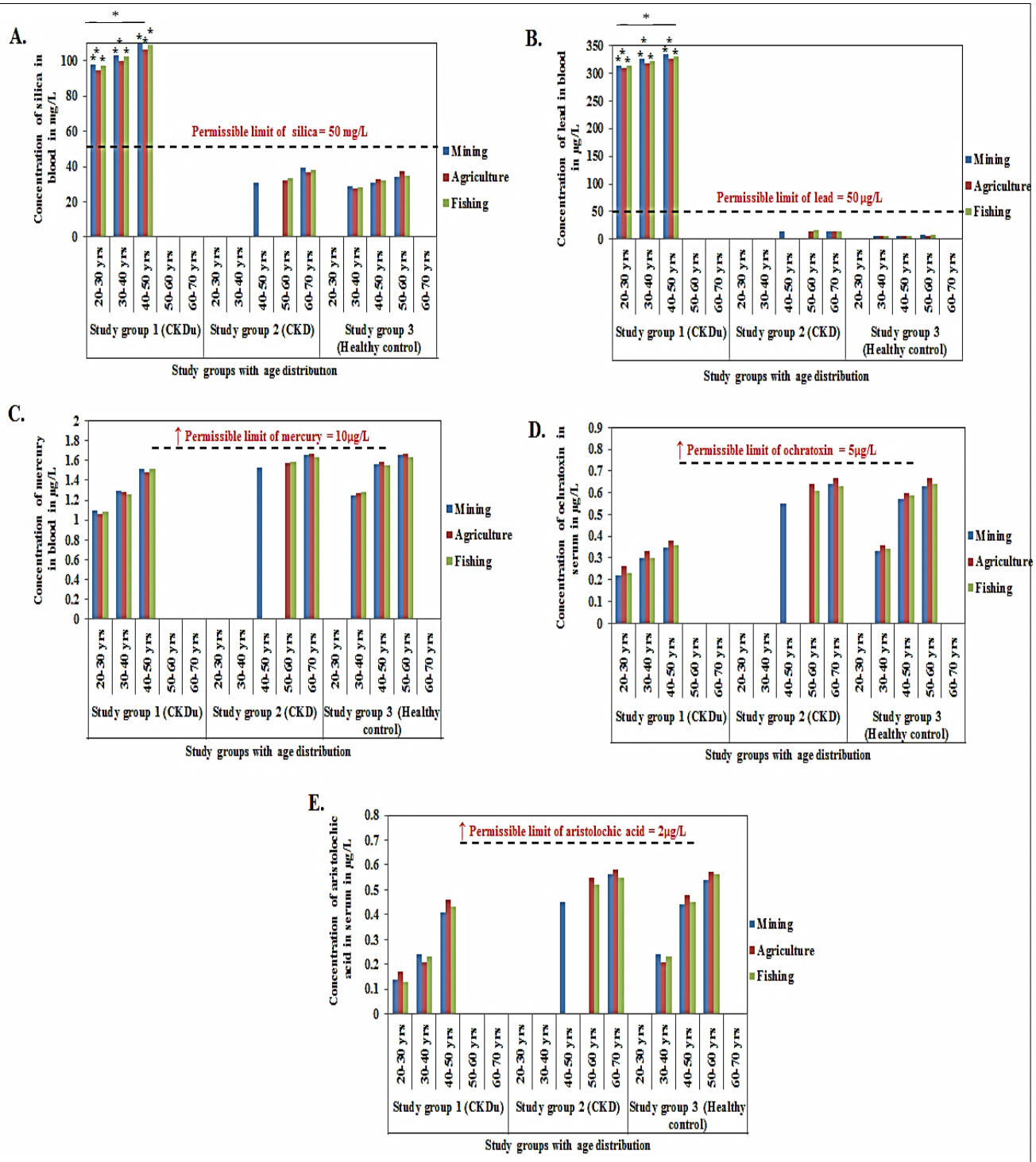


Figure 3.3.3: Occupation based stratification of the levels of analysed nephrotoxins in the blood of the entire study population for identification of occupational risk groups that are susceptible to elevated nephrotoxin exposure and associated CKDu development.

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology.Study group(cohort) 1, 2 & 3 consists of CKDu affected,diabetes/hypertension induced CKD affected & healthy controls subjects respectively. The total number of subjects in study groups 1,2 and 3 are 114,28 and 124 respectively.In this,the number of subjects involved in mining previously(M), agriculture(A) and fishing(F) occupations in study group one, two and three are 87(M)/11(A)/16(F); 4(M)/7(A)/17(F)and 7(M)/55(A)/62(F) respectively. The age based distribution(i.e. the number of subjects in each age category) among the mining(previously involved),agricultural and fishing communities in all three study groups are detailed in the affixed table (**Table 3.3.3)** of this figure. (A)Concentration of silica in the blood of various occupational groups under each age category in all three study groups (B) Concentration of lead in the blood of population sub-groups stratified by occupation and age in all three study cohorts (C) Concentration of mercury in the blood of occupational and age stratified sub-groups of the three study cohorts' population (D) Concentration of ochratoxin in serum of occupational and age stratified sub-groups of the three study cohorts' population (E) Concentration of aristolochic acid in serum of occupational and age stratified sub-groups of the three study cohorts' population. Values are represented as mean of the nephrotoxin concentration estimated independently in subjects involved in different occupations (i.e.mining(previously),agriculture and fishing) belonging to various age groups (i.e. 20-30yrs,30-40yrs,40-50yrs,50-60yrs and 60-70yrs) in each of the three study groups. All concentration values are in µg/L except for silica which is depicted in mg/L.Differences at *p<0.05 were considered to be significant as compared to healthy controls(group 3) & CKD affected group(group 2) and also relative to lower age categories (in a given study group).The WHO established permissible limits in blood for toxins like silica= 50mg/L; lead=50µg/L; mercury=10 µg/L and in serum for ochratoxin=5µg/L; aristolochic acid=2µg/L.Cadmium and Arsenic were present at below detectable levels in the blood of all three study groups' subjects,hence are not represented in the figure.The absence of a bar representation of the levels of a given nephrotoxin for a particular occupational category under a given age group indicated lack of subjects pursing that occupation at that particular age category. Absence of occupational bias in the nephrotoxin exposure was observed which was apparent from comparable levels of a given toxin being reported in the blood of all occupational groups in the 3 study cohorts.Herein the silica & lead levels in CKDu affected subjects(study group 1) were found to remarkably surpass their respective WHO set permissible limits in all age groups with significantly($p<0.05$) raised levels being noted in the higher age group of 40-50 yrs as compared to lower ages, portraying heightened nephrotoxin accumulation with higher exposure(i.e. age) due to their bioaccumulative potencies. The details on the range of minimum to maximum values of the concentration of each nephrotoxin & prevalence distribution (%) of the nephrotoxin concentration in various ranges for different occupational groups under individual age categories in all three study groups are listed in **Table 3.3.3**. For more details of the levels of each nephrotoxin, kindly refer Figure3.3.1and Table 3.3.1.

Table 3.3.3: Occupation based stratification of the prevalence of the levels of various nephrotoxins of the entire study population for identification of occupational risk groups prone to higher nephrotoxin exposure and associated CKDu development.

Age in years	Study group	Occupation	Number of subjects in each occupational category for a particular age group	Nephrotoxin analysed														
				Blood lead			Blood mercury			Blood silica			Serum ochratoxin			Serum aristolochic acid		
				mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)	mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)	mean in mg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in mg/L)	mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)	mean in µg/L (min-max)	prevalence	Annnotation for the ranges of the biomarker levels (in µg/L)
20-30 yrs	1	M	2	315.2 (315.4-316.7)	G=1.7%	A=5.5-6.0	1.09 (1.0-1.17)	A=1.7%	A=0.95-1.15	96.9 (95.3-99.1)	E=1.5%; F=0.2%	A=27-30	0.22 (0.20-0.27)	A=1.7%	A=0.2-0.3	0.14 (0.10-0.17)	A=1.7%	A=0.1-0.2
		A	2	315.1 (315.3-316.6)	G=1.7%	B=6.0-6.5	1.06 (0.99-1.14)	A=1.7%	B=1.25-1.35	96.5 (69.0-98.7)	E=1.5%; F=0.2%	B=30-33	0.26 (0.22-0.30)	A=1.7%	B=0.3-0.4	0.17 (0.10-0.20)	A=1.7%	B=0.2-0.3
		F	2	314.9 (315.1-316.4)	G=1.7%	C=6.5-6.9	1.08 (0.97-1.14)	A=1.7%	C=1.35-1.55	96.8 (95.2-99.0)	E=1.5%; F=0.2%	C=33-36	0.23 (0.20-0.28)	A=1.7%	C=0.5-0.6	0.13 (0.10-0.15)	A=1.7%	C=0.4-0.5
	2	M	-	-	-	D=15.0-15.1	-	-	D=1.55-1.65	-	-	D=36-39	-	-	D=0.6-0.7	-	-	D=0.5-0.6
		A	-	-	-	E=15.1-15.4	-	-	E=1.65-1.75	-	-	E=95-98	-	-		-	-	
		F	-	-	-	F=15.4-15.8	-	-		-	-	F=98-100	-	-		-	-	
	3	M	-	-	-	G=315-317	-	-		-	-	G=100-102	-	-		-	-	
		A	-	-	-	H=317-319	-	-		-	-	H=102-104	-	-		-	-	
		F	-	-	-	I=319-322	-	-		-	-		-	-		-	-	
30-40 yrs	1	M	35	318.3 (317.3-319.0)	H=40.2%		1.29 (1.25-1.34)	B=40.2%		100.3 (98.8-100.9)	F=40.2%		0.35 (0.32-0.39)	B=40.2%		0.24 (0.22-0.30)	B=40.2%	
		A	4	318 (317.0-319.7)	H=38.6%		1.28 (1.24-1.33)	B=38.6%		100.0 (98.5-100.8)	F=38.6%		0.31 (0.30-0.36)	B=38.6%		0.21 (0.20-0.27)	B=38.6%	
		F	5	317.9 (317.0-319.6)	H=37.9%		1.26 (1.22-1.31)	B=37.9%		100.1 (98.6-100.9)	F=37.9%		0.34 (0.31-0.38)	B=37.9%		0.23 (0.21-0.29)	B=37.9%	
	2	M	-	-	-		-	-		-	-		-	-		-	-	
		A	-	-	-		-	-		-	-		-	-		-	-	
		F	-	-	-		-	-		-	-		-	-		-	-	
	3	M	3	6.2 (6.0-6.4)	B=22.8%		1.25 (1.24-1.33)	B=22.8%		28.2 (27.2-30.0)	B=22.8%		0.33 (0.30-0.37)	B=22.8%		0.24 (0.20-0.27)	B=22.8%	
		A	21	6.1 (5.9-6.3)	A=2.3%; B=22.1%		1.27 (1.26-1.35)	B=24.5%		27.8 (27.0-29.7)	B=24.5%		0.36 (0.33-0.39)	B=24.5%		0.28 (0.24-0.30)	B=24.5%	
		F	28	5.9 (5.7-6.1)	A=2.4%; B=23.4%		1.28 (1.27-1.36)	B=25.8%		28.1 (27.1-29.9)	B=25.8%		0.34 (0.31-0.38)	B=25.8%		0.26 (0.22-0.29)	B=25.8%	
40-50 yrs	1	M	50	320.5 (319.3-322.4)*	I=57.4%		1.52 (1.47-1.55)	C=57.4%		102.7 (101.1-103.9)*	G=2.1%; H=55.3%		0.56 (0.52-0.60)	C=57.4%		0.41 (0.40-0.45)	C=57.4%	
		A	5	320.3 (319.1-322.4)*	I-55.4%		1.48 (1.42-1.52)	C-55.4%		102.4 (100.8-103.6)*	G=1.9%; H=53.5%		0.53 (0.50-0.58)	C-55.4%		0.46 (0.42-0.50)	C-55.4%	
		F	9	320.1 (319.0-322.2)*	I=56.2%		1.51 (1.45-1.54)	C=56.2%		102.6 (101.0-103.7)*	G=1.2%; H=55.0%		0.55 (0.51-0.59)	C=56.2%		0.43 (0.40-0.47)	C=56.2%	
	2	M	2	15.05 (15.0-15.1)	D=5.7%		1.53 (1.52-1.54)	C=5.7%		30.5 (30.4-30.6)	B=5.7%		0.55 (0.54-0.56)	C=5.7%		0.45 (0.44-0.47)	C=5.7%	
		A	-	-	-		-	-		-	-		-	-		-	-	
		F	-	-	-		-	-		-	-		-	-		-	-	
	3	M	2	6.5 (6.4-6.7)	A=1.5%; B=30.5%		1.56 (1.53-1.60)	C=1.5%; D=30.5%		30.7 (30.0-32.2)	B=32%		0.57 (0.51-0.60)	C=32%		0.44 (0.40-0.48)	C=32%	
		A	28	6.2 (6.1-6.4)	A=32.9%		1.59 (1.56-1.62)	C=1.2%; D=32.9%		31.1 (30.2-32.6)	B=32.9%		0.60 (0.54-0.61)	C=32.9%		0.48 (0.44-0.50)	C=32.9%	
		F	22	6.4 (6.3-6.6)	A=1.2%; B=31.5%		1.55 (1.52-1.58)	C=1.2%; D=31.5%		30.9 (30.0-32.5)	B=32.7%		0.59 (0.53-0.60)	C=32.7%		0.45 (0.41-0.49)	C=32.7%	
50-60 yrs	1	M	-	-	-		-	-		-	-		-	-		-	-	
		A	-	-	-		-	-		-	-		-	-		-	-	
		F	-	-	-		-	-		-	-		-	-		-	-	
	2	M	-	-	-		-	-		-	-		-	-		-	-	
		A	3	15.3 (15.1-15.5)	D=2.1%; E=38.1%		1.57 (1.56-1.64)	D=40.2%		32.0 (30.5-32.98)	B=40.2%		0.64 (0.61-0.68)	D=40.2%		0.55 (0.52-0.58)	D=40.2%	
		F	8	15.1 (15.0-15.3)	D=1.9%; E=39.3%		1.59 (1.58-1.66)	D=41.2%		32.3 (30.8-33.0)	B=41.2%		0.61 (0.60-0.65)	D=41.2%		0.52 (0.50-0.55)	D=41.2%	
	3	M	2	6.8 (6.7-6.9)	C=35.5%		1.66 (1.65-1.71)	E=35.5%		33.4 (33.0-35.3)	C=35.5%		0.63 (060-0.67)	D=35.5%		0.54 (0.51-0.57)	D=35.5%	
		A	6	6.7 (6.6-6.8)	C=37.6%		1.67 (1.66-1.71)	E=37.6%		33.7 (33.2-35.6)	C=37.6%		0.67 (0.63-0.70)	D=37.6%		0.57 (0.54-0.60)	D=37.6%	
		F	12	6.5 (6.4-6.7)	C=38.3%		1.63 (1.62-1.68)	E=38.3%		33.6 (33.1-35.5)	C=38.3%		0.64 (0.62-0.68)	D=38.3%		0.56 (0.52-0.58)	D=38.3%	
60-70 yrs	1	M	-	-	-		-	-		-	-		-	-		-	-	
		A	-	-	-		-	-		-	-		-	-		-	-	
		F	-	-	-		-	-		-	-		-	-		-	-	
	2	M	2	15.5 (15.4-16.0)	E=1.9%; F=53.6%		1.69 (1.67-1.71)	E=1.9%; F=53.6%		38.1 (36.3-39.0)	D=55.5%		0.64 (0.60-0.68)	D=55.5%		0.56 (0.53-0.60)	D=55.5%	
		A	4	15.2 (15.1-15.7)	E=1.7%; F=52.9%		1.66 (1.64-1.70)	E=1.7%;F =52.9%		37.8 (36.0-38.7)	D=53.6%		0.67 (0.62-0.70)	D=53.6%		0.52 (0.50-0.58)	D=53.6%	
		F	9	15.4 (15.3-15.9)	E=1.5%; F=51.8%		1.65 (1.64-1.68)	E=1.5%; F=51.8%		38.0 (36.2-38.9)	D=53.3%		0.63 (0.60-0.66)	D=53.3%		0.55 (0.52-0.61)	D=53.3%	
	3	M	-	-	-		-	-		-	-		-	-		-	-	
		A	-	-	-		-	-		-	-		-	-		-	-	
		F	-	-	-		-	-		-	-		-	-		-	-	

Abbreviations: A-agriculture; CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; F-fishing; M-mining. Values are represented as mean with the range of minimum to maximum concentrations of each nephrotoxin depicted as well. All

concentration values are in µg/L except for silica which is depicted in mg/L. The prevalence of the nephrotoxin concentration in various ranges for each age and sex category of the three study groups are represented by prevalence % and annotated by uppercase letters (i.e. A to I) for convenience. The annotations for each estimated nephrotoxin varies and have been independently described. The prevalence % of a particular study group denotes the proportion of subjects from the total number in its particular occupational category (i.e. mining/farming/fishing) of a specific age groups bearing the nephrotoxin at a given concentration range. The total number of subjects involved in the mining, farming and fishing occupations for study group 1 are 87, 11 and 16 respectively. For study group 2, the total number of subjects involved in the mining, farming and fishing occupations are 4,7 and 17 respectively. For study group 3, the total number of subjects involved in the mining, farming and fishing occupations are 7,55 and 58 respectively. For example, the prevalence % annotation of 'A' in the case of blood lead denotes the percentage of the total number of subjects in the respective study group involved in a particular occupation(with the total number of the subjects engaged in a particular occupation being different in all of the three study groups) of a particular age group possessing levels in the range of 5.5-6.0 µg/L. Differences at *$p<0.05$ were considered to be statistically significant as compared to the healthy controls(group 3) & CKD affected group(group 2) and also relative to the lower age categories (in a given study group). No occupational bias in the nephrotoxin exposure was noted as represented from similar levels of toxins being reported in the blood of all occupational groups in all three study cohorts.Moreover, all of the nephrotoxin concentrations in the subjects of the three study groups were prevalent at higher ranges of their levels with increasing age but the differences in the higher age groups (i.e.40-50yrs in case of study group 1 and 60-70 yrs in study group 2) were not statistically significant ($p>0.05$) as compared to their respective lower age groups. However, silica and lead levels among the CKDu affected subjects (study group 1) were largely surpassing their respective WHO established permissible levels in all age groups with their significantly ($p<0.05$) highest concentration range noted in the older individuals in the age group of 40-50 years as compared to lower age groups, demonstrating nephrotoxin accumulation with increased exposure (i.e. age) due to their bioaccumulative potencies. For details of each nephrotoxin, kindly refer Table 3.3.1.

However,levels of nephrotoxins-Hg, OTA and AA although prevalent in their higher ranges with rising age to a uniform extent in all occupational categories in each study groups (**Figure 3.3.3 & Table 3.3.3)** were established to be well below their respective WHO permissible level even in the highest age groups for all occupations (i.e. 40-50 yrs in study group 1 and 60-70 yrs in study groups 2) signifying no contribution of these nephrotoxins in CKDu development of Canacona (Gruia et al.,2018; Nanayakkara et al.,2014; Pavlovic, 2014; Rango et al.,2015; Vlahos et al., 2018;Wanigasuriya et al.,2008; Weaver &Jaar,2015).

On the contrary the levels of silica and Pb in the blood were homologous in all the occupational categories of the CKDu affected subjects and were found to be significantly ($p<0.05$) exceeding their respective WHO established permissible levels in all the age groups to a comparable extent in each occupational cohort with the highest range of their levels noted in the oldest age group of the CKDu affected subjects i.e. 40-50 yrs **(Figure 3.3.3 & Table 3.3.3)**. Thus depicting a similar degree of the extent of prolonged and higher rate of these nephrotoxins exposure coupled with increased accumulation of silica and lead in all the occupational groups of these CKDu affected subjects. Thus justifying the absence of an occupational risk-factor or predisposition towards CKDu development in Canacona owing to similar blood levels of causative nephrotoxins i.e. silica and lead noted in all of its the occupational categories,further indicating of a common source of exposure to these nephrotoxins in all these occupational groups (i.e.mining, agriculture & fishing) (Ekong et al., 2006; Khandare et al.,2015; McClean et al.,2012; Levine et al.,2016; Nanayakkara et al., 2014; Rango et al.,2015; Ganguli,2016; Tatapudi et al.,2018; Vlahos et al.,2018; Weaver and Jaar, 2015). These findings further confirmed that etiology of CKDu in Canacona is highly linked to environmental factors specifically nephrotoxins exposure (i.e.silica and Pb) mostly associated with drinking water as the elimination of occupational risk negates the exposure to

nephrotoxins via air (Gifford et al.,2017; Lusco et al.,2017; Rajapakse et al., 2016; Ratnayake et al.,2017). This was backed by non-functionality of the granite mine present in the vicinity of CKDu hit area for past 10 yrs that reduces predisposition of exposure to occupational toxins via air among the highly prevalent previous miners of group1 (The Hindu, 2017).

Overall, these findings were contradictory to a majority of the previously reported observations of the CKDu analysis in Central America which stated that CKDu possesses a high tendency to affect the agricultural workers or the farming community owing to the high risk of exposure of this group to various renal damage inducing causal factors like dehydration, heat stress and nephrotoxic pesticides (Abiola,2017; Almaguer et al.,2014; Lusco et al.,2017). However, our results were also found to be in accordance with the findings from other related studies of the CKDu scenario in Sri Lanka and India specifically Andhra Pradesh which supported a non-occupational risk in the disease etiology (Ekong et al.,2006; Khandare et al.,2015; McClean et al.,2012; Levine et al.,2016; Nanayakkara et al., 2014; Rango et al.,2015; Ganguli,2016; Tatapudi et al.,2018; Vlahos et al.,2018; Weaver and Jaar,2015). Therefore suggesting that despite CKDu being an environmentally induced disease, its etiological origin (i.e. causal factors) will inevitably possess regional differences owing to the variations in geography, geology,occupational constitution and lifestyle habits in these regions, thus explaining the difference in etiologies of the CKDu cases reported globally (Gifford et al.,2017;Lunyera et al.,2016).

Furthermore, the results from this occupational based prevalence distribution of various biomarkers of nephrotoxin exposure (**Figure & Table 3.3.3)** completely refuted the involvement of an occupational risk in the development of glomerular dysfunction based CKD in the non-endemic group 2 of the taluka as well. These findings paralleled the study of effect of various occupations on the pathologies of diabetic & hypertensive glomerular nephropathies by Smith et al. (2011). Thus providing confirmatory evidence to the role of traditional causative factors (i.e. diabetes & hypertension) in induction of CKD in this area.

3.3.3.3 Association between various biomarkers of nephrotoxin exposure (i.e. the levels of the nephrotoxins in the blood) of the study population via correlation assessments.

The relationship or association between various measured nephrotoxins in the blood (i.e. biomarkers of nephrotoxin exposure) were analysed by calculation of Pearson's correlation coefficients (r) with their respective p-values for identifying a common source of exposure to the nephrotoxins (Lunyera et al., 2016), the results of which are presented in **Table 3.3.4.**

Table 3.3.4: Correlation assessments between the mean levels of various biomarkers of nephrotoxin exposure (i.e. the blood nephrotoxin levels) in the entire study population of Canacona taluka for identification of the common source of origin (if any) of these nephrotoxins exposure.

Nephrotoxin in blood	Study group (total number of subjects)	Correlation coefficients [r] *(p values)* Nephrotoxin in blood						
		Silica	Pb	Cd	As	Hg	Ochratoxin	Aristolochic acid
Silica	1	1						
	(N=114)	-						
	2	1						
	(N=28)	-						
	3	1						
	(N=124)	-						
Pb	1	0.954	1					
	(N=114)	*0.027*	-					
	2	0.099	1					
	(N=28)	*0.116*	-					
	3	0.109	1					
	(N=124)	*0.086*	-					
Cd	1	NC	NC	NC				
	(N=114)	NC	NC	NC				
	2	NC	NC	NC				
	(N=28)	NC	NC	NC				
	3	NC	NC	NC				
	(N=124)	NC	NC	NC				
As	1	NC	NC	NC	NC			
	(N=114)	NC	NC	NC	NC			
	2	NC	NC	NC	NC			
	(N=28)	NC	NC	NC	NC			
	3	NC	NC	NC	NC			
	(N=124)	NC	NC	NC	NC			
Hg	1	-0.095	-0.109	NC	NC	1		
	(N=114)	*0.098*	*0.14*	NC	NC	-		
	2	-0.074	-0.099	NC	NC	1		
	(N=28)	*0.127*	*0.113*	NC	NC	-		
	3	-0.041	-0.065	NC	NC	1		
	(N=124)	*0.128*	*0.078*	NC	NC	-		
Ochratoxin	1	-0.078	-0.067	NC	NC	0.016	1	
	(N=114)	*0.112*	*0.089*	NC	NC	*0.116*	-	
	2	-0.055	-0.037	NC	NC	0.029	1	
	(N=28)	*0.144*	*0.056*	NC	NC	*0.102*	-	
	3	-0.022	-0.013	NC	NC	0.035	1	
	(N=124)	*0.156*	*0.134*	NC	NC	*0.089*	-	
Aristolochic acid	1	-0.055	-0.069	NC	NC	0.011	0.039	1
	(N=114)	*0.135*	*0.146*	NC	NC	0.123	*0.103*	-
	2	-0.041	-0.052	NC	NC	0.035	0.056	1
	(N=28)	*0.078*	*0.099*	NC	NC	0.106	*0.077*	-
	3	-0.013	-0.019	NC	NC	0.048	0.087	1
	(N=124)	*0.123*	*0.145*	NC	NC	0.203	*0.069*	-

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; NC:- not calculated correlation coefficient owing to the below detectable levels of one of the group of nephrotoxins in the blood. 'N' in each study group depicts the number of subjects in that group from whom the blood samples for nephrotoxin analysis were procured Values are represented as Pearson's correlation coefficients (r) with their respective p-values also calculated and italicized for identification of statistically significant associations (if any) (viz. common exposure source) present between any given two nephrotoxins. Differences at $p<0.05$ were considered to be statistically significant correlations, which are highlighted in bold. a 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, positive, strong and statistically significant ($p<0.05$) associations were noted between lead (Pb) and silica levels in the blood of the CKDu affected subjects signifying a common source of exposure of these nephrotoxins among these subjects which was most likely to be nephrotoxin enriched groundwater, as WHO surpassing significantly higher levels of the aforementioned nephrotoxins were detected in the groundwater regularly consumed in this region. For units of each individual nephrotoxin concentrations, kindly refer Table 3.3.1 of this section.

As indicated in **Table 3.3.4**, strong and significant ($p<0.05$) correlations were noted between the levels of Pb and Silica ($r=0.954$, $p=0.027$) in the blood of the CKDu affected subjects (i.e.study group 1) signifying a common source of origin of these nephrotoxins in the blood. This common source of nephrotoxins exposure was most likely to be consumption of untreated nephrotoxin contaminated drinking groundwater (Flora et al., 2012; Jayasumana et al., 2015b; Khandare et al., 2015; Levine et al., 2016; Lusco et al., 2017; Nanayakkara et al; 2014; Radovanovic et al.,1991; Sankhla,et al., 2016; Weaver & Jaar, 2015; Wimalawansa, 2016) as significantly higher levels of the silica and lead were noted in the groundwater of the CKDu affected region as compared to the lower and WHO established safe levels of these

nephrotoxins reported in the groundwater consumed by the general CKD affected non-endemic and healthy control regions(as described in sections 3.1.3.3-3.1.3.4 of this chapter). On the other-hand, insignificant ($p>0.05$) and negligible associations were noted between blood silica and lead levels in the general CKD affected and healthy control groups indicating the absence of these nephrotoxin enriched source of exposure in both the groups.

Additionally, non-significant ($p>0.05$) and inverse association was noted between the levels of Hg and Pb (Levine et al., 2016) and independently with silica levels in the blood CKDu affected subjects (study group 1) indicating two major different contributing sources of exposure of these three nephrotoxins.The former could have emerged from consumption of minimally contaminated fish with Hg (Faragher and DeHaan,2018), as BDL of Hg were detected in groundwater of this region and latter nephrotoxins in the blood(i.e. silica and Pb) could have most likely seemed to have arisen majorly from consumption of Pb and silica enriched groundwater (as high levels of both these nephrotoxins were detected in the same (Abiola,2017;Flora et al.,2012; Khandare et al.,2015;Levine et al.,2016;Sankhla ,et al., 2016).

Furthermore, insignificant and negligible correlations ($p>0.05$) were noted between blood ochratoxin and aristolochic acid levels despite both of them being contaminants of cereal foods, thus signifying a varied origin of these nephrotoxins in the exposure source, with the former arising from a fungal contaminating species (Duarte et al.,2010; Malir et al., 2016) & the latter arising from plant contaminating organism (Debelle et al.,2008; Michl et al., 2013).

As expected non-significant ($p>0.05$)and negative correlations were noted between OTA and AA with the heavy metals (i.e. Pb) and silica in the blood signifying two completely diverse sources of origin of these group of nephrotoxins with the former group (OTA & AA) arising from food contamination especially cereal grain contamination and the latter group(i.e. heavy metals& silica) arising from groundwater contamination (Khandare et al.,2015; Nanayakkara et al.,2014; Obrador et al.,2017; Stiborová et al.,2016).These correlation trends between Hg and Pb, OTA and AA and ochratoxin/aristolochic acid and heavy metal (Pb) and silica were replicated at a comparable extent in general CKD affected (group 2) & healthy control group (group 3) signifying differential origin of these nephrotoxins groups in these cohorts.

3.3.3.4 Relationship of the levels of nephrotoxin exposure in the blood with the estimated daily intake of the nephrotoxin from various sources of their exposure (i.e. untreated groundwater and food) in the study population via correlation assessments.

The association between the various measured nephrotoxins in the blood (i.e. biomarkers of nephrotoxin exposure) and their daily intake from their respective potential sources of

exposure viz.groundwater and food were estimated by calculation of Pearson's correlation coefficients (r) with their corresponding p-values for identifying the major contributing source (i.e. food or water) of nephrotoxin exposure in the blood (Rango et al., 2015), the results of which are presented in **Table 3.3.5.**

Table 3.3.5: Correlation assessments between the mean levels of various biomarkers of nephrotoxin exposure (i.e. the blood nephrotoxin levels) and their respective intake from the two major exposure sources viz. groundwater and food in the entire study population of Canacona taluka for identification of the probable source of exposure of the individual nephrotoxins.

CKD status		CKDu endemic cases		CKD non-endemic cases		No CKD prevalence (Healthy controls)	
Nephrotoxin in the blood with its exposure source	EDI intake of resepctive nephrotoxin from its exposure source	Study group 1 [N(males)=58; N(females)=56; N(total)=114]		Study group 2 [N(males)=15; N(females)=13; N(total)=28]		Study group 3 [N(males)=62; N(females)=62; N(total)=124]	
		r-value	*p-value*	r-value	*p-value*	r-value	*p-value*
Silica (blood)	Water	**0.953**	*0.015*	0.006	*0.045*	0.011	*0.031*
	Food	NA	NA	NA	NA	NA	NA
Lead (blood)	Water	**0.907**	*0.027*	0.009	*0.037*	0.017	*0.046*
	Food	0.102	*0.043*	0.019	*0.027*	0.043	*0.039*
Cadmium (blood)	Water	NC	NC	NC	NC	NC	NC
	Food	NC	NC	NC	NC	NC	NC
Arsenic (Blood)	Water	NC	NC	NC	NC	NC	NC
	Food	NC	NC	NC	NC	NC	NC
Mercury (blood)	Water	NC	NC	NC	NC	NC	NC
	Food	0.009	*0.041*	0.012	*0.031*	0.015	*0.047*
Ochratoxin (serum)	Water	NA	NA	NA	NA	NA	NA
	Food	0.013	*0.027*	0.024	*0.048*	0.033	*0.033*
Aristolochic acid (serum)	Water	NA	NA	NA	NA	NA	NA
	Food	0.009	*0.039*	0.018	*0.04*	0.027	*0.019*

Abbreviations: CKD-chronic kidney disease; CKDu-chronic kidney disease of unknown etiology; NA-not applicable for correlation assessments as the stated nephrotoxins are not present in the given exposure sources(viz. groundwater of food); NC- not calculated correlation coefficient owing to the below detectable levels of the nephrotoxin present in either the blood or the exposure source(i.e. food or groundwater). 'N' in each study group depicts the number of subjects in that group from whom the blood samples for nephrotoxin analysis were procured Values are represented as Pearson's correlation coefficients (r) with their respective p-values also calculated and italicized for identification of statistically significant associations (if any) viz. the probable exposure source (food /groundwater) of each individual nephrotoxin. Differences at $p<0.05$ were considered to be statistically significant correlations. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. Strong associations (correlations) are highlighted in bold that accurately confirm the most likely exposure source (i.e. groundwater or food) of the given nephrotoxin(s). As indicated in the results, significant associations ($p<0.05$) were noted between the blood levels of Pb and silica in the blood and the levels of the respective in water and to a certain extent in food(for lead exposure) in all the three study groups suggesting these exposure sources to be responsible for the origin of these nephrotoxins in the blood. But the strength of association (indicated by the value of the correlation coefficients with higher values denoting higher association) between the blood lead and silica levels and the levels of lead and silica in the groundwater consumed in the CKDu affected group(study group 1) were remarkably higher (highlighted in bold) than that witnessed with the concentrations in the food ingested by the same group(for lead exposure).Thus depicting that the source of origin of the significantly elevated levels of lead and silica in the blood of these subjects most probably arises from the chronic consumption of the nephrotoxin enriched groundwater in this region (indicated from significantly higher and WHO permissible limit surpassing levels of silica and lead detected in the same as described in section 1 of this chapter) rather than from the ingestion of the JCEFA established safe levels of minimally contaminated food (for lead exposure).Whereas the strength of association between the levels of lead in the blood and its levels in the food were comparatively higher than that with levels in the water in the diabetic and hypertensive CKD-affected(study group 2) and healthy control subjects(study group 3) suggesting that the most probable source of exposure in these cohorts were most likely to be consumption of negligibly contaminated food rather than groundwater. Moreover weak and significant($p<0.05$) associations were noted between the levels of Hg, ochratoxin and aristolochic acid and the levels of the respective in food at a comparable extent in all the three study groups indicating that all the three study cohorts possessed a common exposure source of these nephrotoxins i.e. ingestion of JCEFA established non-toxic levels of minimally contaminated food (as described in section 2 of this chapter) owing to similar food eating habits of the three groups arising from procurement of their respective food from a common source, hence level of contamination and associated exposure of these nephrotoxins through the food was comparable. For units of each individual nephrotoxin concentrations in blood and water and food, refer Table 3.3.1 of this current section, Table 3.1.4 of 1stsection and Tables 3.2.2 to 3.2.7 of 2nd section of this chapter respectively.

As indicated in **Table 3.3.5**, positively strong and significant ($p<0.05$) associations were noted between Pb in the blood and Pb intake from water ($r=0.907$, $p=0.027$) and food ($r=0.102$, $p=0.043$) but the strength of association between the exposure levels in the blood with the water was found to be comparatively higher than with the intake from food (Jayasumana et al., 2015 a and b; Levine et al., 2016; Nanayakkara et al; 2014).

Alternatively significant associations were observed between silica in the blood and EDI of silica from the water ($r=0.953$, $p=0.015$), with simultaneous non-significant ($p>0.05$) cum negligible associations noted between from silica in the blood and silica intake from food in the CKDu affected group (study group 1) (Khandare et al., 2015; Radovanovic et al., 1991).

These association results signified consumption of untreated nephrotoxin enriched groundwater and not food ingestion to be the major source of exposure of these subjects to the CKDu causative nephrotoxins viz. lead and silica in the Canacona taluka. These findings were backed by the observance of significantly ($p<0.05$) higher and WHO limit surpassing levels of silica noted in the groundwater (i.e. 115.5 mg/L) consumed by this CKDu affected regions along with its elevated daily intakes levels from the groundwater exceeding the WHO established tolerable limits as compared to the remarkably lowered values noted in the diabetic/hypertensive CKD affected and healthy control regions, thereby significantly contributing to the increased level of silica exposure (i.e. 100.2 mg/L) in the blood of these regions (Khandare et al., 2015; Radovanovic et al., 1991; Stiborova et al., 2016). Alternatively although the levels of lead in the groundwater were at the WHO established borderline levels and the levels of lead in the food were significantly below the JCEFA established safe levels which resulted in the associated intakes of lead from both food and water to be well within the JECFA set tolerable dietary limit, the exposure levels in the blood (306.5 µg/L)of these CKDu affected subjects significantly ($p<0.05$) exceeded the WHO permissible ranges(50 µg/L).This could be attributed to the inherently high bioacccumulative tendencies of lead to deposit in the major target organs specifically kidney (and to a small extent in the bone) even at lowered levels of exposure, which is frequently remobilized from the reserves in the kidney and the bone into circulation owing to the reabsorptive tendencies of these organs that resulted in increased levels of the same in the blood (Gunatilake et al., 2015; Jayasumana et al., 2015 a and b; Levine et al., 2016; Nanayakkara et al; 2014; Sanoff et al., 2010;Weaver and Jaar, 2015;Wimalawansa, 2016). These findings were in agreement with the previously reported studies of CKDu, wherein exposure of the chronic kidney disease affected subjects to lowered levels of lead (6-8 µg/L) through chronic consumption of drinking water had resulted in blood lead concentrations in the range of 200-400 µg/L, which

was reported to cause the development of prominent Pb inclusion bodies in the proximal tubular cells (on renal biopsy) at such levels,which ultimately manifested in development of interstitial fibrosis and tubular dysfunction.Thus signifying Pb to be a prominent nephrotoxin responsible in Canacona's CKDu development on chronic exposure (CDC, 2015; Fadrowski et al., 2013; Lin et al., 2006; NIH, 2012; Rastogi, 2008; Wijkström et al., 2018).

The elevated levels of silica and borderline levels of lead in the groundwater and the associated increased intake of these nephrotoxins in CKDu affected region of Canacona taluka were an outcome of the acid mine drainage from the non-operational granite mine located in the vicinity of the CKDu affected region into this region's aquifer that caused the acidic leaching of the trace geogenic element viz. silica and Pb (which majorly constitutes the aquifers bedrock) into the groundwater (Flora et al.,2012; Khandare et al., 2015; Khan et al., 2015; Levine et al.,2016; McCarthy,2011; Sankhla,et al., 2016;) consequently enriching it with silica and Pb, thereby resulting in increased intake in this region.

Moreover the elevated levels of lead in this region that contributed to the increased intake could also have been a consequence of the acidic groundwater leaching the lead from the inferior PVC well-casing enriched in lead stabilisers (LEAD Action News, 2010; Parker et al., 1990) that was found to be prevalent in wells of this CKDu affected region (as described in section 1 of this Chapter). Such elevated leachings of Pb and silica in the groundwater were not evident in the CKD-non-endemic group and the healthy control regions of the taluka owing to the neutral pH of the groundwater in these regions accounted to the absence of acid mine drainage due to the increased geographical distance from the non-operational mine (Flora et al., 2012; Sankhla et al., 2016).

Overall, these findings were in line with the generally reported trend of the major source of silica and lead exposure in the CKDu affected developing countries like India (i.e.Andhra Pradesh) (Khandare et al., 2015; Tatapudi, et al., 2018), Sri Lanka (Ekong et al., 2006; Jayasumana et al., 2015 a and b; Levine et al., 2016; Nanayakkara et al; 2014) and the Balkan region (Goldsmith and Goldsmith,1993; Hotz et al., 1995; Khandare et al., 2015; Markovic and Lebedev, 1965; Markovic; 1968 and 1974; Markovic and Arambasic, 1971; Ng et al., 1992; Radovanovic et al., 1991; Stiborova et al., 2016; Sunethra et al., 2018) to be via consumption of contaminated drinking water and not ingestion of food

Alternatively weak and significant ($p<0.05$) associations were noted at a comparable extent between Pb in blood and Pb intake from water and food along with silica in blood and silica intake from water and food in both the general CKD affected group and healthy controls group, depicting that the blood lead levels and blood silica levels in both these groups were

derived from exposure to lead and silica via intake from food and water, but the levels of exposure were well below the WHO established safe levels due to absence of a significant contaminating source (like acidic groundwater in the CKDu affected region), suggesting no cause for concern (Khandare et al., 2015; Levine et al., 2016)

On other hand, significantly ($p<0.05$) weak and negligible associations were noted at a comparable extent in all of the three study groups between the Hg in the blood and Hg intake from food with no correlations detected between Hg in blood and intake from water owing to the below detectable levels of Hg present in the groundwater of all these regions (as described in section 3.1.3.3 of this Chapter), thus not contributing to intake via groundwater consumption. Thus signifying that the minimal, comparable, non-toxic and WHO established safe level of Hg exposure in all of these three study groups subjects were mainly derived from chronic consumption of food specifically fish negligibly contaminated with Hg (as described in section 3.2.2.3.1.1.4 of this Chapter), that did not suffice in inducing significant amount of nephrotoxicity in either of the groups. These findings were in agreement with the general source of Hg intake and exposure to be via the diet especially through fish ingestion with contaminated drinking water consumption contributing very negligibly to the intake and exposure levels (Almaguer et al., 2014; Correa-Rotter et al., 2014; Jayatilake et al., 2013; McClean et al., 2012; Wesseling et al., 2013).

Similarly significantly ($p<0.05$) weak and minimal correlations were also noted between ochratoxin in the blood and intake from food and aristolochic acid in the blood and intake from food in all of the three study cohorts with absolutely no inevitable correlations observed between these blood nephrotoxin levels and the intake from groundwater as these nephrotoxins are major contaminants of the food grains i.e. cereals and pulses, thus these nephrotoxin exposure in the blood was not expected to be associated with the consumption of drinking groundwater.However,levels of exposure were comparable, non-toxic and present at WHO established safe ranges in all of the three study groups, thus ruling out any significant contribution of these aforementioned toxins in inducing nephrotoxicity or renal damage in either of the groups (Obrador et al. 2017; Stiborová et al., 2016; Wanigasuriya et al., 2008).

These findings were in agreement with observations of the previous studies that negated the role of Hg, ochratoxin and aristolochic acid in CKDu causation in these regions (Almaguer et al., 2014; Correa-Rotter et al., 2014; Jayatilake et al., 2013; Khandare et al., 2015; Levine et al., 2016; McClean et al., 2012; Nanayakkara et al., 2014; Obrador et al. 2017; Stiborová et al., 2016; Wanigasuriya et al., 2008; Wesseling et al., 2013).

3.3.3.5 Effect of heavy metals (i.e. Pb, Cd, As and Hg) and metalloid (viz. silica) on the essential element profile of the study population

Heavy metals and metalloids [viz. silica specifically silicon (Si)] chemically behave in a similar manner wherein both exhibit long half-lives due to their higher chemical stabilities which confers strong accumulative tendencies in biological organism.Hence these chemical similarities between heavy-metals and metalloid confer homology in mechanism of interactions of the same with essential elements in humans. Hence was determined in the current study as well (Jan et al.,2015;Lentini et al., 2017;Matović et al., 2015;Orr and Bridges, 2017;Sergent et al.,2017;Sponholtz et al.,2016;Tsuchiya et al.,2017; Xu et al., 2018). It has been well reported that there exists a co-dependent relationship between the nephrotoxic heavy metal (i.e.Pb, Cd, As, Hg) and metalloid (viz.silica specifically silicon) accumulation and essential element homeostasis wherein the toxic metal & metalloid accumulation can influence essential element homeostasis or level of essential metals can determine the extent of toxic metals & metalloid accumulation in the body (Goyer, 2016). Hence it becomes mandatory to assess levels of essential elements in blood during analysis of nephrotoxin exposure in an exposed population in order to check susceptibility for heavy metal & metalloid accumulation and its induced toxic effects(Levine et al.,2016).Hence,in this study levels of major essential elements i.e.Zn, Cu, Fe, Mn, Se,Ca in blood were analysed **(Figure 3.3.4 & Table 3.3.6)** & their relation with accumulation of heavy metals (i.e.Pb, Cd, As, Hg) & metalloid (i.e.silicon) were examined by correlation analysis **(Table 3.3.7).**

As indicated in **Figure 3.3.4 & Table 3.3.6**, levels of all essential elements were within their suggested limit in diabetes & hypertensive CKD cases (group 2) & healthy control (group 3) indicating no involvement of metal and metalloid (specifically silica) toxicity (Goyer,2016). Contrarily, levels of all essential elements in the blood were significantly($p<0.05$) well below their respective WHO set recommended limits in CKDu affected subjects,clearly signifying induction of heavy-metal and metalloid (specifically silica) induced nephrotoxicity among these subjects (Jayatilake et al.,2013; Levine et al.,2016; Khandare et al., 2015; Radovanovic et al.,1991). It has been well reported that heavy metals and metalloid (specifically silica) being homologous in their valencies with most essential elements tends to compete with metal binding sites of the latter's transporter that are involved in uptake, transport and absorption of these essential elements into blood. Thus increased presence of one or more heavy metals and metalloid (specifically silica) in the human body greatly influences the rate of absorption of these essential elements and hence results in decreased levels of the latter in blood circulation (Carson,2018;Gil and Hernández,2015;Nordberg, 2016;Sergent et al.,2017).

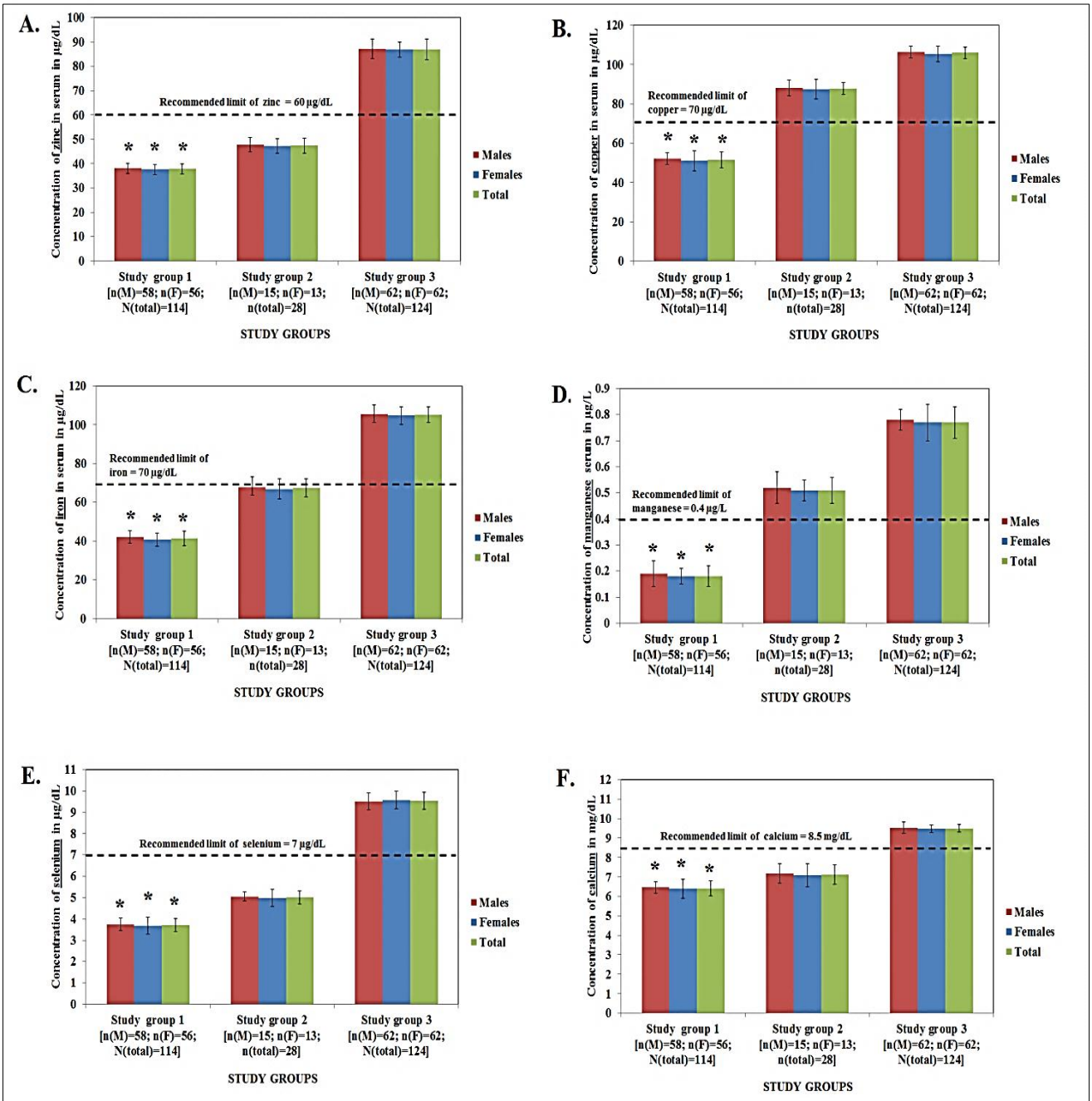


Figure 3.3.4. Mean levels of the essential elements in the serum of both sexes of all three study groups viz. CKDu affected group, diabetes and hypertensive CKD affected group and healthy controls

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; M-male; F-female. Data are derived from two independent experiments with each essential element of an individual experiment measured in duplicates.Study group 1, 2 & 3 consists of CKDu affected,diabetes/hypertension induced CKD affected & healthy controls subjects respectively.n(M) & n(F) in each study group denotes the total number of males & females respectively from whom blood samples(specifically serum) for analysis were obtained.n(M) +n(F)=N(total) of a given study group which represents the total number of subjects in that group.(A)Concentration of zinc in the serum of the subjects of all three study groups;(B)Concentration of copper in the serum of the study subjects;(C)Concentration of iron in the serum of the study subjects;(D) Concentration of manganese in the serum of the study subjects;(E) Concentration of selenium in the serum of the study subjects;(F) Concentration of calcium in the serum of the study subjects.Values are represented as mean±SD(standard deviation) of the essential element level estimated independently in the male subjects, female subjects and both sexes combined(i.e. total number of subjects) in all three study groups All concentration values are in µg/dL except for manganese depicted in µg/L and calcium in mg/dL. Differences at *p<0.05 were considered to be significant. The WHO established recommended limits in blood for zinc=60µg/dL; copper=70µg/dL;iron= 70µg/dL; manganese= 0.4µg/L; selenium=7 µg/dL; calcium=8.5mg/dL. Significantly lowered levels of all essential elements were noted in the blood(specifically serum) of CKDu affected subjects(study group 1) as compared to diabetes or hypertensive CKD affected group(study group 2)and healthy controls(study group 3) and were also found to be well below their respective WHO recommended limits thus indicating retardation of intestinal absorption of these essential elements into blood circulation by high levels of nephrotoxic heavy metal and metalloid viz. lead and silica respectively that were present in the blood of these subjects.The absorption inhibition was possibly attributed to the high levels of lead and silica competitively binding to the metal transporters involved in intestinal uptake and absorption of these essential element (due to similarities in the structure & valencies of heavy metals & essential elements) thus causing competitive exclusion of these essential elements from intestinal absorption resulting in decreased levels of the same in the blood of these subjects.The essential element deficiency in CKDu affected subjects(study group 1) could have contributed to the worsening of skeletal disorders and bone pains noted in these subjects as these elements at optimal levels serve as metal cofactors for enzymes involved in bone mineral metabolism that is required for absorption and deposition of calcium & phosphate in the bone matrix.Hence deficiency of the same could have caused disruption of bone mineralization and calcium homeostasis causing further skeletal damage.The below optimal levels of essential element in these CKDu affected subjects could have further contributed to the aggravation of the renal tubular damage as lowered levels of the same failed to restrict the bioavailability of silica & lead toxins by forming inert and insoluble metal complexes with these toxins when present at optimal levels which contributed to the increased toxin accumulation and associated induction of tubular damage. Moreover this essential elements' deficiency failed to provide anti-oxidant protection against the oxidative damage inflicted by lead heavy metal as these essential elements at optimal levels serve as metal cofactors for antioxidant enzymes like superoxide dismutase and glutathione peroxidase.Thus increased the susceptibility of CKDu affected subjects (study group1) to increased oxidative tubular damage by the nephrotoxins. The details on the range of minimum to maximum values of the levels of each essential element & prevalence distribution(%) of the essential element concentration in various ranges for each sex and both sexes combined(i.e.total number of subjects) in all three study groups are listed in **Table 3.3.6.**

Table 3.3.6: Mean and range concentrations of the essential elements estimated in the blood in both sexes of the entire study population and comparison of their levels with the respective WHO established normal reference ranges

CKD status		CKDu endemic cases				CKD non-endemic cases				No CKD prevalence(Healthy controls)				WHO established permissible limits
Essential element	Gender	Study group 1 [N(males)=58; N(females)=56; N(total)=114]				Study group 2 [N(males)=15; N(females)=13; N(total)=28]				Study group 3 [N(males)=62; N(females)=62; N(total)=124]				
		Mean	Range (min-max)	Prevalence (%)	Annnotation for the ranges of the esential element	Mean	Range (min-max)	Prevalence (%)	Annnotation for the ranges of the esential element	Mean	Range (min-max)	Prevalence (%)	Annnotation for the ranges of the esential element	
Zinc (serum)	M	38.1±1.04 *Δ	(37.3-40.4)	A=65.3%; B=25.9%; C=8.8%	A=37-38; B=38-39; C=39-40	47.7±2.02	(45.6-48.0)	A=3.5%; B=22.9%; C=73.6%	A=45-46; B=46-47; C=47-48	87.1±3.03	(85.5-87.9)	A=5.6%; B=40.6%; C=53.8%	A=85-86; B=86-87; C=87-88	Between 60-130 µg/dL
	F	37.6±1.02 *Δ	(37.0-39.3)	A=63.5%; B=28.2%; C=8.3%		47.2±2.04	(45.1-47.6)	A=3.0%; B=23.6%; C=73.4%		86.8±3.01	(85.0-87.0)	A=6.9%; B=42.4%; C=50.7%		
	Total	37.8±1.03 *Δ	(37.0-40.4)	A=64.4%; B=27.0%; C=8.5%		47.4±2.03	(45.1-48.0)	A=3.2%; B=23.2%; C=73.5%		86.9±3.02	(85.0-87.9)	A=6.20%; B=41.5%; C=52.2%		
Copper (serum)	M	52.0±2.03 *Δ	(50.8-56.1)	A=70.2%; B=26.8%; C=3.0%	A=50-52; B=52-54; C=54-56	88.1±3.03	(84.6-88.9)	A=3.2%; B=31.3%; C=68.5%	A=83-85; B=85-87; C=87-89	106.4±2.02	(102.8-106.9)	A=1.8%; B=40.1%; C=58.1%	A=101-103; B=103-105; C=105-107	Between 70-150 µg/dL
	F	51.1±5.1 *Δ	(50.1-55.5)	A=68.5%; B=28.3%; C=3.2%		87.4±5.05	(83.5-88.1)	A=2.9%; B=34.1%; C=64.0%		105.5±4.01	(101.9-106.1)	A=1.6%; B=38.1%; C=60.3%		
	Total	51.5±4.07 *Δ	(50.1-56.1)	A=69.35%; B=27.5%; C=3.1%		87.7±3.04	(83.5-88.9)	A=3.0%; B=32.7%; C=66.2%		105.9±3.02	(101.9-106.9)	A=1.7%; B=39.1%; C=59.2%		
Iron (serum)	M	41.9±2.5 *Δ	(41.3-45.3)	A=75.1%; B=21.3%; C=3.6%	A=40-42; B=42-44; C=44-46	67.7±4.1	(62.6-67.8)	A=2.0%; B=45.3%; C=52.7%	A=62-64 B=64-66; C=66-68	105.6±3.5	(101.5-106.0)	A=1.5%; B=30.3%; C=68.2%	A=100-102; B=102-104; C=104-106	60-170 µg/dL
	F	40.8±3.0 *Δ	(40.1-44.5)	A=72.3%; B=25.3%; C=2.4%		66.9±5.3	(62.1-67.1)	A=1.5%; B=47.8%; C=50.7%		104.8±4.6	(101.1-105.5)	A=1.0%; B=31.2%; C-=67.3%		
	Total	41.3±2.7 *Δ	(40.1-45.3)	A=73.7%; B=23.3%; C=3.0%		67.3±4.7	(62.1-67.8)	A=1.7%; B=46.5%; C=51.7%		105.2±4.0	(101.1-106.0)	A=1.2%; B=30.7%; C=67.7%		
Manganese (serum)	M	0.19±0.05 *Δ	(0.18-0.22)	A=60.3%; B=39.7%	A=0.17-0.20; B=0.20-0.23	0.52±0.06	(0.49-0.53)	A=40.1%; B=59.9%	A=0.47-0.50; B=0.50-0.53	0.78±0.04	(0.75-0.79)	A=35.6%; B=64.4%	A=0.73-0.76; B=0.76-0.79	0.4-0.85 µg/L
	F	0.18±0.03 *Δ	(0.17-0.21)	A=58.9%; B=41.1%		0.51±0.04	(0.48-0.52)	A=44.3%; B=56.7%		0.77±0.07	(0.74-0.78)	A=38.4%; B=62.6%		
	Total	0.18±0.04 *Δ	(0.17-0.22)	A=59.6%; B=40.4%		0.51±0.05	(0.48-0.53)	A=42.2%; B=58.3%		0.77±0.06	(0.74-0.79)	A=37%; B=63.5%		
Selenium (serum)	M	3.75±0.3 *Δ	(3.69-4.11)	A=63.7%; B=32.9%; C=3.4%	A=3.6-3.8; B=3.8-4.0; C=4.0-4.2	5.05±0.2	(4.67-5.11)	A=1.1%; B=28.6%; C=70.3%	A=4.5-4.7; B=4.7-4.9; C=4.9-5.1	9.51±0.4	(9.11-9.57)	A=4.3%; B=40.6%; C=55.1%	A=9.0-9.2; B=9.2-9.4; C=9.4-9.6	Between 7-15 µg/dL
	F	3.68±0.4 *Δ	(3.60-4.09)	A=61.7%; B=35.5%; C=2.8%		4.98±0.4	(4.60-5.05)	A=0.9%; B=30.3%; C=68.8%		9.58±0.4	(9.19-9.62)	A=4.6%; B=36.9%; C=58.5%		
	Total	3.71±0.3 *Δ	(3.60-4.11)	A=62.7%; B=34.2%; C=3.1%		5.01±0.3	(4.60-5.11)	A=1.0%; B=29.4%; C=69.5%		9.54±0.4	(9.11-9.62)	A=4.4%; B=38.7%; C=56.8%		
Calcium (serum)	M	6.46±0.2 *Δ	(6.34-6.78)	A=70.5%; B=25.3%; C=4.2%	A=6.3-6.5; B=6.5-6.7; C=6.7-6.9	7.17±0.4	(7.08-7.51)	A=2.0%; B=44.9%; C=53.1%	A=6.6-6.8; B= 6.8-7.0 C=7.0-7.2	9.53±0.2	(9.18-9.59)	A=1.1%; B=30.5%; C=68.4%	A=9.0-9.2; B=9.2-9.4; C=9.4-9.6	Between 8.5-10.5 mg/dL
	F	6.39±0.4 *Δ	(6.32-6.72)	A=67.1%; B=29.1%; C=3.8%		7.09±0.5	(7.00-7.44)	A=2.4%; B=47.3%; C=50.3%		9.48±0.1	(9.13-9.54)	A=1.5%; B=33.4%; C=65.1%		
	Total	6.42±0.3 *Δ	(6.32-6.78)	A=68.8%; B=27.2%; C=4.0%		7.13±0.4	(7.00-7.51)	A=2.2%; B=46.1%; C=51.7%		9.50±0.1	(9.13-9.59)	A=1.3%; B=31.9%; C=66.7%		

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; M-male; F-female; WHO-world health organization. Data are derived from two independent experiments with each essential element of an individual experiment measured in duplicates. 'N' in each study group depicts the number of subjects in that group from whom the blood samples for essential element analysis were procured Values are represented as mean±SD with the range of minimum to maximum concentrations of each essential element depicted as well. All concentration values are in µg/dL except for manganese depicted in µg/L and calcium in mg/dL. The prevalence of the essential element concentration in various ranges for each sex category of the three study groups are represented by prevalence % and annotated by uppercase letters(i.e. A to C) for convenience. The annotations for each estimated essential element varies between the elements and the study groups as well and have been independently described for each of the study groups for a particular element. The prevalence % of a particular study group denotes the proportion of subjects from its total number in their respective sex categories (N for males and N for females being different in all the study groups) bearing the essential element at a given concentration range. The total number of males(M)/females(F) in study group one, two and three are 58(M)/56(F); 15(M)/13(F) and 62(M)/62(F) respectively. For example, the prevalence % annotation of 'A' in the case of serum zinc levels estimated in study group 1 denotes the percentage of the total population of study group 1 in a particular sex category possessing concentrations at the range of 37-38µg/dL. Differences at *$p<0.05$ were considered to be statistically significant as compared to the true controls(study group 3). Differences at $^{\Delta}$ $p<0.05$ were considered to be

statistically significant as compared to the non-endemic CKD cases(study group 2). The values highlighted in bold denote the levels of all of the essential elements in the blood (specifically the serum) that were significantly lower than their respective WHO established normal permissible range being prevalent at their respective lowest concentration ranges(depicted from increased prevalence % for a particular range) and were reduced only in the CKDu endemic group(study group 1) depicting inhibition of absorption of these elements into blood circulation by the nephrotoxic heavy metal and metalloid (viz. lead and silica respectively that were present at significantly elevated levels of exposure in blood).This absorption inhibition was attributed to possible competition of the metal binding sites in the transporters involved in uptake, absorption and distribution of these essential element owing to the homology in the valencies and structure of the essential elements and the heavy metal or metalloid.

Interactions between the heavy metals [Pb or Cd (major interactions); Hg or As(minor interactions and metalloid (specifically silica)] with the essential elements-Zn and Cu have been limitedly documented in some of the studies related to CKDu affected subjects exposed to these nephrotoxic heavy metals in Sri Lanka and the Uddanam region of Andhra Pradesh (India) (Gunatilake et al., 2015; Jayatilake et al., 2013; Jayasumana et al., 2015a; Khandare et al.,2015; Levine et al., 2016; Nanayakkara et al; 2014; Radovanovic et al., 1991; Sanoff et al., 2010; Weaver and Jaar, 2015; Wijkström et al., 2018; Wimalawansa, 2016). Interactions between these heavy metals or metalloids and essential element viz. Zn or Cu metabolism in the body were related to shared tendency of the heavy metals, metalloid and the essential elements to trigger metallothionein synthesis and competitively bind to the cation- binding thiol sites of metallothioneins. Metallothioneins are metal and metalloid binding proteins which are inherently enriched in the proximal tubular compartment of the kidney being produced under conditions of excessive metal and metalloid stress that helps in the detoxification of these metals and metalloid from the circulation. Thus under conditions of excessive heavy-metal accumulation specifically of Pb or Cd and metalloid deposition (specifically of silica) causes these heavy metals and metalloids to signal the induction of the metallothioneins wherein the intrinsic preferential selectivity of the essential elements for the metallothoneins induces the essentials elements to compete with the heavy metals and metalloids for the binding to the metallothioneins that ultimately causes the formation of essential element and metallothionein complexes which are consequently eliminated from the body in the urine thereby resulting in decreased levels of Zn and Cu under higher heavy metal and metalloid accumulation conditions (Díaz-Gómez et al., 2017; Dashnyam et al., 2017; Gil and Hernández, 2015; Kowalska et al., 2015; Nordberg et al., 2014; Nordberg and Nordberg, 2016; Rahman et al., 2017). Moreover Pb and Cd tends to compete with the metal-binding sites of the metal-transporter-2 (MT-2) which characteristically regulates the uptake and trafficking of these divalent essential metal ions i.e. Zn and Cu, thus further aggravating the decreased absorption of these essential elements into circulation (Carson, 2018; Díaz-Gómez et al., 2017; Dashnyam et al., 2017; Maret, 2017; Nordberg et al., 2014; Nordberg and Nordberg, 2016; Sergent et al., 2017; Yang and Shu, 2015). These negative interactions

between Pb and Zn; Pb and Cu; Silica and Zn and Silica and Cu explains the decreased levels of Zn and Cu noted in the blood of the CKDu affected subjects **(Figure 3.3.4 & Table 3.3.6)** owing to the enhanced accumulation of Pb and silica in these subjects as evidenced from the higher levels of these nephrotoxins in the blood noted in the current study. This was further supported by the observance of significantly ($p<0.05$) negative correlations between Pb-Zn ($r=-0.901$, $p=0.041$), Pb-Cu ($r=-0.752$, $p=0.023$); Silica-Zn ($r=-0.863$, $p=0.023$) and Silica-Cu ($r=-0.715$,$p=0.015$) in the blood of the CKDu affected subjects as indicated in **Table 3.3.7**, depicting negative interactions between this heavy metal (viz. lead), metalloid (viz.silica) and the essential elements (viz. Zn and Cu) (Levine et al., 2016; Khandare et al., 2015). This significant decline in the levels of essential elements viz. Zn and Cu in response to chronic and increased lead and silica exposure were in agreement with the values noted in a similar long-term exposure of independent CKDu cases to the high levels of lead and silica nephrotoxin respectively through chronic groundwater consumption in Sri Lanka, the Uddanam region of Andhra Pradesh (another Indian state) and some parts of Central America (Gunatilake et al., 2015; Jayatilake et al., 2013; Jayasumana et al., 2015a; Khandare et al., 2015; Levine et al., 2016; Nanayakkara et al; 2014; Radovanovic et al., 1991; Sanoff et al., 2010; Weaver and Jaar, 2015; Wijkström et al., 2018; Wimalawansa, 2016).

The existence of interactions between the heavy metals [i.e. Pb or Cd (majorly); As or Hg (minorly)], metalloid (specifically silica) and the essential elements viz. Fe and Mn metabolism have been considerably reported.It has been demonstrated that both in experimental animals and in humans that absorption of the heavy-metals (specifically of Pb and Cd and to a negligible extent of Hg and As)and metalloid (specifically silica) from the intestinal tract was found to be inversely related to blood ferritin levels and Fe stores in the body (Carson,2018; Dashnyam et al.,2017; Goyer,2016; Jones et al.,2017; Klotz and Göen, 2017; Nordberg et al.,2014). Additionally, addition of Fe or Mn into the diet was observed to be effective in preventing signs of heavy-metal and silica induced nephrotoxicity (i.e.Pb and Cd nephrotoxicity) in pigs, rats & sheep as opposed to other conditions that causes increased absorption and accumulation of these heavy metals and silica in these animals.The mechanism underlying the ability of these essential elements to retard the absorption of these nephrotoxic heavy metals and metalloid (viz. silica) is not clear till date (Caito et al., 2017; Gajek et al.,2013;Gil and Hernández,2015;Mozrzymas,2018; Nordberg and Nordberg, 2016).

Table 3.3.7: Correlation assessments between the mean levels of various biomarkers of nephrotoxin exposure(i.e. the blood nephrotoxin levels) and the essential element concentrations(i.e. serum essential elements levels) in the entire study population of Canacona taluka for analysis of the effect of the nephrotoxin on the essential element profile of the study population.

Nephrotoxin in blood	Study group (total number of subjects)	Correlation coefficients [r] *(p values)* Essential elements					
		Zn	Cu	Fe	Mn	Se	Ca
Silica	1	**-0.863**	**-0.715**	**-0.805**	**-0.887**	**-0.941**	**-0.856**
	(N=114)	0.023	0.015	0.037	0.021	0.017	0.015
	2	-0.013	-0.009	-0.021	-0.017	-0.0008	-0.001
	(N=28)	0.147	0.112	0.089	0.101	0.212	0.077
	3	-0.021	-0.017	-0.009	-0.005	-0.027	-0.011
	(N=124)	0.131	0.098	0.071	0.063	0.145	0.136
Pb	1	**-0.901**	**-0.752**	**-0.799**	**-0.874**	**-0.924**	**-0.946**
	(N=114)	*0.041*	*0.023*	*0.041*	*0.023*	*0.039*	*0.031*
	2	-0.019	-0.013	-0.004	-0.007	-0.016	-0.008
	(N=28)	*0.078*	*0.102*	*0.323*	*0.058*	*0.212*	*0.172*
	3	-0.002	-0.02	-0.006	-0.009	-0.005	-0.037
	(N=124)	*0.41*	*0.181*	*0.122*	*0.137*	*0.156*	*0.099*
Cd	1	NC	NC	NC	NC	NC	NC
	(N=114)	NC	NC	NC	NC	NC	NC
	2	NC	NC	NC	NC	NC	NC
	(N=28)	NC	NC	NC	NC	NC	NC
	3	NC	NC	NC	NC	NC	NC
	(N=124)	NC	NC	NC	NC	NC	NC
As	1	NC	NC	NC	NC	NC	NC
	(N=114)	NC	NC	NC	NC	NC	NC
	2	NC	NC	NC	NC	NC	NC
	(N=28)	NC	NC	NC	NC	NC	NC
	3	NC	NC	NC	NC	NC	NC
	(N=124)	NC	NC	NC	NC	NC	NC
Hg	1	-0.012	-0.015	-0.027	-0.038	-0.029	-0.044
	(N=114)	*0.117*	*0.136*	*0.241*	*0.211*	*0.345*	*0.099*
	2	-0.018	-0.025	-0.031	-0.03	-0.037	-0.061
	(N=28)	*0.219*	*0.311*	*0.25*	*0.078*	*0.31*	*0.084*
	3	-0.015	-0.019	-0.039	-0.041	-0.033	-0.055
	(N=124)	*0.076*	*0.097*	*0.104*	*0.091*	*0.175*	*0.333*
Ochratoxin	1	-0.0009	-0.0003	-0.0006	-0.0002	-0.0011	-0.0004
	(N=114)	*0.125*	*0.117*	*0.144*	*0.289*	*0.362*	*0.245*
	2	-0.0005	-0.0004	-0.0009	-0.0002	-0.0006	-0.0003
	(N=28)	*0.114*	*0.098*	*0.074*	*0.157*	*0.356*	*0.279*
	3	-0.0002	-0.0008	-0.0004	-0.0001	-0.0006	-0.0003
	(N=124)	*0.075*	*0.094*	*0.215*	*0.314*	*0.128*	*0.305*
Aristolochic acid	1	-0.0006	-0.0003	-0.0009	-0.0007	-0.0007	-0.0004
	(N=114)	*0.321*	*0.195*	*0.411*	*0.117*	*0.165*	*0.264*
	2	-0.0011	-0.0004	-0.0009	-0.0005	-0.0006	-0.0002
	(N=28)	*0.201*	*0.121*	*0.213*	*0.097*	*0.06*	*0.077*
	3	-0.0016	-0.0009	-0.001	-0.0003	-0.0012	-0.0006
	(N=124)	*0.068*	*0.201*	*0.097*	*0.114*	*0.273*	*0.149*

Abbreviations: CKD-chronic kidney disease; CKDu- chronic kidney disease of unknown etiology; NC- not calculated correlation coefficient owing to the below detectable levels of the nephrotoxin present in the blood. 'N' in each study group depicts the number of subjects in that group from whom the blood samples for nephrotoxin and essential element analysis were procured Values are represented as Pearson's correlation coefficients (r) with their respective p-values also calculated and italicized for identification of statistically significant associations (if any) viz. the effect of the given nephrotoxin on the essential element levels in the three study cohort. Differences at $p<0.05$ were considered to be statistically significant correlations. 'r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. All the associations between the nephrotoxin and the essential elements were negative due to the intrinsic inhibitory effect of the nephrotoxin on the essential element profile. The strength of association (indicated by the value of the correlation coefficient(r)) varied between the groups of the nephrotoxin and essential elements with higher values denoting stronger inhibitory effects of the former on the latter. As indicated in the results, strong significant($p<0.05$) and negative associations(correlations) (highlighted in bold) were noted between the blood lead and silica levels with each individual essential element estimated in the blood of the CKDu affected group(study group 1) depicting strong inhibition of absorption of these essential elements into blood circulation by these two toxic nephrotoxic heavy metal and metalloid (viz. lead and silica respectively that were present at significantly elevated levels of exposure in blood).This absorption inhibition was attributed to possible competition of the metal binding sites in the transporters involved in uptake, absorption and distribution of these essential element owing to the homology in the valencies and structure of the essential elements and the heavy metal or metalloid. Contrarily, weak, negative and insignificant($p<0.05$) associations were also noted between each nephrotoxin with the individual essential element profile of the diabetic and hypertensive CKD-affected(study group 2) and healthy control subjects(study group 3) signifying inhibitory effect of the nephrotoxins on the essential element but at a very negligible extent [indicated from insignificant and minimal 'r' values noted between these groups(nephrotoxin v/s essential element)] owing to the negligible levels of nephrotoxin exposure at WHO established safe levels prevalent in these groups which fails to strongly retard the absorption of these essential elements into circulation. For units and values of each individual nephrotoxin and essential element concentrations, kindly refer Table 3.3.1 and Table 3.3.6 of this section.

Some recent investigations on the basis of the relationship between these heavy metals, metalloid (silica) and essential elements suggest the existence of a common metal/metalloid transporter for all of these divergent categories of the chemical element that control their metal and metalloid uptake and trafficking. One such transporter believed to play a major role in the transport of the aforementioned metals and metalloids is DMT1 (divalent metal transporter 1). The presence of a shared metal/metalloid transporter between these heavy metals, silica metalloid and the essential elements (i.e. Fe and Mn), inevitably triggers the heavy metals and silica under increased accumulation to increasingly compete with these essential elements for the metal binding sites of transporter causing the significant exclusion of these essential metals from binding which ultimately decreases the absorption and uptake of these elements into the blood stream (Nordberg et al., 2014; Maret, 2017; Sergent et al., 2017;Yang and Shu, 2015).

This inhibitory effect of the heavy metals (especially Pb and Cd and to minor extent-Hg and As) and the metalloid (specifically silica) on the essential elements viz. Fe and Mn explains the significantly ($p<0.05$) reduced levels of the latter observed in the blood of the CKDu affected subjects of the Canacona taluka **(Figure 3.3.4 & Table 3.3.6)** attributed to the increased exposure and accumulation of lead and silica in these subjects depicted from increased blood levels of these nephrotoxins in the current study. This was further supported by observance of significantly ($p<0.05$) negative correlations between Pb-Fe ($r=-0.799$, $p=0.041$), Pb-Mn ($r=-0.874$, $p=0.023$),Silica-Fe ($r=-0.805$, $p=0.037$) & Silica-Mn ($r=-0.887$, $p=0.021$) in the blood of CKDu subjects as indicated in **Table 3.3.7**,depicting negative association between Pb, silica & these essential elements (Levine et al., 2016; Khandare et al., 2015). This significant reduction in blood levels of essential elements viz. Fe and Mn in response to prolonged and increased lead and silica exposure were in agreement with the values noted in related independent chronic exposure of the CKDu cases in Sri Lanka and the Uddanam region of Andhra Pradesh to high levels of the lead and silica nephrotoxin respectively through drinking water (Gunatilake et al., 2015; Jayatilake et al., 2013; Jayasumana et al., 2015a;Khandare et al., 2015; Levine et al., 2016; Nanayakkara et al; 2014;Radovanovic et al., 1991;Sanoff et al., 2010;Weaver and Jaar, 2015;Wijkström et al.,2018;Wimalawansa, 2016).

Negative associations between Se, heavy metals like Pb, Cd and Hg have been well reported wherein an increased intake of Se has been shown to reduce the toxicity of these heavy metals by increased production of selanoproteins (with anti-oxidative properties) and forming inert and insoluble metal complexes (metal-Se complexes) that prevent the effective action of

the toxic ionic form of the heavy metals due to the trapping of the metal in the combined form. However on excessive heavy metal accumulation, the heavy metals avert the complex formation with selenium as it remarkably exceeds the normal levels of selenium present in the blood ,allowing it to simultaneously triggers the reduction in the intestinal absorption of selenium by competing for the Se binding and uptake sites, thereby consequently resulting in increased excretion of selenium from the body and decreased levels of the same in the blood (Carson, 2018; Gajek et al., 2013; Goyer, 2016; Jan et al., 2015; Mozrzymas , 2018; Nordberg et al., 2014; Nordberg and Nordberg , 2016; Prashanth et al., 2015; Wu et al., 2016). However the inhibitory absorptive effect of selenium on silica still needs to be investigated as no research has been done in this field. This inhibitory effect of the heavy metals on the absorption of Se at high heavy metal concentrations explains the significantly decreased levels of Se noted in the blood of the CKDu affected subjects **(Figure 3.3.4 & Table 3.3.6)** accredited to increased and prolonged exposure to lead and possibly silica resulting in enhanced lead & silica accumulation in these affected subjects as evident from increased blood levels of these nephrotoxins in the current study.This was further supported by existence of significantly ($p<0.05$) inverse correlations between Pb-Se ($r=-0.924$, $p=0.039$) and Silica-Se ($r=-0.941$, $p=0.017$) in the blood of the CKDu affected subjects as indicated in **Table 3.3.7**, depicting the negative relationship between this heavy metal (i.e. Pb) and metalloid (i.e. silica) and the essential element (i.e. Se). Due to the negative associations obtained between silica and selenium levels in the blood of the CKDu affected subjects in the current study, we assume that possibly silica also has an inhibitory effect on the absorption of selenium in a mechanism similar to the heavy metal lead owing to similarities in the electronic and structural configurations of this heavy metal (lead) and metalloid (silica) which could have contributed to the decreased levels of selenium in the blood of the affected subjects. However further investigation is warranted to significantly prove this assumption.

These findings of the remarkable reduction in the blood levels of selenium in response to chronic and increased lead and possibly silica exposure were in agreement with the values noted in a similar prolonged exposure of the independent CKDu cases in Sri Lanka and Andhra Pradesh to elevated levels of lead and silica nephrotoxin respectively through drinking water (Gunatilake et al., 2015; Jayatilake et al., 2013; Jayasumana et al., 2015a; Khandare et al., 2015; Levine et al., 2016; Nanayakkara et al; 2014; Radovanovic et al., 1991; Weaver and Jaar, 2015; Wijkström et al., 2018).

The inverse associations between the heavy metals (specifically Pb and Cd) and the metalloid (specifically silica) with the essential element Ca has been well described in two of the major

target organs of the heavy metals viz. kidney and bone. In the bone, these divalent heavy-metal ions (viz. Pb and Ca) and metalloid (i.e.silica) on excessive accumulation in the body can effectively compete for the calcium binding sites due to similarities in the structure and valencies of these heavy metals and metalloid with the essential element (calcium), which causes the replacement of calcium from the bone structure and concurrent calcium mineral loss. This Ca loss causes severe disturbances in the calcium mineral homeostasis resulting in the disruption of calcification, decalcification and bone remodeling which ultimately manifests in various skeletal disorders (Assi et al., 2016; Caito et al., 2017;de Souza et al.,2018; García-Esquinas et al.,2015; Gil et al.,2015; Khandare et al., 2015; Klotz and Goen, 2017; Maret, 2017; Saravanabhavan et al.,2017; Tomaszewska et al., 2016; Wu et al., 2016).

This skeletal damage is further aggravated by the action of these heavy metals and metalloid in the kidney. In the kidney, these heavy metals (i.e. Pb and Cd) and metalloid (i.e. silica) can interfere with the activation of vitamin D (i.e. conversion of vitamin D to 1,25-dihydroxyvitamin D, its active form, which takes place primarily in renal tubule) resulting in Vitamin D deficiency. Vitamin D deficiency reduces the intestinal absorption of calcium and phosphate leading to overstimulation of parathyroid hormone (PTH) causing increased bone turnover and further loss of Calcium from the bones to maintain the optimum levels; which impairs Ca homeostasis. This ultimately leads to defective bone mineralization, reduction in the bone density and apatite thereby further aggravating the severity of the skeletal damage inflicted (Buser et al.,2016; Flora et al., 2012; Khandare et al.,2015; Kim et al., 2015; Ruiz et al., 2016; Sabath and Robles-Osorio, 2012; Tchounwou et al.,2012; Weaver and Jaar,2015).

This previously reported negative interaction of the heavy metals (i.e. Pb) and metalloid (i.e. silica) with the essential element (i.e. Ca) explains the significantly lowered levels of calcium noted in the blood of the CKDu affected subjects **(Figure 3.3.4 & Table 3.3.6)** attributed to prolonged exposure of these subjects to the higher levels of the heavy metal viz. lead and silica metalloid through the chronic consumption of drinking water that causes the increased accumulation of Pb and silica in these subjects which simultaneously triggers the loss of calcium from the blood. This was further supported by the existence of significantly ($p<0.05$) negative correlations noted between Pb-Ca ($r=-0.946, p=0.041$) and Silica-Ca ($r=-0.856$, $p=0.015$) in the blood of the CKDu affected subjects as indicated in **Table 3.3.7**, depicting the inverse relationship between this heavy metal (i.e. Pb), metalloid (i.e. silica) and the essential element (i.e. Ca).

These findings of the remarkable reduction in the blood levels of calcium in response to chronic and increased lead and silica exposure were in agreement with the values noted in a

similar prolonged exposure of the independent CKDu cases in Sri Lanka and Andhra Pradesh to elevated levels of the lead and silica nephrotoxin through drinking water consumption (Gunatilake et al., 2015; Jayatilake et al., 2013; Jayasumana et al., 2015a; Khandare et al., 2015; Kupferman et al., 2016; Levine et al., 2016; Nanayakkara et al; 2014; Radovanovic et al., 1991; Weaver and Jaar, 2015; Wijkström et al., 2018).

In totality, the reduced levels of these essential elements (i.e. Zn, Cu, Fe, Mn, Se) in the blood of CKDu affected subjects in this study **(Figure 3.3.4 & Table 3.3.6**) further conferred an increased vulnerability of the kidneys in these subjects to the oxidative damage primarily induced by the heavy metals (i.e. Pb) as part of their major toxicological mechanism.This increased predisposition to oxidative damage is accounted to these groups of essential elements viz. Mn, Zn, Cu, Se and Fe majorly constituting chemical structure of anti-oxidant defense enzymes i.e.Superoxide dismutase and glutathione peroxidase as metal cofactors.

Alternatively this heavy metal (viz.lead) also possesses an intrinsic tendency to bind to the thiol and sulfhydryl groups of the glutathione anti-oxidant enzyme thereby further disrupting the structural and functional integrity of this enzyme and the overall anti-oxidant balance which additionally renders the kidney (specifically the proximal tubular cells) more susceptible to oxidative injuries (Carson, 2018; Díaz-Gómez et al., 2017; Goyer, 2016; Jan et al., 2015; Khandare et al., 2015; Mozrzymas , 2018; Nordberg et al., 2014; Nordberg and Nordberg, 2016; Prashanth et al., 2015; Wu et al., 2016).

However, whether oxidative stress induction is a part of the toxicological mechanism of silica induced nephrotoxicity as well reported in the heavy metals needs to be investigated. Hence was pursued in this PhD research wherein through in-vitro nephrotoxicity analysis of silica on human proximal tubular cells (HK cells), we attempted to elucidate the cellular and molecular toxicological mechanisms underlying silica induced renal toxicity. This has been described in detail in Chapter 4 of this thesis.

Thus the instigation of these reduced levels of the essential elements (viz. Zn, Cu, Mn, Se and Fe) in CKDu affected subjects of the Canacona taluka **(Figure 3.3.4; Tables 3.3.6 and 3.3.7)** due to the heavy metal (viz. Pb) exposure could have majorly disrupted the anti-oxidant defenses of the kidney (specifically the proximal tubular compartment). This compromised anti-oxidant defenses stimulated the induction of heavy-metal triggered oxidative stress mediated pathway of apoptosis and inflammation, that cumulatively resulted in the disruption of renal tubular function via development of tubular atrophy and fibrosis, which ultimately advanced into chronic renal failure (viz. CKDu) (Abiola, 2017; Gamboa et al., 2016; Gifford et al., 2017; Matovic et al., 2015; Weaver and Jaar, 2015).

Due to the similar negative associations obtained between silica and the essential elements (viz. Zn, Cu, Mn, Se, and Fe) just like the interactions between lead and these aforementioned essential metals in the current study, we assume silica might also be inducing nephrotoxicity via the oxidative stress pathway. But this needed further investigations and confirmation, and hence was pursued through in-vitro nephrotoxicity analyses of silica on human renal proximal tubular cells (described in Chapter 4 of this thesis).

3.3.3.6 Dose-response relationship obtained between levels of nephrotoxin exposure and CKDu development (i.e.renal tubular dysfunction) in Canacona via correlation analysis.

In order to confirm the etiological contribution of these 2 potential causal nephrotoxins viz. silica and Pb in CKDu causation (i.e.tubular dysfunction) in the taluka it is mandatory to assess the dose response relationship between the estimated exposure levels of these 2 nephrotoxins and the induced renal damage effect (i.e. tubular injury).The exposure levels of nephrotoxins can be best analysed by measuring levels of biomarkers of nephrotoxin exposure in the blood that is estimating the levels of parent nephrotoxin itself in the blood,as blood serves as a good indicator of the internal dose of nephrotoxin exposure (Jayasumana et al.,2016; Moody et al.,2018; Rango et al.,2015; Sellamuthu et al., 2011a and b).Hence nephrotoxin levels in blood were analysed in this study wherein significantly ($p<0.05$) higher levels of silica and Pb were noted in CKDu affected subjects blood that surpassed WHO permissible levels as compared to non-affected healthy control group of this taluka, which deemed these nephrotoxins as causals for CKDu development in the taluka(please refer section 3.3.3.1 of this chapter).These nephrotoxins role in CKDu causation can only be confirmed on analysis of the association of their exposure levels with induction of tubular dysfunction in these subjects (Lunyera et al.,2016).The level of this inflicted tubular injury can be accurately diagnosed by measurement of specific indicators or biomarkers of the same in the urine (Jayatilake et al.,2013). Tubular damage specific markers include uBCR (urinary b2M to creatinine ratio) and uNCR (urinary NAG to creatinine ratio)which depicts levels of low-molecular weight proteins (b2M) and tubular specific enzyme (NAG) respectively,that significantly escalate in urine on tubular injury due to nephrotoxin induced disruption in intrinsic tubular reabsorption of these proteins into plasma (Laws et al.,2016; Ramírez-Rubio et al.,2015; Wanigasuriya et al.,2017). Although involvement of nephrotoxins in triggering tubular dysfunction is well-reported,its role in induction (if any) of glomerular pathology needs to be analysed in order to confirm the lack of nephrotoxins contribution in glomerular injury induction with its sole role restricted to tubular injury manifestation.Glomerular injury

is best analysed by measurement of specific markers that include uPCR (urinary protein/ creatinine ratio), uACR (urinary albumin/creatinine ratio), uAPR (urinary albumin/protein ratio and uAlb/b2M ratio (urinary albumin/b2M ratio) which indicates proteins levels majorly albumin,that rise in the urine owing to diabetes/hypertension induced glomerular damage, which under good-health is averted due to these protein's high molecular weight that prevents filtration into urine (Fisher et al.,2013; Solini et al.,2014; Levey et al,2015;Warady et al.,2015). Thus measurement of tubular and glomerular injury markers in urine was also conducted in this study population previously to assess the type and extent of renal damage inflicted (detailed in Chapter 2 i.e. Table 2.8) (Jha et al., 2014; Lunyera et al., 2016;Saucedo et al, 2018) and levels of these biomarkers were used in this correlation analysis.Therefore,a correlation analysis between levels of biomarkers of nephrotoxin exposure (i.e.internal nephrotoxin dose or blood nephrotoxin levels) and biomarkers of renal dysfunction (tubular & glomerular) was conducted in order to evaluate and establish the dose-response relationship between the levels of nephrotoxin exposure and prevalence of renal tubular dysfunction;whose results are presented in **Table 3.3.8.** This analysis will validate contribution of these potential causal nephrotoxins viz.silica and Pb at the prevalent exposure levels in CKDu induction (i.e.tubular pathology) in Canacona and negate their involvement in glomerular injury induction (McClean et al.,2012; Rajapakse et al.,2016).

As indicated in **Table 3.3.8**, significantly positive($p<0.05$) and greater(r value of >0.5) dose-effect relations were noted between Pb exposure levels in blood and renal tubular injury indicators viz.uBCR ($r=0.937, p=0.037$) and uNCR ($r=0.901, p=0.018$) among CKDu patients, indicating a significant contribution of Pb nephrotoxin in etiological development of the CKDu in Canacona at the prevalent high exposure levels.These results were in agreement with values obtained in dose response relationship analysis of Pb with CKDu development in Sri Lanka and Central America (Brooks, 2009; Gunatilake et al., 2015; Jayasumana et al., 2015a & b; Jayasumana et al., 2016; Laws, 2015; Laws et al., 2015; Levine et al., 2016; McClean et al., 2012; Nanayakkara et al., 2012 a & b; Nanayakkara et al; 2014).This trend of strong correlations of Pb with tubular dysfunction indicators resonated in association of blood silica levels with the same dysfunction indicators,wherein highly significant ($p<0.05$) and strong correlations were observed between increased levels of silica exposure in blood and elevated intensities of tubular damage biomarkers viz. uBCR ($r=0.974, p=0.015$) and uNCR ($r=0.923, p=0.036$) in these CKDu patients.

Table 3.3.8: Correlation assessments between mean concentrations of various biomarkers of nephrotoxin exposure (i.e. the blood nephrotoxin levels) and the average urinary based biomarkers of renal dysfunction of the Canacona taluka's study population for analysis of the contribution (if any) of the blood level of nephrotoxin exposure in the induction of tubular or glomerular pathology.

Nephrotoxin in blood	Study group (total number of subjects)	Correlation coefficients [r] *(p values)*					
		Urinary nephropathy specific selectivity indices					
		uPCR	uACR	uBCR	uNCR	uAPR	uAlb/b2M ratio
Silica	1	-0.185	-0.129	**0.974**	**0.923**	-0.107	-0.134
	(N=114)	*0.123*	*0.079*	*0.015*	*0.036*	*0.09*	*0.189*
	2	-0.175	-0.109	0.009	0.017	-0.112	-0.145
	(N=28)	*0.101*	*0.212*	*0.087*	*0.147*	*0.112*	*0.099*
	3	-0.018	-0.023	0.012	0.022	-0.041	-0.025
	(N=124)	*0.145*	*0.136*	*0.231*	*0.098*	*0.071*	*0.163*
Pb	1	-0.123	-0.108	**0.937**	**0.901**	-0.089	-0.114
	(N=114)	*0.098*	*0.078*	*0.037*	*0.018*	*0.085*	*0.103*
	2	-0.164	-0.124	0.005	0.009	-0.133	-0.157
	(N=28)	*0.323*	*0.058*	*0.183*	*0.069*	*0.178*	*0.102*
	3	-0.02	-0.015	0.005	0.008	-0.027	-0.016
	(N=124)	*0.147*	*0.156*	*0.109*	*0.41*	*0.181*	*0.122*
Cd	1	NC	NC	NC	NC	NC	NC
	(N=114)	NC	NC	NC	NC	NC	NC
	2	NC	NC	NC	NC	NC	NC
	(N=28)	NC	NC	NC	NC	NC	NC
	3	NC	NC	NC	NC	NC	NC
	(N=124)	NC	NC	NC	NC	NC	NC
As	1	NC	NC	NC	NC	NC	NC
	(N=114)	NC	NC	NC	NC	NC	NC
	2	NC	NC	NC	NC	NC	NC
	(N=28)	NC	NC	NC	NC	NC	NC
	3	NC	NC	NC	NC	NC	NC
	(N=124)	NC	NC	NC	NC	NC	NC
Hg	1	-0.109	-0.124	0.021	0.015	-0.107	-0.099
	(N=114)	*0.078*	*0.069*	*0.096*	*0.109*	*0.117*	*0.089*
	2	-0.121	-0.145	0.014	0.01	-0.132	-0.085
	(N=28)	*0.103*	*0.141*	*0.076*	*0.058*	*0.141*	*0.103*
	3	-0.018	-0.029	0.008	0.004	-0.033	-0.027
	(N=124)	*0.139*	*0.103*	*0.098*	*0.147*	*0.115*	*0.063*
Ochratoxin	1	-0.094	-0.115	0.017	0.019	-0.123	-0.102
	(N=114)	*0.289*	*0.337*	*0.245*	*0.125*	*0.127*	*0.144*
	2	-0.112	-0.145	0.025	0.036	-0.154	-0.143
	(N=28)	*0.156*	*0.078*	*0.356*	*0.279*	*0.114*	*0.098*
	3	-0.009	-0.014	0.039	0.045	-0.021	-0.011
	(N=124)	*0.314*	*0.156*	*0.305*	*0.075*	*0.099*	*0.215*
Aristolochic acid	1	-0.078	-0.109	0.011	0.008	-0.135	-0.117
	(N=114)	*0.155*	*0.164*	*0.311*	*0.107*	*0.221*	*0.185*
	2	-0.087	-0.131	0.015	0.02	-0.157	-0.136
	(N=28)	*0.112*	*0.103*	*0.165*	*0.108*	*0.096*	*0.089*
	3	-0.016	-0.009	0.035	0.027	-0.007	-0.003
	(N=124)	*0.078*	*0.101*	*0.088*	*0.121*	*0.174*	*0.127*

Abbreviations: CKD-chronic kidney disease, CKDu- chronic kidney disease of unknown etiology; uPCR-urinary protein to creatinine ratio; uACR- urinary to albumin ratio; uBCR- β2-microglobulin to creatinine ratio; uNCR-N-acetyl-β-D-glucosaminidase to creatinine ratio; uAPR-albumin to protein ratio; uAlb/b2M ratio-urinary albumin to β2-microglobulin ratio. N' in each study region depicts the number of subjects in that study group from whom the blood samples were procured for estimation of the nephrotoxin and the nephropathy selectivity indices. Values are represented as Pearson's correlation coefficients (r) with their respective p-values below 'italicized' for identification of statistically significant associations(if any) between the any given two parameters [i.e. the nephrotoxin exposure level in the blood and the variations(increase or decrease) in the tubular or glomerular nephropathy specific biochemical markers/selectivity indices] to identify the effect of the level of nephrotoxin exposure on the manifestation of the tubular pathology in the CKDu affected group. r' value in the range from 0 to + 1 bearing respective p-values<0.05 were considered as statistically significant positive associations. 'r' value in the range from 0 to -1 bearing respective p-values<0.05 were considered as statistically significant negative associations. As indicated in the results, strong and significant($p<0.05$) associations(highlighted in bold) were noted between the level of lead and silica nephrotoxin exposure and the tubular nephropathy selectivity indices (i.e.uBCR and uNCR) in the study group 1(comprising of CKDu endemic cases) suggesting the strong contribution of these nephrotoxins at such high levels of exposure in the blood (internal doses) in the induction and aggravation of enhanced renal damaging effects specifically tubular dysfunction, that is typical of the manifestation of the CKDu pathology endemic in this group. Thus signifying the presence of a strong and significant dose effect relationship between these two high nephrotoxins (viz. silica and lead) internal doses and the manifestation of increased renal tubular functional and structural damage at such levels of exposure.Therefore confirming the significant and remarkable contribution of these nephrotoxins viz. lead and silica in the etiological development of the CKDu disease in the Canacona taluka, with major and high levels of exposure of these nephrotoxins being routed from prolonged consumption of untreated nephrotoxin contaminated groundwater. On the other hand, insignificantly negligible and weak associations ($p>0.05$) were noted between internal levels of exposure of the remaining nephrotoxins(in blood) viz. Hg,OTA and AA and biomarkers of tubular injury viz.. uBCR and uNCR in all 3 study groups i.e. CKDu affected group(study group1), diabetic and hypertensive CKD-affected(study group 2) and healthy control(study group 3),indicating no role of these nephrotoxins in development of either CKDu or general CKD in the taluka.Thus further supporting the strong involvement of silica and lead nephrotoxins and diabetes and hypertension causal factors in the manifestation of the CKDu pathology and the general CKD trend in the Canacona taluka respectively. This was further supported by existence of negative and insignificant ($p>0.05$) relationships between internal exposure of all nephrotoxins and biomarkers of glomerular damage(viz..uPCR,uACR,uAPR ,uAlb/b2M) at a homologous extent in all 3 study cohorts, indicative of lack of role of these nephrotoxins in induction of glomerular injury in all study cohorts owing to the inherent potency of the nephrotoxins to solely cause tubular damage. For units and values of each individual biomarker of nephrotoxin exposure (i.e. blood nephrotoxin levels) and the biomarkers of tubular and glomerular dysfunction kindly refer Table 3.3.1 of the current section and Table 2.8 of chapter 2 of this thesis respectively.

These findings were in agreement with results of silica toxicity animal studies wherein a high dose-response link of chronically high silica exposure levels in blood (stemming from chronic polluted drinking water intake) in induction of renal tubular damage specifically chronic tubulointerstitial nephritis was obtained as evident from urinary excretion of tubular injury markers (b2M, NAG) increasing in strong relation with rising blood silica exposure levels (Dobbie and Smith; 1982; Markovic and Arambasic; 1971; Newberne and Wilson, 1970). Our results were also consistent with findings from epidemiological and biochemical analyses of CKDu cases in the Uddanam region (Andhra Pradesh) (Tatapudi et al., 2018) and Balkan region (Europe) (Radovanovic et al., 1991). Here also a good correlation was noted between chronic intake of groundwater containing high silica levels i.e. 92.3-98.3 mg/L by CKDu patients that translated into high exposure levels in blood (i.e. 89.3-93.1 mg/L) which ultimately resulted in significant increase of urinary tubular injury markers(i.e.b2M, NAG) with corresponding decrease of the same noted in blood (due to urine excretion), which highlighted silica's strong role in tubular pathological manifestation.In unity with findings of these animal and human epidemiological based silica dose response correlation analysis,our study also highlighted causal role of higher and chronic silica nephrotoxin exposure in blood in induction of severe tubular injuries associated with CKDu pathogenesis in Canacona.

This strong contribution of Pb and silica in development of the tubular dysfunction in CKDu affected subjects was further supported by insignificantly ($p>0.05$) weak and negligible correlations noted between blood Pb and silica levels with tubular injury markers measured in diabetic/hypertensive CKD patients (group 2) and healthy controls (group 3).Thus depicting no role of Pb or silica in causation of CKD noted in group2,thus supporting CKD causality to be linked to typical risk factorsviz.diabetes,hypertension (Smith et al.,2011). Furthermore, insignificantly ($p>0.05$) negligible correlations were noted between blood Hg, OTA, AA exposure levels with tubular damage markers at comparable extents in all 3 groups indicating no role of these toxins in CKDu (of group 1) or CKD (of group 2) causation (Gunatilake et al., 2015; Jayasumana et al., 2015a; Nanayakkara et al., 2012; McClean et al., 2012; Nanayakkara et al; 2014; Levine et al., 2016). Contrarily,insignificantly inverse ($p>0.05$) relations were noted between blood exposure levels of all nephrotoxins and glomerular injury markers (viz. uPCR, uACR, uAPR, uAlb/b2M) at similar extents in all 3 study groups.These findings were consistent with the study of glomerular and tubular proteinuria cases in relation to heavy metals exposure wherein no significant link between heavy metal exposure and increase in glomerular proteinuria was noted (Smith et al.,2011). Thus signifying no causal role of these toxins in glomerular injury induction which is consistent with inherent toxicity

mechanism of these nephrotoxins known to target and attack renal proximal tubular cells owing to these cells functional role in toxin elimination. Overall these correlation findings established a strong dose response relationship between the level of silica and Pb exposure in blood and enhanced induction of renal tubular damage associated with CKDu pathology in Canacona.Thus confirming the significant role of these nephrotoxins viz. Pb and silica in the etiological development of CKDu in the Canacona, with high levels of these nephrotoxins exposure being routed from chronic intake of untreated toxin contaminated groundwater.

3.3.4 Conclusion

As per our knowledge this is the 1st report to decipher causative nephrotoxins responsible for the manifestation of endemically high incidence of this strange form of chronic kidney disease viz. CKDu disease observed in Canacona which has not been elucidated till date. Literature states that in order to accurately identify and confirm the role of the probable nephrotoxins (as established from the environmental monitoring of the exposure sources-water and food) in the development of a particular renal pathology (i.e. tubular dysfunction in this case), it becomes mandatory to assess the internal dose of nephrotoxin exposure in the blood (i.e. nephrotoxin concentrations in the blood) and relate these levels (via correlation assessments) to the intensities of the nephrotoxin induced renal damage effects viz. renal tubular injuries by measurements of urinary tubular dysfunction biomarkers/indicators.

Hence,the internal levels of nephrotoxin exposure (in the blood) specifically silica and Pb exposure (i.e.biomarkers of nephrotoxin exposure) and degree of induced renal tubular injury (i.e. biomarkers of effect) were measured and the dose-response relationship between the two was successfully demonstrated in the current study. From the results of the current study, we have successfully established that prolonged exposure to remarkably elevated levels of silica and lead routed through its major exposure source viz. long-term consumption of untreated nephrotoxin enriched groundwater (established in the first section of this chapter) resulted in significantly higher internal doses of silica and lead exposure (visible from the increased levels of blood silica and lead measured) in the CKDu affected subjects that surpassed the WHO permissible threshold which consequently induced a series of renal tubular damaging effects (evident from significantly increased levels of tubular dysfunction biomarkers) that ultimately disrupted the tubular structural and functional integrity which cumulatively exaggerated into the manifestation of chronic tubulointerstitial nephritis, the major hallmark of CKDu. This nephrotoxin (silica and lead) induced tubular damage was further aggravated

due to the increased vulnerability of the kidneys of the CKDu affected subjects to the nephrotoxin induced oxidative effects which was conferred upon by the decreased essential element profile (viz. Zn, Cu, Mn, Fe and Se) witnessed in these subjects. These essential elements under good health conditions ideally provide renal anti-oxidant protection as it majorly constitutes the structural framework of these major anti-oxidant enzymes viz. superoxide dismutase and glutathione peroxidase. This significantly and abnormally lowered essential element profile in the blood of these CKDu affected subjects stemmed from the retardation of absorption of these essential elements by the competing toxic heavy metals and metalloid (viz. lead and silica) for the metal binding sites at the major transporters (in the intestinal tract) involved in uptake,absorption and distribution in the blood therefore cumulatively conferred the increased susceptibility of these CKDu subjects to oxidative damage of the kidney (specifically the proximal tubular portion) induced by the heavy metal and metalloid viz. Pb and silica respectively as a part of their toxicity mechanism. Furthermore, through this study we confirmingly ruled out the potential contribution of other known nephrotoxins viz. cadmium, mercury, arsenic, ochratoxin and aristolochic acid in causation of CKDu in Canacona. This conclusion stemmed from the presence of negligible levels or WHO established safe and non-toxic levels as applicable of these aforementioned nephrotoxin exposure in the blood and in its potential sources of exposure viz. groundwater and food, which moreover was comparable between the CKDu affected and non-affected healthy subjects, thereby safely negating their involvement in CKDu causation in this taluka.

Chapter 4

Elucidation of the cellular and molecular renal toxicity mechanisms of an 'emerging environmental nephrotoxin - Silica' by 'in-vitro' cytotoxicity studies

4.1 Introduction

In recent times, silica has gained attention as a nephrotoxin apart from its pulmonary toxin status owing to its induced renal pathological manifestations on long-term exposure. These pathological alterations are typical of chronic tubulo interstitial nephritis, the major histo-pathological manifestation of CKDu development (Ganguli, 2016; Millerick-May et al., 2015). This scientific evidence was substantially backed by our previous investigation (described in sections 1 and 3 of Chapter 3) of the CKDu cases in the Canacona taluka, which stated silica exposure through continuous contaminated groundwater consumption to be potentially responsible for the development of CKDu in this region. Thus highlighting the need for understanding the toxicity mechanistic aspects of this emerging nephrotoxin.

As described earlier silica abundantly constitutes the earth's crust but lacks bioavailability under ideal environmental conditions categorizing it as a trace geogenic-compound (Cornelis and Delvaux, 2016). However the bioavailability when enhanced by anthropogenic invasion of major exposure matrices (i.e. air and water) as explained in section 1 of Chapter3 can inflict various pathological conditions like pulmonary-disorders (i.e. silicosis, bronchitis) and environmentally induced Chronic Kidney Disease of tubular pathological origin (CKDu). Thus,endowing silica with emerging contaminant status (Pollard,2016;Vupputuri et al.,2012.)

Over the years, epidemiological studies have primarily focused on the induction of respiratory diseases on silica exposure (Kawasaki, 2015; Sen et al., 2016) neglecting the plausibility of developing extra-pulmonary toxic-effects. However, limited studies have recently shifted attention to risk-assessment of CKDu development (tubular nephropathy) in conjunction with silica exposure (Mohner et al.,2017; Sponholtz et al.,2016). For instance, data-analysis of five cohort studies by Sponholtz (2016) stated that silica-exposed subjects displayed a 55.1% risk of developing CKDu and 21.8% risk of fatality consequential of chronic renal failure. Moreover, clinical presentation & histopathological alterations reported from these silica exposed case-control studies were typical of chronic tubulointerstitial nephritis. Thus, these clinical observations bore similarities with heavy-metal elicited CKDu manifestation (Lentini et al.,2017), suggesting plausibility of direct nephrotoxicity induction.

Hence reported evidence had prompted us to hypothesize that silica on gaining entry into the blood-stream [via anthropogenically contaminated exposure routes (air/ groundwater)], could inflict renal-toxicity by damaging the kidney's nephrotoxin-susceptible compartment viz. proximal-tubule. Proximal tubule is located in the corticular region of the kidney and serves as a major target of xenobiotic injuries (like nephrotoxins, in this case-silica), owing to its

intrinsic reabsorption potency, unceasing exposure to circulating-substances and toxin-clearing abilities (Ahmed et al.,2013).These elicited tubular injuries could gradually intensify on prolonged exposure to elevated silica dosages that can eventually exaggerate into chronic tubulo-interstitial nephritis (CTN). CTN primarily contributes to CKDu development and advancement that ultimately manifests as irrevocable renal failure (Nakagawa et al., 2015).

The above mentioned hypothesis was also substantially deduced from the preliminary silica induced nephrotoxicity analyses conducted in animal models (*in-vivo*).These studies stated that observed renal histo-pathological alterations comprised of tubular atrophy and interstitial fibrosis which were typical of CTN (Dobbie and Smith,1982; Markovic and Arambasic, 1971). This evidence was supported by biochemical examination of CKDu cases in Canacona (described in section2.3.4 of Chapter2), wherein trend in serum markers (i.e.serum creatinine, urea, bicarbonates, phosphate) & urinary biomarkers (i.e. b2M, NAG) were representative of CTN, revealing CTN to be the renopathological presentation of silica induced nephrotoxicity.

Although, epidemiological analyses and *in-vivo* animal studies have highlighted CTN to be the primary histo-pathological observation of silica triggered renal toxicity; *in-vitro* or *in-vivo* studies explaining the cellular- and molecular-mechanisms of silica-induced nephrotoxicity were lacking. Using this knowledge, the current chapter aimed to understand the cellular and molecular toxicological mechanisms of silica induced nephrotoxicity as a function of dose and time using kidney's nephrotoxin susceptible cells viz. normal human renal proximal tubular cells (HK-cells) as an *in-vitro* model (Li et al.,2017). For this, proximal-tubular cytotoxicity outcomes following chronic exposure of HK-cells to increasing silica doses were analyzed via a panel of assessments comprising of cell-viability (Adan et al.,2016), mitochondrial-integrity (Verdugo et al.,2016), oxidative-damage (Gamboa et al.,2016), cell-cycle arrest (Halasi et al,2013), inflammatory responses (Ernandez and Mayadas,2016), genomic-damage (Glei et al.,2016) and apoptotic-pathway regulation (Verdugo et al.,2016). Furthermore, this study additionally aimed to rule out the anti-tumorigenic potential of silica by analysis of toxicity inflicted (if any) on the carcinoma-equivalent of proximal-tubular cells viz. A498 cells. Moreover, this study attempted to gather causal evidence for the role of silica in CKDu manifestation and development among the residents of Canacona taluka.

4.2 Material and Methods

4.2.1 Chemicals and Reagents

Cell-culture media viz.Dulbecco' modified eagle medium(DMEM),phosphate buffered saline

(PBS, 10x), 0.25% trypsin (1x), fetal bovine serum (FBS), bovine serum-albumin (BSA) and antimycotic-antibiotic solution were procured from Invitrogen (Carlsbad, California, USA). Sodium meta-silicate (for silica-stock preparations) was purchased from Merck-Millipore (Millipore, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide (MTT), 2'-7'-dichlorofluorescin-diacetate (DCFH–DA), RNase-A, AnnexinV-FITCconjugate,JC-1(5, 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanide iodine), propidium-iodide, probe were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Protein assay kit was obtained from Thermo-Scientific (Waltham, MA, USA). SOD & catalase colorimetric kits were obtained from Cell-Biolab (San-Diego, CA, USA). Cytokines sandwich-ELISA kits viz. IL-1β, IL-2, IL-6, TNF-α & TGF-β were acquired from Enzo Life-science (Farmingdale, NY, USA). Comet-assay kit was obtained from Trevigen Inc (Gaithersburg, MA, USA). LDH and GSH-photometric, checkpoint ELISA-kits (viz. Chk1, cyclin-B1, Cdc25, Cdk1, Chk2), apoptotic-initiators ELISA-kits (viz. p53, cytochrome-C, bax, bcl-2) & caspase-activities colorimetric-kit (caspase 3,8, 9) were from Abcam (Cambridge, MA, USA).Ultrapure deionized-water was obtained using Milli-Q system (Millipore, MA, USA).The chemicals utilized for this study were from commercial sources and of the highest purity available.

4.2.2 Cell-culture and silica exposure

HK (Human-kidney) cells are widely used for *in-vitro* toxicological studies due to possession of functional characteristics of proximal-tubular cells (Li et al.,2017). Its carcinoma-equivalent (A498) was utilized as a comparator model for cell-type dependent nephrotoxicity evaluation. All the cell-types were procured from National Centre for Cell-Sciences (NCCS, Pune, India) and used between 10-20 passages. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution at 37°C and 5% CO_2 atmosphere. On attaining 85% confluence, cells were harvested utilizing trypsin (0.25%) and passaged into appropriate tissue-culture plates based on the experiment-type. Cells were allowed to undergo surface-attachment for 24 h prior to silica treatment. Silica stock solution (10000 mg/L) was prepared by dissolution of sodium meta-silicate (0.0473 g) in DMEM (1ml) followed by filter-sterilization. Based on the experiment-type, stock-solution was appropriately diluted to desired concentrations (20-120 mg/L) and applied to cells for varied exposure-period (ranging from 1-15 days). Cells untreated with silica were used as controls for each assay.

4.2.3 Assessment of Cytotoxicity

4.2.3.1 Cell-viability analysis

Our previous study of CKDu etiology in Canacona taluka of south Goa(described in sections 1 and 3 of Chapter 3 of this thesis) had established silica exposure(through drinking silica contaminated groundwater from bore-wells) at a toxic concentration range of 100-120 mg/L to be responsible for the high-incidence of CKDu in that region (Mascarenhas et al.,2017). The nephrotoxicity of this concentration range was initially determined from the highly significant amount of human renal proximal-tubular cell-death that was observed *in-vitro* on prolonged exposure to this range. The toxicity of this silica concentration range was further confirmed from the observance of similar mean silica levels (115.5 mg/L, refer Table 3.1.5 of section1, chapter3) in the potable groundwater of CKDu-affected regions (Mascarenhas et al., 2017). Thus, to validate the toxicity of silica levels present in the groundwater of the CKDu-hit region, the blood of affected individuals who regularly consumed the contaminated groundwater were also analyzed for silica (specifically its major constituting element-silicon) (Mascarenhas et al.,2017) by Inductively Coupled Plasma Atomic-Emission spectroscopy (ICP-AES) method (Bercowy et al., 2016). The details of which are mentioned in the section 3 of Chapter 3 (specifically paragraph number 3.3.2.4.4.2). From our previous-results, the estimated silica (silicon) levels in blood of CKDu-affected individuals (please refer Table 3.3.1 of section3, Chapter 3) were found to be as high (100.2 mg/L) as were the levels in groundwater of this CKDu-region. Thus, our former findings had justified the role of this nephrotoxic silica dose (100 mg/L) in CKDu development. This observation was additionally substantiated by the complete absence of CKDu occurrence in the region containing sub-toxic concentrations (i.e. below 100 mg/L) of silica in potable-groundwater (i.e.13.5 mg/L, Table 3.1.5 of section 1,Chapter 3) and in the blood (30.7 mg/L, Table 3.3.1 of section 3, Chapter 3) of the residing healthy (non-affected) individuals. Thus, highlighting that chronic exposure to nephrotoxic silica range of 100-120 mg/L is clinically linked to induction of renal damage & related CKDu development (Mascarenhas et al.,2017).The nephrotoxic potential exhibited by 100 mg/L silica in Canacona was consistent with studies by Markovic (1968), Markovic and Arambasic (1971); Radovanovic et al. (1991) on CKDu cases in Yugoslavia (Balkan, Europe). In this study as well, a similar range of silica levels (100 mg/L) in drinking-water and blood of diseased individuals were responsible for this region's high CKDu incidence.

To validate our previous-findings (Mascarenhas et al., 2017) in the current-study, a preliminary cell-viability analysis of HK and A498-cells following exposure to sub-toxic (20-80 mg/L) and toxic (100-120 mg/L) concentrations was evaluated using MTT-assay (Adan et

al., 2016). The silica concentration range of 0-120 mg/L was determined according to average human-intake data known to cause nephrotoxicity (Markovic; 1968; Markovic and Arambasic. 1971; Radovanovic et al., 1991) and by primarily considering the previously proposed nephrotoxic dose of 100 mg/L silica as the reference dosage (Mascarenhas et.al, 2017). Thus doses higher (maximally toxic- 120 mg/L) and lower (sub-toxic- 20-80 mg/L) than the nephrotoxic reference dosage (100 mg/L) were chosen as test-concentrations for the preliminary verification of silica inflicted dose and time-dependent proximal-tubular cytotoxicity (manifested as HK-cell death) by the MTT-assay.

For this, $1x10^4$ cells/well were plated in 96-well plates and grown overnight in 5% CO_2 atmosphere at 37°C. Ensuing 24 h cell-attachment, the cells were exposed to silica doses of 20-120 mg/L for eight passages or sub-cultures (equivalent to 1, 3, 5, 7, 9, 11, 13 and 15 days respectively). After each exposure-period, the culture-medium was substituted with 1 mg/ml MTT solution and incubated for 2.5h in 5% CO_2 atmosphere. Consequently, the resulting formazan-products (MTT-metabolite) were solubilized in 150 µL DMSO and absorbance quantified at 570 nm using microplate-reader (MultiSkanFC, Thermo-Fisher Scientific, MA, USA). Absorbance of control cells denoted 100% cell-viability. Cell-survival was estimated from the relative-absorbance and represented as a percentage of untreated cells. In this preliminary cytotoxicity analysis **(Figure 4.1 A-B)**, no significant changes in the viability of renal-proximal tubular HK-cells were witnessed on dosing with sub-toxic silica concentrations (i.e. 20-80 mg/L) for an extended exposure period (of 15 days).However, a highly significant ($p<0.05$) decrease in cell-viability was noted on chronic exposure of HK-cells to toxic silica concentration range of 100-120 mg/L, with absolutely no cells surviving beyond 7 days exposure-period. Thus for further toxicological-assessments, exposure-period and dosages were restricted to 1-7 days and three doses [i.e.80 mg/L (sub-toxic), 100 mg/L (toxic) and 120 mg/L (highly toxic)] respectively. This was attributed to observance of highly significant and insignificant toxicity in HK-cells on chronic toxic and sub-toxic dosing respectively. These assessments could not be conducted beyond 7 days of exposure to toxic silica doses of 100 & 120 mg/L owing to total absence of viable HK-cells at these conditions.

4.2.3.2 Lactate-dehydrogenase (LDH) assay

Release of intracellular lactate-dehydrogenase in the cell-medium signifies compromised cell-membrane integrity leading to irreversible cell-death (Adan et al.,2016). LDH leakage in extracellular-medium was estimated using LDH assay-kit, according to manufacturer's instructions. For this, both cell-types were cultured in a 24-well plate and treated with

different silica-doses (sub-toxic- 80 mg/L & toxic- 100 &120 mg/L) for a period of 1-7 days respectively. Following each exposure period, 100µL of the treated cell-medium was utilized for colorimetric measurements of LDH activity at λ_{max}=450nm using a micro-plate reader (MultiSkanFC, ThermoFisher, MA, USA).The absorbance measured represented the amount of NAD^+ reduced to NADH, by LDH, serving as direct indicator of LDH-activity. The activity was calculated as IU/L in the extracellular-medium and represented as % of control.

4.2.4 Intracellular reactive-oxygen species (ROS) detection

The silica induced cytotoxicity effects could be exhibited through oxidative-stress or apoptosis induction mediated by excessive ROS generation. Hence intracellular ROS production was estimated by an oxidation-dependent fluorescent-sensor viz. 2',7'-dichloro fluorescin-diacetate (DCFH-DA) which reacted with ROS to form dichlorofluorescein (DCF), a fluorescent compound. ROS production was estimated in HK and A498 cells treated with silica-doses (80–120 mg/L) for 1-7 days in a 24-well plate. After exposure, cells were washed with PBS twice, followed by loading with 1ml of 35 µM DCFH-DA and incubated for 30 min at 37°C.Cells were disrupted via alkaline-treatment and spun down at 2000g for 6 min. Fluorescence intensity of the supernatant (200 µL) was measured using microplate-fluorimeter (Fluoroskan FL, Thermo-Fisher, MA, USA) at $\lambda_{ex}/\lambda_{em}$=495/529 nm. The levels were depicted as % of fluorescence output relative to the untreated wells (Liu et al., 2017).

4.2.5 Analysis of oxidative-stress biomarkers

Oxidative-stress manifests when ROS production overpowers the cell's anti-oxidant defense mechanism resulting in manifold toxic-effects like membrane lipid-peroxidation (LPO), DNA & protein damage. Thus assessment of biomarkers viz. malondialdehyde (end product of LPO) & anti-oxidant defense system [glutathione (GSH), superoxide-dismutase (SOD), catalase enzymes] indicates extent of oxidative-damage inflicted (Gamboa et al.,2016).

4.2.5.1 Cell-extract preparation

HK and A498-cells at a final density of ~5x 10^6 in a 90 mm culture-dish were treated with silica concentrations ranging from 80-120 mg/L for 1-7 days. After dosing, cells were scraped and rinsed with ice-cold 1X-PBS (twice) followed by lysis in cell-extraction buffer [20 mM Tris-HCl (pH 7.5), 1% Triton,1 mM Na_2EDTA, 150 mM NaCl , 2.5 mM PMSF and 2.5 mM sodium-pyrophosphate]. The lysate was incubated for 30 min (on ice) and after

centrifugation (at 12,000g and 4°C for 15 min), the supernatant (cell-extract) was stored at 20°C until experimental-analysis (Costa et al., 2016). Protein-levels were estimated via Pierce protein-assay kit (Thermo-Scientific, MA, USA) in accordance with Bicinchoninic-acid (BCA) protein colorimetric-method (Smith et al., 1985) utilizing BSA-standards.

4.2.5.2 Membrane lipid-peroxidation assay

MDA levels (end-product of lipid-peroxidation) were assessed by a colorimetric Oxiselect TBARS-assay kit (MDA-quantitation) (Cell Biolabs, CA, USA) according to manufacturer's guidelines. The product-absorbance was quantified by micro-plate photometer (MultiSkanFC ThermoFisher, MA, USA) at 532 nm and values were listed as nmolMDA/mg protein

4.2.5.3 Intracellular reduced GSH measurements

Reduced GSH levels were estimated by glutathione colorimetric assay-kit according to manufacturer's directives. The absorbances were measured by microplate-photometer (Multi SkanFC, Thermo-Fisher, MA, USA) at 412 nm &GSH-levels listed as nmolGSH/mg protein.

4.2.5.4 SOD and catalase enzyme levels estimation

SOD and catalase enzyme-activities were determined using colorimetric SOD-activity and Catalase-activity assay-kits respectively, according to the manufacturer's protocols. A unit of SOD enzyme-activity was calculated as the quantity of enzyme utilized for 50% inhibition of chromogenic production (absorbance measured at 450 nm) in 1 min and represented as Units/min/mg protein of specific-activity. Additionally catalase activity was depicted as 1 µmole H_2O_2 decomposed/min/mg protein (absorbance quantified at 570 nm) which is an upshot of its hydrogen-peroxide lysing capacity in 1min residence time.

4.2.6 Assessment of inflammation potential

Inflammation, a major immunotoxicity mechanism is pivotal to the development of environmental-toxin induced nephrotoxicity. It is majorly mediated by pro-inflammatory (viz.IL-1β, IL-2 and IL-6) and fibrosis inducing cytokines (i.e. TNF-α and TGF-β) (Ernandez and Mayadas, 2016). These cytokines were quantified in the current-study by their respective colorimetric sandwich-ELISA kits according to the manufacturer's instructions. Cell-supernatants required for the assays were obtained as described above from HK and A498 cells following silica dosing (80-120 mg/L) for 1-7 days. The product-absorbance was quantified by the microplate-photometer (MultiSkanFC, Thermo-Fisher, MA, USA) at 450

nm. The quantities of these pro-inflammatory and fibrogenic cytokines were calculated from their standard-plots and depicted as pg/ml or ng/ml accordingly.

4.2.7 DNA-damage estimation

DNA strand breakage is a major outcome of imbalanced ROS homeostasis which can be detected by Single-cell gel-electrophoresis (viz. Comet-assay).DNA damage inflicted in HK and A498 cells following silica treatment (80-120 mg/L) for 1-7 days was determined by the Comet-assay kit according to manufacturer's guidelines.20µL of exposed cell-dispersion (in PBS) was mixed with 0.5% low-melting agarose (80 µL) & dropped onto the comet-slide for solidification at 4°C (for 15 min).This was followed by immersion of the slides in ice-cold lysis-buffer (containing 100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris pH-10, 1% TritonX-100 and 10% DMSO) for 2.5 h. Subsequently, the slides were transferred to an alkaline-solution for 1h at room-temperature. The slides were ran in a horizontal gel-electrophoresis unit (20 V, 300 mA, 4°C, 30 min)containing basic electrophoresis-buffer (comprising of 1 mM Na_2EDTA, 300 mM NaOH, and 0.2% DMSO, pH-13) for induction of DNA-uncoiling and conversion of alkali-sensitive sites to single-stranded nicks. This was followed by neutralization in Tris-buffer (0.4M, pH 7.5) for 20 min and staining with propidium-iodide (0.1 mg/ml, 50 µL). 100 cells/slide were randomly visualized under the rhodamine/red-filter ($\lambda_{ex}/\lambda_{em}$= 535/617nm) of fluorescence-microscope (OlympusIX51, Tokyo, Japan). The visualized cells were evaluated by Komet-7.0 image-analysis software (Kinetic imaging Ltd., Andor technology, Belfast, U.K.) for calculating tail-DNA% (DNA-damage indicator) (Glei et al., 2016).

4.2.8 Flow-cytometric evaluation of DNA-content and cell-cycle phase arrest analysis

DNA breakage causes excessive reduction of DNA content, which demonstrates a characteristic increase in sub-G1 peak (apoptotic-population) on flow-cytometric analysis of the stressed cell's DNA. Our previous study (Mascarenhas et al.,2017) quantified the cell-cycle phase distribution of HK and A498-cells following silica exposure (80- 120 mg/L) for 1-7 days via flow-cytometric evaluation of their PI-stained DNA content. Cell-fractions in subG1, S, G1, & G2-M phases were obtained using BD Cell-Quest and Mod-Fit LT software included in FACS-Calibur flow-cytometer (Becton-Dickinson,CA,USA) at $\lambda_{ex}/\lambda_{em}$= 488/620 nm.

4.2.9 G2/M-checkpoint pathway assessment

Elevated DNA-damage typically induces responsorial activation of G2/M-checkpoint arrest. Hence critical regulators of this checkpoint-pathway like anti-mitotic player viz. Chk1 (checkpoint-kinase 1) and pro-mitotic players viz. Cdc25 (cell-division control-protein-25), Cyclin-B1 and Cdk1 (cyclin-dependent kinase 1) (Bonventre, 2014) were estimated by their respective colorimetric sandwich-ELISA kits, in accordance with the manufacturer's guidelines. Moreover, the levels of G1-checkpoint mediator viz.Chk2 (that is activated by neoplasticity inducing double-stranded DNA-damage) were also assessed by the ELISA technique to rule out silica's carcinogenic potential (Halasi et al., 2013). Cell-extracts for the assays were isolated as detailed above from HK and A498-cells following chronic exposure (1-7 days) to silica doses (80-120 mg/L). The absorbance was optically quantified at 450nm in a micro-plate photometer (MultiSkanFC, ThermoFisher, MA, USA).The protein levels of each G2/M-arrest mediators were calculated from their standard curves and denoted as pg/ml.

4.2.10 Cellular-death pathway assessment (Annexin V/FITC-PI apoptosis-assay)

Persistent oxidative and DNA-damage on exceeding the cellular-repair capacity potentiates into cellular-death. However, the pathway followed (apoptosis or necrosis) was assessed by Annexin-V/FITC-PI dual-labeling. Annexin-V-FITC greatly attracts the phosphatidylserine (membrane-phospholipid) that is externalized during early-apoptosis. Concurrently PI (vital dye) distinguishes dead cells (consequential of late-apoptosis or necrosis) from early-apoptotic cells. As reported in our previous investigation (Mascarenhas et al., 2017), HK and A498-cells dosed with silica (80-120 mg/L) for 1-7 days were analyzed by flow-cytometery following dual-staining with Annexin V/FITC and PI.

4.2.11. Evaluation of mitochondrial-pathway of apoptosis

On establishment of apoptosis as the primary mechanism of cellular-death, its pathway of action viz. mitochondrial-activated (intrinsic) or death-receptor mediated (extrinsic) was explored by assessment of various markers of the mitochondrial-apoptotic route. The pivotal step in the mitochondrial-pathway is ROS stimulated mitochondrial membrane-permeabilisation which reduces the membrane-potential to stimulate the cytosolic efflux of inter-membranous protein viz. cytochrome-c. This efflux is further facilitated by DNA-damage responsive p53-mediated activation and suppression of pro-apoptotic (Bax) and anti-

apoptotic (Bcl2) effectors respectively. The released cytochrome-c ultimately triggers the caspase-cascade for potentiating apoptotic cell-death (Verdugo et al., 2016).

4.2.11.1 Mitochondrial membrane-potential (MMP) detection

MMP was determined using JC-1 probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi dazolylcarbocyanide-iodine. This sensor being a cationic-dye undergoes reversible red to green fluorescence transformation with diminishing membrane-potential as cells convert from healthy to apoptotic state. Healthy cells accumulate the dye in mitochondria (red) whereas apoptotic cells in cytoplasm (green), thereby depicting MMP change via a green/red intensity ratio (Verdugo et al.,2016).For this, HK & A498-cells were chronically dosed (7 days) with different silica concentrations (80-120 mg/L).Following each silica-treatment, the stressed cells (10000cells) were incubated with JC-1 (10 µg/mL) for 15 min and evaluated by flow-cytometry (BD FACS-Calibur, CA, USA).The red and green fluorescence-output were quantified at $\lambda_{ex}/\lambda_{em}$=488/525nm& $\lambda_{ex}/\lambda_{em}$=488/590nm respectively & ratio was calculated.

4.2.11.2 Detection of p53, cytochrome-c, Bax and Bcl-2 by ELISA

The p53, cytochrome-c, bax and bcl-2 protein levels were estimated by their respective colorimetric sandwich-ELISA kits according to manufacturer's instructions. Cell-lysates required for the immunoassays were isolated as described above from HK and A498-cells following chronic (1-7 days) exposure to various silica doses (80-120 mg/L).The protein levels of the lysates were normalized by standard BCA method. The product-absorbance measurements were conducted by a micro-plate photometer at 450 nm (MultiSkanFC, Thermo Fisher, MA, USA).The levels of these pro-apoptotic inducers were estimated from their standard plots and represented as ng/ml.

4.2.11.3 Assessment of caspase-3, -8 and -9 activities

Caspase-8 and -9 induces death-receptor and mitochondrial mediated apoptosis respectively while caspase-3 functions as an effector-caspase for both these pathways (Verdugo et al., 2016). Hence, the activities of caspases (i.e. 3, 8 and 9) were determined to confirm the mitochondrial-pathway by their substrate-specific spectrophotometric assay kits according to the manufacturer's guidelines. Cell-lysates required for the assay were obtained from HK and A498-cells following chronic dosing (1-7 days) with different silica concentrations (80-120 mg/L). The absorbance was optically quantified at 405nm by a microplate-photometer

(MultiSkanFC, ThermoFisher, MA, USA).The levels of each caspase were computed from their standard curves and represented as ng/ml.

4.2.12 Statistical analysis

All experiment types (e.g. cell-viability, oxidative-stress, DNA damage etc) were triplicated for better data reproducibility. The sample-size (N) used for each experiment (e.g. cell-viability, oxidative-stress etc.) was 12 treated cell-samples per silica concentration at each exposure time-point (i.e. 1 day or 3 days or 5 days or 7 days).This sample size was calculated by multiplying number of cell-sample replicates analyzed per silica concentration (at each exposure time-point) in a single experiment (i.e. 4 cell-replicates/silica concentration/ experiment) with the number of repetitions of the individual experiment on different days (i.e. 3 times). Thus, sample-size (N)= 4 cell-samples replicates per silica concentration (x) 3-times which gives a total of 12 treated cell-samples per silica dose at any given time-point.

The data for each experimental type (e.g. cell-viability, oxidative-stress etc.) was represented as mean ± S.E of three independent experiments unless specified. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnett's test was applied post-hoc using the SPSS statistics (Version 20.0 for windows) software. Data-analyses results with p values <0.05 were deliberated significant in comparison with untreated groups.

4.3 Results and Discussions

Silica, a trace geogenic-compound is gaining emerging-contaminant status owing to manifold health-hazards inflicted when available to human-beings via anthropogenic disturbances (OSHA, 2016). The primary focus of the silica-imposed health-effects has majorly been pulmonary-disorders (Kawasaki, 2015). Recently, few studies (Markovic, 1968; Markovic and Arambasic,1971; Radovanovic et al.,1991; Vupputuri et al.,2012) including our previous exploration (Mascarenhas et al.,2017) described in sections 1 & 3 of Chapter3, have highlighted a causal association between silica and CKDu development, but failed to elucidate mechanisms of silica induced renal-toxicity. Hence, this study was conducted to understand cellular & molecular toxicological mechanisms of silica nephrotoxicity.

Kidney particularly proximal-tubular cells are crucially involved in renal-physiology maintenance. Additionally, these cells are focal-targets of nephrotoxins accounted to its role in re-absorption, filtration, and toxin-clearance. Thus proximal-tubular cells serve as critical tools for nephrotoxicity assessment (Li et al., 2017). Hence, the current-study employed

human renal proximal-tubular cells viz.HK-cells and its malignant-equivalent i.e.A498 *as in-vitro* prototypes for elucidating mechanisms underlying silica induced renal toxicity.

4.3.1 Dose and time dependent cell-density reduction and LDH release

To gain a primary insight into the mechanisms of silica induced renal-pathogenesis specifically towards proximal-tubular cells; preliminary cytotoxicity-markers such as inhibition of cell-proliferation and membrane-damage were assessed.

Proximal-tubular cells are intrinsically enriched in mitochondria owing to its high-energy requirement for reabsorption and toxin-clearing activities (Li et al., 2017). Thus the toxicity of a nephrotoxin can be accurately gaged by its ability to induce mitochondrial-dysfunction in such cells (Gamboa et al., 2016). Moreover, mitochondrion being the respiratory-center actively controls major cellular-functions such as cell-proliferation, protection from oxidative-stress/ inflammation/ DNA-damage and regulation of apoptotic cell-death. Hence, injuries to the mitochondria directly disrupt normal cellular-functioning which eventually results in cell-proliferation arrest & associated apoptotic cell-death (Zhan et al., 2013). Therefore, assessment of a marker of living mitochondria with intact functional integrity (viz. mitochondrial-dehydrogenase dependent NADPH-coupled oxido-reduction activity) via MTT-assay serves as a critical measure for nephrotoxin induced disturbances of cellular-functions and its associated alterations of cell-survival (Che et al., 2013). For this, the cell-viability of human proximal-tubular cells viz.HK cells and its carcinoma-complement viz. A498 cells were preliminarily analyzed by the MTT-assay following 24h exposure to sub-toxic doses (20-80 mg/L) and toxic silica concentrations (100-120 mg/L) **(Figure 4.1 A).** Since no significant decrease in cell-number was noted following 24 h exposure of both cell-types to the highest toxic dose (120mg/L), the exposure-period was prolonged to 15 days. As indicated in **Figure 4.1 A-B**, the cell-density of carcinoma control-A498 exposed to the toxic dose (120 mg/L) remained unaffected even at the highest exposure-period (15 days) owing to heightened protection of the carcinoma-cells (A498) from the silica-toxin. This could be an outcome of its smaller doublings and greater metabolic-activity that plummets the effective toxin-action period. The silica-toxin resistance was reinforced by stronger toxin-efflux responses by endogenously present multi-drug resistance pump (Luczak & Zhitkovich, 2015). Concurrently, an insignificant negligible decrease in cell-viability of HK-cells was observed on prolonged exposure (15 days) to sub-toxic doses (20-80 mg/L) **(Figure 4.1 A-B)**.This indicated that sub-toxic silica doses did not inflict severe mitochondrial-injuries sufficient to disrupt the normal cellular-functions, which permitted the retention of their survival capacity.

The lack of significant toxicity at these sub-toxic doses could be an upshot of the functional retention of the cell's intrinsic toxin-clearing capacity (possibly executed by metallothioneins proteins) that remained unaffected due to the reduced detoxification demand created by such lower silica-toxin concentrations (Shibutani et al., 2000). Metallothioneins are metal-stress proteins that are inherently enriched in proximal-tubular cells owing to its role in scavenging and detoxification of nephrotoxic metals from these cells (George et al., 2017).Thus, these tubular detoxification-mechanisms could have prevented the intracellular silica-toxin accumulation and subsequent induction of toxin-triggered mitochondrial-damage and associated cell-death (Chargui et al., 2011; Shibutani et al., 2000).

Contrarily, the viability of HK-cells significantly decreased ($p<0.05$) on prolonged exposure (7 days) to higher toxic doses (100 and 120 mg/L) **(Figure 4.1 A)**, demonstrating a dose and time-dependent cytotoxic effect. This was evident from the significant and drastic reduction in number of viable cells from 75.5% in 80 mg/L (sub-toxic) dosed cell-groups to 27.4% ($p=0.033$) and 21.1% ($p=0.011$) in 100 mg/L and 120 mg/L exposed cell-groups respectively, following 7 days exposure. The severe toxicity exhibited at these high-doses could be an outcome of the highly compromised toxin-clearing capacity of these cells at such doses. This could be attributed to the toxin-accumulation surpassing the protective toxin-clearing capacity (plausibly facilitated by intrinsic metal-stress responsive proteins viz. metallothioneins) that possibly resulted in an intracellular saturation with the silica-toxin, which inflicted severe mitochondrial injuries and tubular cell-demise (Boonprasert et al., 2016). This dose-dependent cytotoxicity was further justified from the complete absence of viable-cells on prolonged dosing (beyond 7 days) to 100 and 120 mg/L silica **(Figure 4.1 A-B),** indicating the maximum attainment of toxicity at the 7^{th} day**.** Thus, these observations collectively provided supporting evidence to our preliminary study that suggested 100 mg/L silica to be the chronic renal-toxicity inflicting inhibitory-dose (at which greater than 50 % proximal-tubular cell-density reduction can be achieved). Additionally, it demonstrated that chronic sub-toxic dosing (20-80 mg/L) did not induce significant nephrotoxicity manifested by the lack of substantial (>50%) amount of tubular cell-death. Thus for further analyses of the silica induced cellular and molecular-toxicity mechanisms, a sub-toxic dose (80 mg/L) and a highly toxic dose (120 mg/L) at a ±20 mg/L interval to the proposed nephrotoxic-dose (100 mg/L) were chosen, with exposure-period restricted to 7 days. Thus the MTT results clearly demonstrated that silica inhibited cell-proliferation and survival of proximal-tubular cells by distressing mitochondrial structural and functional integrity as a function of dose & time.

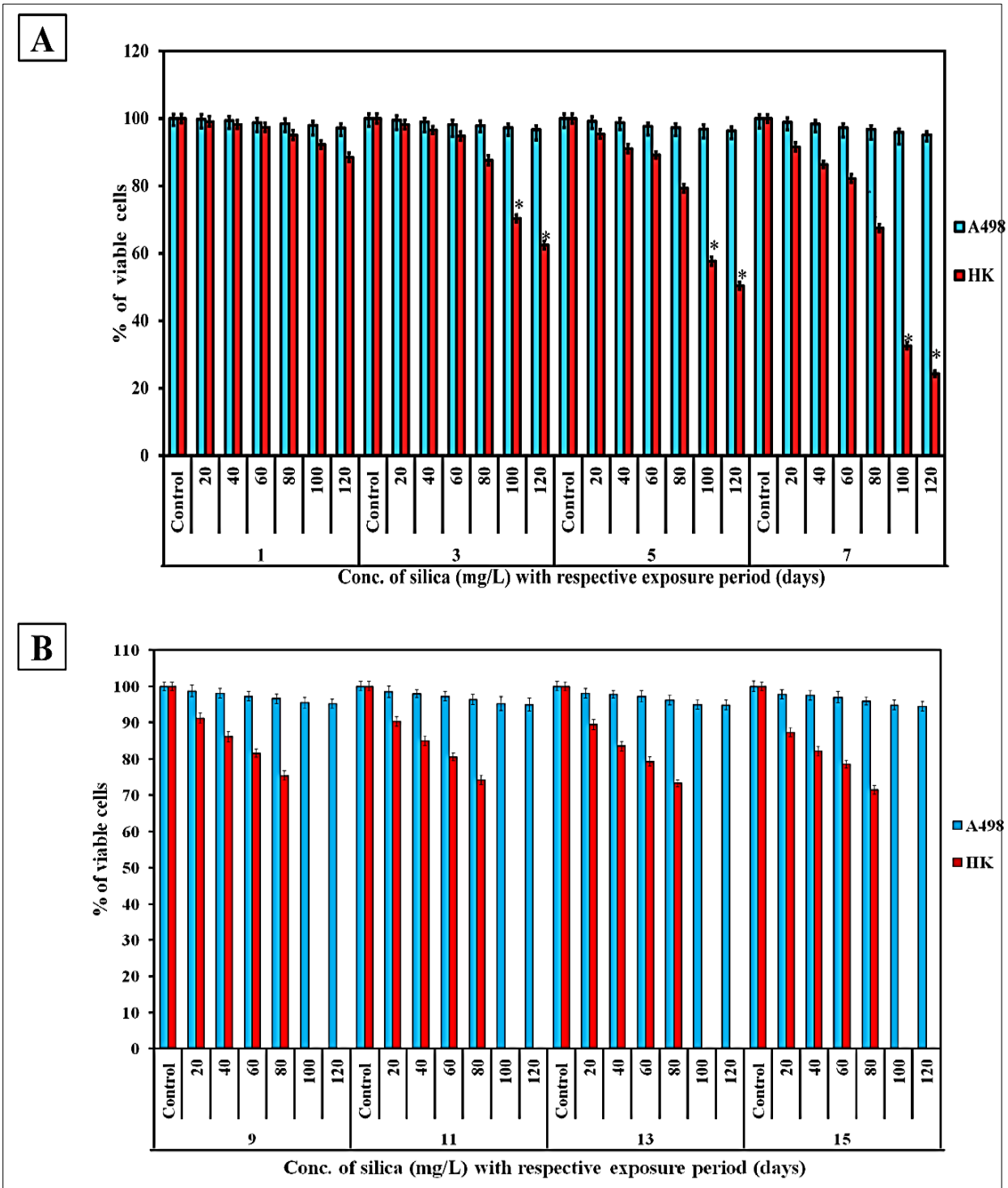


Figure 4.1. Cytotoxicity of HK and A498 cells induced by silica over a chronic exposure (1-15 days).
Percentage of surviving cells in HK and A498 cell-groups following dosing with sub-toxic (20-80 mg/L) and toxic silica doses (100-120 mg/L) for a period of 1-7 days(A) and 9-15 days(B) as assessed by MTT assay. Values are represented as a % of viable cells relative to the untreated groups against varied silica concentrations at each time point for either cell type. The results depicted that silica induced dose, time and cell-type dependent cytotoxicity. A significant ($p<0.05$) decrease in cell-viability(drastically reduced to 27.4% and 21.1%) as compared to the control groups were noted in chronically-exposed (7 days) groups of HK cells to 100 and 120 mg/L silica respectively (A). However, an insignificant ($p>0.05$) negligible decrease in the cell-viability of HK-cells was observed on chronic sub-toxic dosing (20-80 mg/L) with silica despite a prolonged exposure of 15 days (B). This was evident from the inconspicuous alterations in the cell-viability noted from 75.5 % on 7 day dosing (A) to 71.6 % on 15 day dosing (B). This lack of toxicity could be attributed to the cell's inherent detoxification potency (aided by metallothioneins) remaining unaffected by such low silica toxin-doses that prevented excessive intracellular toxin accumulation and subsequent induction of persistent mitochondrial damage associated cell death. Similarly, no significant toxicity was noted in the carcinoma counterpart (A498 cells) as well (A and B). This could be attributed to the inherent resistance of the malignant-cells to toxin (silica) owing to its shorter doublings that diminishes the effective toxin-action period. Data is described as mean ± S.E from three autonomous experiments. *$p < 0.05$ in comparison with the untreated cells was considered significant. Error bars illustrate standard error of less than 5%.

Moreover, MTT results were consistent with amplified plasma-membrane damage, reflected from significantly ($p<0.05$) increased LDH-release (by 125 & 152%) on7days exposure of HK-cells to toxic silica doses of 100 & 120 mg/L as compared to untreated cells **(Figure 4.2).**

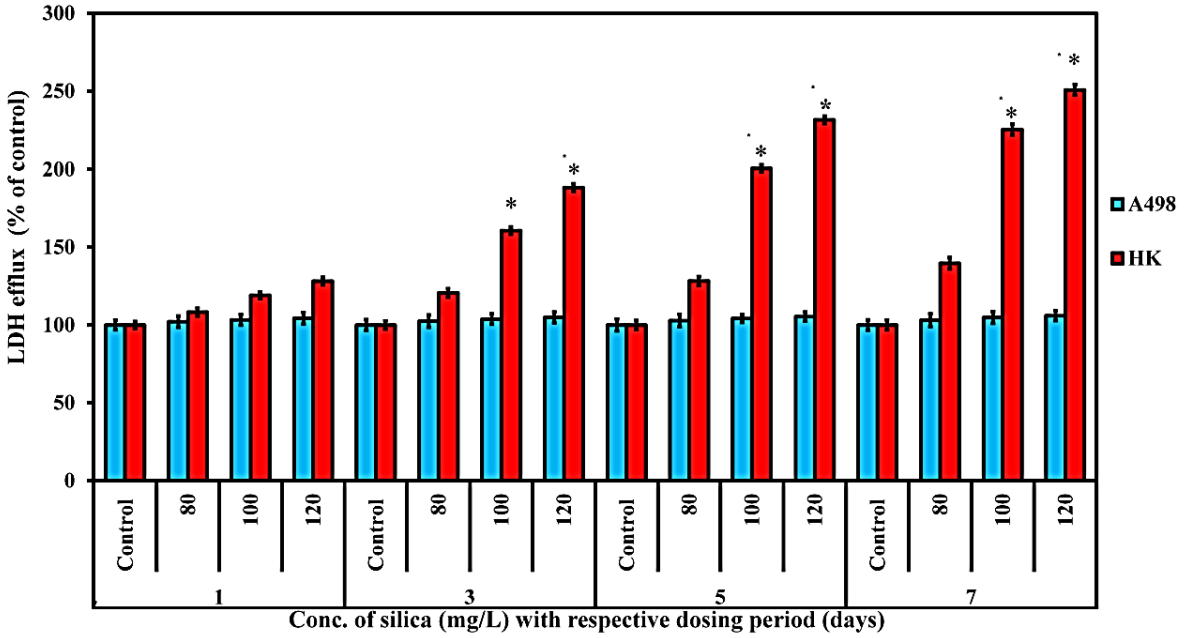


Figure 4.2. LDH leakage in HK and A498 cells induced by silica over a long-term exposure (1-7 days)
LDH release from HK and A498 cells treated with sub-toxic (80 mg/L) and toxic silica doses (100-120 mg/L) for 1-7 days. Values are depicted as % of relative untreated groups against varied silica concentrations at each time point for either cell type. The results depicted that silica induced dose, time and cell-type dependent cytotoxicity. A significant ($p<0.05$) increase in LDH efflux (by 125% &152 %) as compared to the control groups were noted in chronically-exposed (7 days) groups of HK cells to 100 and 120 mg/L silica respectively. This could be ascribed to the enhanced permeability of the cellular membrane resulting from silica induced ROS generation that inflicted severe oxidative membrane damage. Contrarily, no significant increase in LDH was noted on sub-toxic dosing (80 mg/L).This lack of LDH rise could be attributed to retention of the cell's intrinsic toxin-clearing capacity at such lower-doses, which prevented excessive intracellular toxin accumulation and subsequent ROS generation thereby averting induction of membrane damage. Similarly, no significant elevation in LDH levels were observed in the carcinoma equivalent (A498 cells) as well, owing to the inherent possession of toxin resistant mitochondria that confers preservation of ROS homeostasis and amplified anti-oxidant protection. Data is described as mean ± S.E from 3 independent experiments.*$p < 0.05$ in comparison with untreated cells was considered significant. Error bars denote standard error of < 5% .

This heightened LDH leakage could be a consequence of ROS induced cell-membrane damage, which highlighted the plausibility of ROS contribution in silica induced renal toxicity-mechanisms. Thus, these observations further justified 100 mg/L to be the nephrotoxic-dose that induced severe disruption of membrane-integrity. Contrarily, no significant effect was noted on sub-toxic silica (80 mg/L) dosing, owing to the efficient toxin-clearance at these doses which could have restricted the toxin incited membrane-damage (Shibutani et al., 2000). This trend resonated in A498-cells as well (**Figure 4.2)**,owing to the carcinoma-cell's innate resistance to ROS elicited membrane-damage, attributed to intrinsic ROS-homeostasis conservation. Thus, adequately negated the anti-tumorigenic potential of silica due to lack of induced A498 cytotoxicity (Choueiri and Motzer, 2017). Overall, these silica induced nephrotoxicity findings were in agreement with mechanisms of renal-toxicity elicited by other environmental-toxins (like heavy-metals) in human proximal-tubular cells (Verdugo et al.,2016) and in rats (*in-vivo*) (Wongmekiat et al., 2018).

4.3.2 Silica induced ROS generation

Proximal-tubular cells being inherently enriched in mitochondria (ROS hub) are more prone to nephrotoxin induced mitochondrial respiratory-dysfunction. This dysfunction disturbs ROS homeostasis which activates incessant ROS generation. Excessive ROS can unceasingly inflict sub-cellular oxidative-injury that eventually triggers apoptotic cell-death (Gorin,2016)

In the current-study, incessant ROS production was observed to be efficiently inhibited **(Figure 4.3A)** in the carcinoma-equivalent (A498-cells).This could be attributed to presence of metabolically-energetic and toxin-resistant mitochondria in these cells, which easily eradicated ROS by enhanced antioxidant activities (Song et al.,2015)(**Figure 4.3 C-E)**. Contrarily, ROS generation gradually intensified in silica treated HK-cells as a function of dose and time**.** As indicated in **Figure 4.3 A**, no significant ROS production was observed on sub-toxic (80 mg/L)dosing of HK-cells for shorter exposure-periods (i.e. 1-5 days).This could be attributed to faster toxin-elimination (possibly mediated by metallothionein toxin-efflux proteins) at this dosage that prevented elicitation of silica induced mitochondrial respiratory-dysfunction (Ruttkay-Nedecky et al., 2013; Shibutani et al., 2000).However, this ROS production marginally-increased [p-value approached the alpha-value(0.005), p=0.055] on prolonging the exposure(for 7 days) to this sub-toxic dose.This could be accredited to gradual accumulation of nominal amounts of residual silica-toxin (after clearance) with time that could have exerted very negligible mitochondrial-injuries (Aleo et al.,2005;Thijssen et al., 2007a). Overall, these time-dependent effects on sub-toxic silica dosing were in agreement with heavy-metal induced tubular cell-responses inflicted under similar conditions (Ruttkay-Nedecky et al.,2013; Thijssen et al.,2007a and b).Also, significant (p=0.011) increase in ROS generation was noted on chronic-dosing of HK-cells with ≥100 mg/L silica, proving this dose to be oxidative-injury inducing nephrotoxic-dose. This excess ROS production could be a consequence of direct or indirect attack of silica on mitochondria (ROS-hub) via disturbance of ATP production & respiratory-chain reactions (Valko et al.,2016). Moreover silica can liberate highly-reactive $OH^{\cdot}$ radicals incessantly from its surface that could have contributed to increased ROS output (Barron et al.,2016). This elevated ROS can ultimately disrupt the redox-balance creating a pro-oxidant environment, which can instigate a series of biological responses like DNA-damage, inflammation & apoptosis (Gorin, 2016). Overall, role of ROS in silica triggered renal-toxicity were consistent with oxidative-damage triggering mechanisms of heavy-metal nephrotoxicity in human proximal tubular-cells (Lee et al., 2017)] and rats (*in-vivo*) (Oyagbemi et al.,2015). Thus highlighting excessive ROS generation to be a critical mediator of silica induced nephrotoxicity.

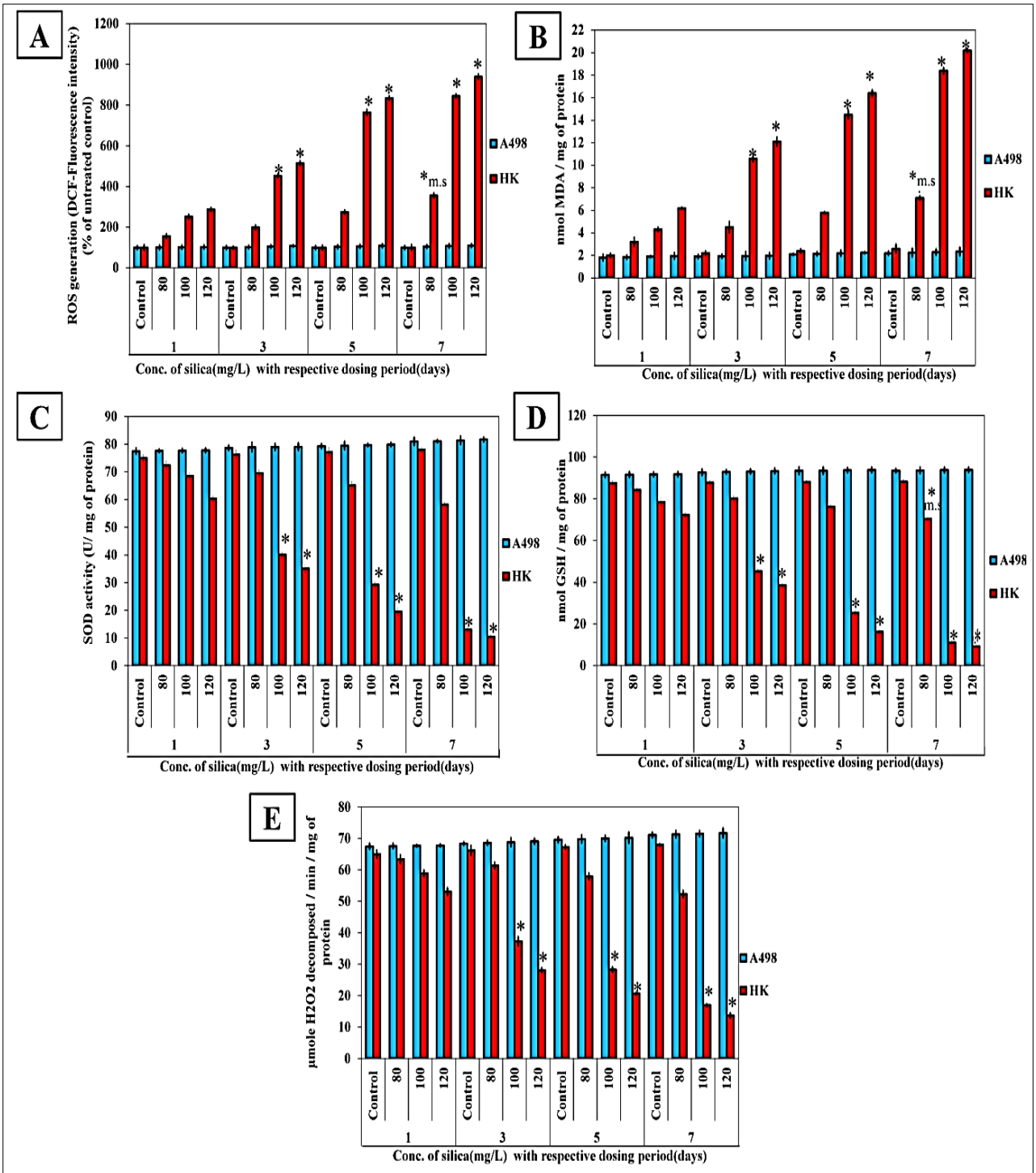


Figure 4.3. ROS induced oxidative-stress and consequent oxidative injuries in HK and A498 cells triggered by silica over a chronic exposure (1-7 days).

Dose and time response based elevation in oxidative-stress was noted in HK cells. This was evident from the continuous silica generated ROS production (A) suppressing the antioxidant defense activities viz. SOD (C), GSH (D) and catalase (E). This excessively generated ROS inflicted manifold toxic injuries, one being oxidative membrane damage (indicated by increased lipid peroxides viz. MDA formation) (B). A significant ($p<0.05$) 8- and 9-fold rise in ROS, 9- and 10-fold elevation in MDA levels along with 6- and 7- fold decrease in SOD, 8- and 9-fold diminution in GSH and 4- and 5-fold reduction in catalase in comparison to untreated controls, were detected in chronically exposed groups (for 7 days) of HK-cells dosed with 100 mg/L and 120 mg/L silica respectively. Marginally significant (p value approaching 0.05) oxidative-stress was noted on chronic sub-toxic (80 mg/L) dosing. This was attributed to the marginally significant ROS generated from the chronically accumulated residual silica-toxin. However, it did not suffice in inducing significant cell-mortality in the long-run (evident from MTT results) attributed to uninhibited cellular-repair at lower-doses that eradicate the mortality inducing intracellular-stressors (e.g. ROS). No significant oxidative-insults and slightly elevated antioxidant levels were observed in its carcinoma equivalent (A498 cells). This was accredited to its inherent heightened ROS clearance and antioxidant-defense protection due to possession of metabolically energetic and toxin resistant mitochondria. Data is denoted as mean ± S.E from three autonomous experiments.*$p < 0.05$ in comparison with the untreated cells was considered significant. *m.s =marginally significant with p values in the range of 0.051-0.059 i.e. approaching the alpha value of 0.05. Error bars illustrate less than 5% deviation in the values.

4.3.3 Silica triggered oxidative injuries

Oxidative-stress manifests when ROS generation surpasses the anti-oxidant defense capacity causing it to inflict various sub-cellular injuries. It is inevitable in cells enriched in mitochondria owing to role of mitochondria in ROS-homeostasis. Thus, this condition predisposes proximal-tubular cells to oxidative-stress which can be triggered majorly by environmental-toxins (Gorin,2016).Oxidative-stress is the major toxicity mechanism adopted by silica for cell-types of varied histological-origins (Murugadoss et al,2017; Pirela et al.,2016). This oxidative-stress mechanism was found to be replicated in the silica induced nephrotoxicity in renal proximal-tubular (HK-cells) as well. This was established from enhanced ROS generation, subsequent lipid peroxidation (LPO), and obliterated antioxidant enzyme-activities (viz. GSH, SOD & catalase activities) noted in HK-cells in a dose and time reliant fashion **(Figure 4.3)**.This incessantly generated ROS (**Figure 4.3 A**) was attempted to be destroyed and converted to molecular oxygen & water by the antioxidants (GSH, SOD, catalase) (Baradaran et al.,2015); but resulted in a wasted effort concluded from exhausted anti-oxidant levels obtained **(Figure 4.3 C-E).** This excessive ROS ultimately inflicted profound plasma-membrane damage especially in metabolically-active mitochondria through lipid-peroxidation forming end-products like malondialdehyde (Gorin, 2016) (**Figure 4.3B).** This membrane-damage was further justified from increased discharge of cytosolic-LDH enzyme (Stepniewska et al.,2015) as indicated in **Figure 4.2**.These oxidative-injuries were found to be highly pronounced ($p<0.05$) from chronic treatment with 100 mg/L silica and above, substantiating this dosage to be the oxidative-destruction inducing nephrotoxic dose. Conversely, no significant oxidative-injuries were noted on sub-toxic (80 mg/L) dosing of HK-cells to silica for reduced exposure-periods (1-5 days). This could be accounted to greater toxin-elimination (possibly assisted by metallothioneins) at such doses that could have prevented the ROS generation and associated oxidative injury (George et al.,2017). However a marginal rise in lipid-peroxidation ($p=0.051$) & decline in antioxidant activities [p (average of anti-oxidant values)= 0.052)] were obtained on prolonged sub-toxic exposure (for 7 days). This could be attributed to role of negligible amounts of chronically accumulated residual toxin in inducing nominal oxidative-stress & anti-oxidant destruction (Thijssen et al, 2007a). Nevertheless, this minimal amount of oxidative-stress generated on chronic sub-toxic silica (80 mg/L) dosing could not induce substantial amounts of sub-cellular (lipids, proteins & DNA) oxidative-injuries, sufficient to trigger cell-death (final nephrotoxicity outcome) in the long-run. This was evident from minimal decline in cell-viability (MTT-results- **Figure4.1**) noted at this dose in spite of lengthening the exposure period beyond 7 days (i.e. for 15 days).

The lack of substantial cell-mortality at this sub-toxic dose could be attributed to the cellular-repair capacity (majorly assisted by repair-enzymes and ROS-scavengers like anti-oxidants & metallothioneins) remaining intact at such low-dosages which was capable of eliminating such mild-levels of cell-death inducing intracellular-stressors (viz. ROS) (Ruttkay-Nedecky et al, 2013; Shibutani et al., 2000). These time-based sub-toxic effects were consistent with heavy-metal induced tubular cell-responses under similar conditions (Chargui et al, 2011)
Therefore, our findings of oxidative-stress significantly induced on toxic (≥100 mg/L) silica dosing were in agreement with oxidative-damage mechanisms of heavy-metal induced renal-toxicity in human proximal-tubular cells (Lentini et al,2017) & rats (*in-vivo*) (Oyagbemi et al, 2015).Thus indicating oxidative-stress to be major instigator of silica induced nephrotoxicity.

4.3.4 Silica exhibited inflammatory potential

Heightened ROS generation in proximal-tubular cells on nephrotoxin-stress has been reported to turn on NLRP3-inflammasome activity in monocytes and macrophages. This activated NLRP3-inflammasome triggers the incessant activation of the inflammatory cascade via pro-inflammatory cum fibrogenic cytokine induction. This persistent inflammation eventually culminates in the development of proximal-tubular interstitial-fibrosis (the hallmark of tubulo-interstitial nephritis/CKDu) (Hutton et al.,2016).To examine this effect in our experimental setting, the appearance of pro-inflammatory & fibrogenic cytokines viz. IL-1β, TNF-α, TGF-β, IL-2 and IL-6 were quantitated in HK and A498 cells following long-term (7 days) silica exposure (80-120 mg/L).The pro-inflammatory cytokine profiles in HK-cells depicted a time & concentration-dependent elevation following silica exposure **(Figure 4.4).** As depicted, significantly ($p>0.05$) greater cytokine levels were obtained on long-term dosing with ≥100mg/L, establishing this dose to be severely immuno-toxic and simultaneously proposing silica's inflammatory potency. Interestingly, this elevated cytokine generation in HK-cells coincided with enhanced ROS-production by silica **(Figure 4.3 A)**, signifying ROS to be the primary-mediator of chronic-inflammation progression (Xu et al.,2015). ROS stimulated the trans-locational activation of cytosolic redox-sensitive transcription-factor viz. NF-Kβ to the nucleus, which subsequently activated the synthesis of pro-inflammatory cum fibrogenic cytokines viz. TNF-α, TGF-β and IL-1β.These cytokines attracted macrophages for injury-clearance; wherein the infiltrated macrophages produced additional cytokines (IL-1β, TNF-α, TGF-β, IL-2 and IL-6) **(Figure 4.4)** to sustain the infiltration and intensify the inflammatory-cascade (Tucker et al.,2015).

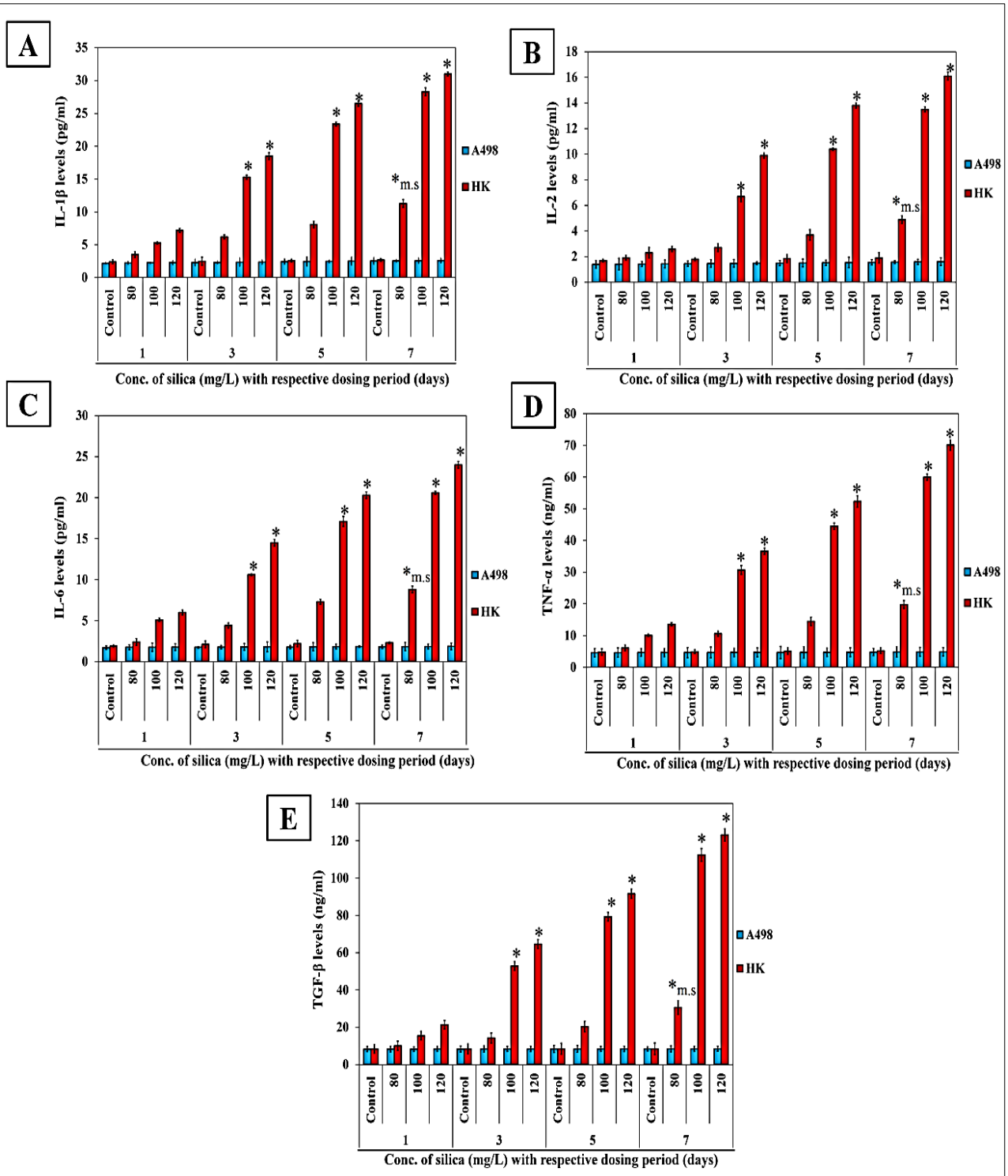


Figure 4.4. Immunotoxicity manifested by inflammatory response in HK and A498 cells triggered by silica over a prolonged exposure (1-7 days).

The incessant ROS generated by silica induced the persistent activation of the inflammatory cascade. This was reflected from the dose and time reliant enhancement ($p<0.05$) in the synthesis of pro-inflammatory cytokines viz. IL-1β (A), IL-2 (B) and IL-6 (C) and fibrogenic cytokines viz. TNF-α (D) and TGF-β (E) in HK cells. A significant fold rise ($p<0.05$) of 10- and 11- in IL-1β, 7- and 8- in IL-2, 9- and 10- in IL-6,11- and 13- in TNF-α and 13- and 14- in TGF-β relative to the control groups were noted in chronically exposed groups (for 7 days) of HK-cells dosed with 100 mg/L and 120 mg/L silica respectively. This persistently induced inflammation ultimately resulted in the development of tubular-fibrosis, the major pathological manifestation of tubulo-interstitial nephritis (CKDu). Contrarily, marginally significant ($p<0.05$) increase in inflammation was noted on prolonged sub-toxic dosing (80 mg/L).This was attributed to the marginally-significant ROS generated from the accumulated residual toxin over-time, that ultimately induced negligible inflammatory-activity. However, this mild-inflammation could not induce significant cell-death in the long-run (evident from MTT-results), owing to the elimination of the mortality inducer viz. ROS by the unaffected cellular-repair at lower-doses (80 mg/L). Lack of significant inflammation was noted in the malignant counterpart (A498 cells) attributed to its intrinsic ROS homeostasis preservation. Results are denoted as mean ± S.E from three autonomous experiments. *$p < 0.05$ in comparison with the untreated cells was considered significant. *m.s =marginally significant with p values in the range of 0.051-0.059 i.e. approaching the alpha value of 0.05. Error bars illustrate <5% deviation in values.

Furthermore, the incessantly released pro-fibrogenic mediators viz. TNF-α **(Figure 4.4 D**); TGF-β **(Figure 4.4 E)** simultaneously initiated an unwarranted repair of inflamed-tissue by activating AKT/mTOR-SMAD signal-transduction pathway. This induced AKT/mTOR signal stimulated the enhanced migration and proliferation of ubiquitous myo-fibroblasts in the tubular-interstitial tissue, which led to persistent deposition of extracellular-matrix (collagen & fibronectin) in the contiguous connective-tissue causing fibrosis. Fibrosis is histopathologically characterized by exaggerated thickening, hardening &scarring of affected tissue that annihilates normal-architecture resulting in irreversible function-loss. It is the dominant pathological-state manifested in chronic tubulo-interstitial nephritis that potentiates from imbalanced tissue-repair (ECM synthesis overriding degradation), which disrupts nephron-homeostasis and consequently induces kidney-dysfunction or CKDu (Amiri, 2016).

The established role of ROS in inflammation activation explains the unaltered cytokine-levels noted on sub-toxic (80 mg/L) dosing of HK-cells with silica for reduced exposure periods (1-5 days). Since, no significant ROS was generated at such doses **(Figure 4.3 A)** attributed to faster toxin-elimination (possibly by metallothioneins toxin-efflux proteins), the induced inflammatory activity was considerably negligible (Ruttkay-Nedecky et al.,2013; Shibutani et al.,2000). However, a marginal-rise [p(average)=0.052] in cytokines levels were noted on sub-toxic (80 mg/L) silica dosing for extended exposure period (7 days). This could be accredited to the mild ROS induced by the negligible levels of chronically accumulated residual-toxin, which could have elicited minimal-inflammatory activity (Thijssen et al., 2007b]. Nevertheless, this minimal immuno-toxicity exhibited on prolonged sub-toxic silica (80 mg/L) dosing could not induce cell-death and associated tubular cell-fibrosis (the ultimate CKDu manifestation) in the long-run. This was witnessed from the retention of cell-viability at this dose **(Figure 4.1)** in spite of prolonged exposure (15 days). This absence of considerable cell-death could be attributed to the cellular-repair capacity (aided by ROS scavengers like metallothioneins and anti-oxidants] remaining intact at such low-concentrations that was capable of eradicating such mild-levels of inflammation and cell-mortality inducing intracellular-stressors (like ROS) (Thijssen et al.,2007a, 2007b). Overall, these time-dependent sub-toxic outcomes were in agreement with tubular cell-responses triggered by sub-toxic concentrations of heavy-metals (Thijssen et al.,2007a, 2007b). Contrarily, unchanged cytokine-levels were noted in malignant-equivalent (A498) **(Figure 4.4)** under similar conditions accounted to its intrinsically rapid ROS metabolism, stronger anti-oxidant defense and maintenance of ROS-homeostasis (Kalluri,2016). Therefore, these findings of enhanced inflammation and subsequent fibrosis inflicted on exposure of

proximal-tubular cells (HK-cells) to toxic silica dose (≥100 mg/L) were consistent with the fibrotic mechanistic-aspects of heavy-metal induced renal-toxicity in human proximal-tubular cells (Chan,2014) and rats (*in-vivo*) (El-Refaiy et al.,2013). Thus, highlighting capacity of silica in inducing fibrosis that majorly contributes to development of chronic tubulo-interstitial nephritis (pathological-manifestation of CKDu) (Chan,2014). Moreover, these results paralleled the prolonged immuno-toxic impact of silica on pulmonary-epithelial cells, which manifested in pulmonary-fibrosis development. Thus justifying the fibrosis-inducing potency of silica in varied cell-types as well (Shimbori et al.,2017).

4.3.5 DNA damage inflicted on silica exposure

Comet-assay was used to explore the key inducer of apoptotic mechanism viz. DNA-damage inflicted on silica exposure. The magnitude of DNA-damage was denoted by % of tail DNA. Tail DNA denotes a single injured DNA strand that trails on electrophoresis of the whole-nucleus, with its length being directly proportional to severity of breakage (Glei et al.,2016).
In the current-study**,** a dose and time-reliant enhancement of DNA-damage (single-strand breaks) **(Figure 4.5 A),** in terms of heightened % of tail-DNA **(Figure 4.5 B)** was evident in silica treated HK cells. This DNA-breakage was highly-significant (p=0.011) from chronic exposure to 100 mg/L silica and above affirming this dose to be genotoxicity inducing nephrotoxic-dose. This dose and time-dependent elevation in single-strand DNA-breakages in HK-cells on silica exposure corresponded to the likewise simultaneous induction of oxidative-stress that inflicted grave genomic-damage. Oxidative-stress in HK-cells manifested from silica induced dose and time-dependent decline in antioxidants and rise in the ROS-production **(Figure 4.3 A)** that created an oxidative-environment for targeting sub-cellular components like DNA. This prolonged attack of ROS on the DNA caused DNA-adduct formation and subsequent strand-breakage, which surpassed the cellular-repair capacity and signaled apoptosis commencement (Jan et al., 2015). The role of oxidative-stress in genotoxicity induction explains the lack of significant DNA-damage in HK cells on sub-toxic silica (80 mg/L) dosing despite prolonged exposure (7 days). This could be attributed to the inadequate amounts of ROS **(Figure 4.3 A)** generated from the negligibly accumulated residual silica-toxin that could not suffice in inducing DNA-injuries, as large-quantities of ROS are required for the same. This genotoxicity was further prevented due to the efficiency of the uninhibited cellular-repair (mediated by DNA-repair enzymes like DNA-polymerase and ROS-scavengers viz.metallothioneins and anti-oxidants) at these lower doses in nullifying the oxidative DNA-damaging effect of the ROS (Isani et al., 2009).

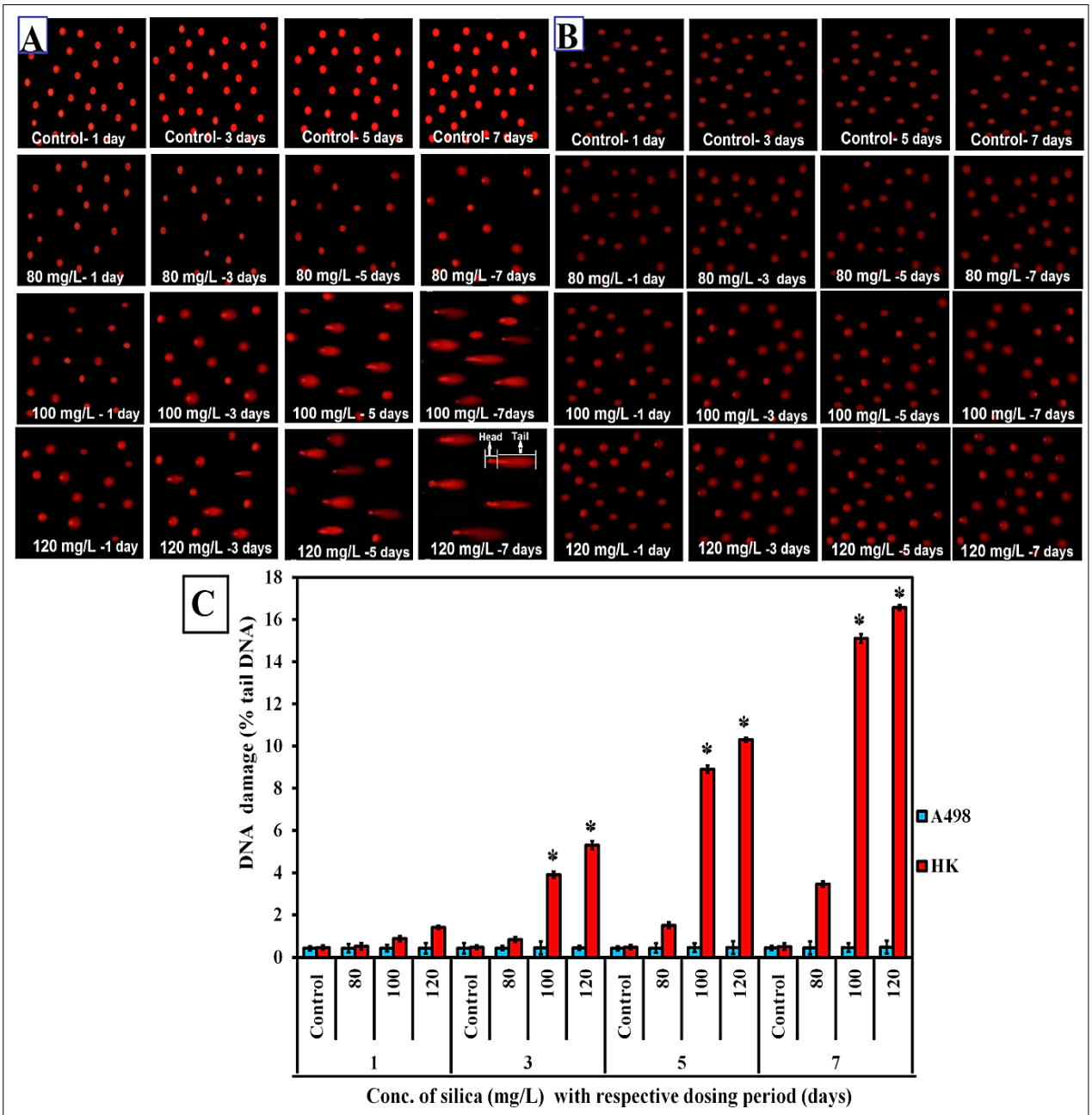


Figure 4.5. Comet assay detecting DNA injuries inflicted on prolonged silica exposure of 1-7days in HK and A498 cells. (A) Comet assay in HK cells (B) Comet assay in A498 cells (C) % of tail DNA in cells"

The silica induced severe oxidative-stress triggered the dose and time-response based escalation in genotoxicity in HK cells (A). This was evident from the continual elevation in the percentage of tail-DNA (C). Tail-DNA symbolizes single DNA strand breaks trailing as a 'tail' on whole-nucleus electrophoresis via the Comet assay (A). This was antagonistic to the untreated controls with conserved structural genomic-integrity represented by intact circular nucleus (A). A significant ($p<0.05$) 30- and 32-fold rise in % of tail-DNA (C) was noted in chronically exposed (7 days) sets of HK-cells treated with 100 and 120 mg/L silica respectively relative to untreated controls. However no significant DNA damage was noted on chronic sub-toxic dosing (80 mg/L).This was attributed to eradication of negligible levels of ROS (DNA-damage inducer) generated by the uninhibited cellular-repair at lower doses.Simultaneously,no significant DNA damage (indicated by round nucleus) was observed in its carcinoma equivalent viz. A498-cells (B) ascribed to its enhanced ROS metabolism and innate anti-oxidant defense averting oxidative-stress induced genomic-injuries. Results of % tail DNA are denoted as mean ± S.E from 3 autonomous experiments.*$p < 0.05$ in comparison with untreated cells was considered significant.Error bars denoted less than 5% deviation in values.

This repair-response was validated by the observance of a significant ($p<0.05$) enhancement in the G2/M arrest **(Table 4.1, Figure 4.6)** for DNA-damage control that prevented the persistent activation of p53-mediated apoptotic cell-death **(Figures 4.7, 4.8 and 4.9)** (Chargui et al.,2011). This lack of cell-death (ultimate outcome of nephrotoxicity) on chronic sub-toxic

dosing was apparent from negligible decrease in cell-viability **(Figure 4.1)** obtained at this dose in spite of a prolonged exposure (for 15 days). This lack of significant DNA-damaging effects noted on sub-toxic dosing were consistent with genotoxic responses obtained on sub-toxic dosing with heavy-metals (Koizumi and Yamada,2003). Concurrently, insignificant alterations in DNA-integrity were detected in its carcinoma-complement (A498-cells) under homologous conditions **(Figure 4.5).** This can be explained by its intrinsic ROS homeostasis preservation and enhanced anti-oxidant protection that failed to elicit DNA-insults. Moreover, the inherent p53-mutation further prevented recognition of DNA-injuries (if any) and subsequent apoptotic-cascade elicitation (Sakthivel and Hariharan,2017).

Therefore, our findings established oxidative-stress to be the primary-mediator of silica induced DNA-damage in proximal-tubular HK-cells on toxic-dosing (≥100 mg/L).These mechanisms were homologous with the DNA-damage mechanism of heavy-metal induced nephrotoxicity in human proximal-tubular cells (Rani et al.,2014) and in rats(*in-vivo*) (Geyikoglu et al.,2013).Thus confirming genotoxicity to be a major mechanism of silica induced nephrotoxicity via induction of apoptotic cell-death.

4.3.6 Silica induced cell-cycle arrest

The preliminary response to environmental-nephrotoxin induced oxidative DNA-damage (single-strand injuries) is G2/M cell-cycle arrest. This arrest is achieved to avert the defective cellular-genome from transitioning into mitosis to preserve genomic-integrity and prevent neoplastic alterations (Nakagawa et al.,2015).

Hence, the cell-cycle arrest was determined on exposure of HK and A498-cells to increasing silica-doses for increased exposure-periods via flow-cytometry **(Table 4.1).** Silica triggered a switch in cell-cycle arrest from G0/G1 to G2/M phase in HK-cells; with simultaneous inactivation of arrest in A498-cells.This inactivation could be explained by endogenous p53-mutation in carcinoma-cells that failed to recognize DNA-injuries (Sakthivel and Hariharan, 2017). Contrarily, a concentration and time-dependent increase in HK-cells arrested in the G2/M-phase following 7 days silica treatment were noted, rising from 14.3% (untreated group) to 23.3%, 46.5% and 54.3% in 80, 100 and 120 mg/L silica exposed cell-groups respectively. This cell-division arrest increased significantly ($p=0.021$, Mascarenhas et al., 2017) on chronic treatment with ≥100 mg/L silica, establishing this dose to be nephrotoxic that severely inhibits cell-division. Besides, no significant arrest was noted on sub-toxic (80 mg/L) treatment of HK-cells for shorter time-periods (1-5 days); accredited to the absence of inducer DNA-damage **(Figure 4.5)** at such doses. This lack of DNA damage was justified

from the insignificant level of injurious ROS generated, owing to the faster toxin-elimination (plausibly by metallothioneins) at lower-doses (Isani et al.,2009).However a significant increase in G2//M arrest was noted [p=0.039,Mascarenhas et al., 2017] on prolonged sub-toxic treatment (7 days). This could be attributed to the need for up-regulation of the endogenous cellular-repair to eliminate the negligibly generated mortality inducing intracellular-stressors (like DNA-damage); to prevent incessant induction of p53-mediated apoptotic death (Chargui et al.,2011;Koizumi and Yamada,2003).The efficiency of this cellular-repair was proved from the nominal decline in cell-viability**(Figure 4.1)** noted at this dose despite the extended exposure (15 days).Overall, these time-dependent effects observed on sub-toxic dosing were homologous to heavy-metal induced tubular cell-responses under similar conditions (Koizumi and Yamada, 2003).

Table 4.1. Cell cycle phase arrest of HEK cells cultured in three different concentrations of silica for a long term (i.e. for four passages).

Passage number	Conc. of silica (mg L^{-1})	% Sub G1	% G1	%S	%G2-M
1	**Control**	3.5±0.8	61.1±0.5	23.1±0.05	10.6 ±1.1
	80	3.8±0.2	58.1±1.2	26.9±0.3	12.2±0.8
	100	4.3±1.1	36.5±0.2	32.1±0.4	14.6±1.3
	120	6.1±1.3	28.2±0.2	38.6±0.3	29.1±0.1
2	**Control**	3.7±0.5	58.1±1.3	26.1±0.5	12.1±0.2
	80	4.2±0.1	41.9±0.3	33.6±1.6	16.3±0.5
	100	7.2±0.4	27.4±1.1	41.3±0.1	24.2±0.4
	120	10.6±1.1	34.8±0.2	18.2±0.1	36.4±0.3
3	**Control**	4.1±0.5	53.6±1.1	28.9±0.1	13.4±0.2
	80	8.4±0.1	42.1±0.5	21.8±1.1	19.6±1.1
	100	13.3±0.2*	38.6±1.1	9.1±0.2	39.1±0.2*
	120	18.4±0.1*	10.8±0.2	22.3±1.1	48.7±0.3*
4	**Control**	3.8±0.2	54.4±0.1	27.5±0.3	14.3±0.1
	80	11.4±0.1*	20.3±1.1	36.2±0.1	23.3±0.4*
	100	19.6±0.2*	19.3±0.2	14.7±0.2	46.5±0.1*
	120	29.4±0.3*	11.1±0.3	5.6±0.1	54.3±0.2*

The percentage of cells arrested at each phase of the cell cycle (G2-M; sub G1; G1 and S) on exposure to increasing concentrations of silica (i.e. 80,100 and 120 mg L^{-1}) and increasing dosage period (as indicated by the passage number) are represented as mean ± SD calculated on three independent experiments.*$p<0.05$ was considered to be significant. A significant increase in the population arrested at the G2-M phase and apoptotic sub-population was seen after the 4^{th} passage in silica at a concentration of 120 mg L^{-1}.

The substantial arrest observed in HK-cells on chronic dosing with high-silica concentrations portrayed that silica suppressed proximal-tubular cell-proliferation by persistently arresting the cell-division at the G2/M-threshold. At this threshold, the silica induced incessant DNA-

insults surpassed the intrinsic repair-potential that ultimately triggered apoptosis. This was evident in the results of our previous-investigation (Mascarenhas et al., 2017), wherein a dose and time-dependent increase in apoptotic sub-G1 population was observed (refer Table 4.1). This prolonged apoptosis can ultimately result in reduction of functional-nephrons causing kidney-dysfunction governing CKDu development (Nakagawa et al.,2015). Simultaneously, the incessant G2/M-arrest contributed to the development of fibrosis(the pathological characteristic of chronic tubulo-interstitial nephritis/CKDu) as well by activating the JNK (c-Jun-NH(2)-terminal-kinase) pathway that positively stimulated generation of fibrogenic-cytokine (TGF-β1) **(Figure 4.4E)** (Yang et al.,2010). Collectively, these findings were consistent with the cell-division arrest mechanism of heavy-metal induced nephrotoxicity in human proximal-tubular cells (Rani et al., 2014) & in mice (*in-vivo*) (Xie and Shaikh, 2006).

4.3.7 Silica elicited DNA damage mediated G2/M checkpoint arrest

The major defense mechanism in response to genotoxic-stress (DNA-injuries) is activation of distinct checkpoint-signaling cascades. These checkpoints initiate repair and prevent advancement of irreversibly injured cells to mitosis via apoptotic cell-death induction. Thereby conferring protection against neoplastic transformation. These checkpoints function by recruiting sensors like 9-1-1 complex on DNA-damage recognition and relay the signals to the respective kinase cascade viz.ATM-Chk2 (for double-stranded breaks) or ATR-Chk1 (for single-stranded breaks) causing a corresponding differential arrest in G1 or G2/M phases (Thomasova and Anders,2014). Chk1 (checkpoint-kinase) is the key-player of G2/M-cascade, as it prevents single-strand DNA-damage from entering mitosis necessitated for genome-integrity preservation. These single-strand DNA breakages specifically attract ATR-kinase at its replication-foci to initiate the DNA-repair. This repair is then initiated by ATR-mediated activation (by phosphorylation) of its downstream kinase-Chk1 **(Figure 4.6A)**, which subsequently arrests the cells at the G2/M-threshold. During the arrest, the activated Chk1 suppresses expression of pro-mitotic phosphatase-Cdc25 **(Figure 4.6 B)** by inhibitory phosphorylation at the S216 amino-acid position. This amino-acid phosphorylation then attracts the 14-3-3 protein that triggers nucleo-cytoplasmic shuttling of Cdc25-phosphatase that fails to activate the nuclear mitotic-kinase (Cdk1) **(Figure 4.6 D)** by removal of Cdk1's inhibitory-phosphate. This inactivated Cdk1 prevents activation of mitotic cyclin-B1 **(Figure 4.6 C)**, attributed to inability of cyclin-B1 to bind to Cdk's active-site owing to inhibitory phosphate induced conformational incompatibility. Thus, resulting in damage-fixation at the G2/M-checkpoint and mitotic-obstruction (Bonventre,2014).

Silica is reported in inflicting toxicity in non-renal cell-types (pulmonary-cells) via DNA-damage induced G2/M-checkpoint arrest mechanism (Gonzalez et al.,2014). However, as per our knowledge, this toxicity-aspect has not been explored in renal proximal-tubular cells, hence was pursued in this study. Thus to confirm the role of DNA-injury triggered G2/M-arrest cascade in nephrotoxicity induction, the activation of G1-checkpoint regulator (Chk2) and G2/M mediators (Chk1, Cdc25, Cdk1 and cyclin-B1) were investigated. A concentration and time-reliant disruption of G2/M-regulation network (**Figure 4.6**) was noted in HK-cells. This was evident from the up-regulation of checkpoint-transducer i.e. Chk1 **(Figure 4.6 A)** and down-regulation of mitotic-inducers viz. Cdc25, cyclin-B1 and Cdk1 **(Figure 4.6 B-D)**. The G2/M-regulation was significantly disturbed ($p<0.05$) on prolonged-exposure to doses ≥ 100mg/L, affirming this concentration to be nephrotoxic that induces severe cell-division inhibition. The enhanced G2/M-arrest further signified single-stranded DNA-injuries to be the primary-inhibitor of cell-proliferation owing to inactivation of G1-checkpoint effector i.e. Chk2 (for double-stranded injury) **(Figure 4.6 E) (**Canaud & Bonventre,2014). Moreover, no significant irregularities in G2/M-cascade were observed on short-term (1-5 days) treatment of HK-cells with sub-toxic silica dose (80 mg/L)This is accredited to absence of significant DNA-damage (that triggers arrest) due to lack of significant ROS generation **(Figure 4.5),** owing to faster toxin-clearance (possibly by metallothioneins) at smaller-doses (Ruttkay-Nedecky et al,2013). However, a significant-increase ($p<0.05$) in G2/M arrest inducers were noted on chronic (7 days) sub-toxic (80mg/L) dosing. This was accredited to activation of intrinsic cellular-repair (primarily assisted by repair-enzymes and ROS-scavengers like metallothioneins and anti-oxidants) for damage-control of the insignificant genomic-injuries generated by negligible ROS (arising from minimal levels of chronically accumulated toxin) at such doses (Koizumi and Yamada,2003).This repair was significantly induced to avert persistent stimulation of p53-mediated apoptotic cell-death (the final outcome of nephrotoxicity) in the long-run (Chargui et al., 2011). The efficacy of this cellular-protection was proved from the negligible drop in cell-number **(Figure 4.1)** observed at this sub-toxic dose, in-spite of chronic exposure (15 days). These time-reliant outcomes on sub-toxic dosing were consistent with tubular cell-responses induced by sub-toxic doses of heavy-metals (Isani et al.,2009). Parallely, the malignant-counterpart (A498-cells) witnessed an absence of disturbances in G2/M-pathway regulation **(Figure 4.6)** under comparable conditions. This was ascribed to endogenous p53 gene-mutation that failed to activate the checkpoint-effector (Chk1) and Cdk-inhibitor viz. p21 which resulted in uncontrolled mitotic-progression (Swift and Golsteyn, 2014).

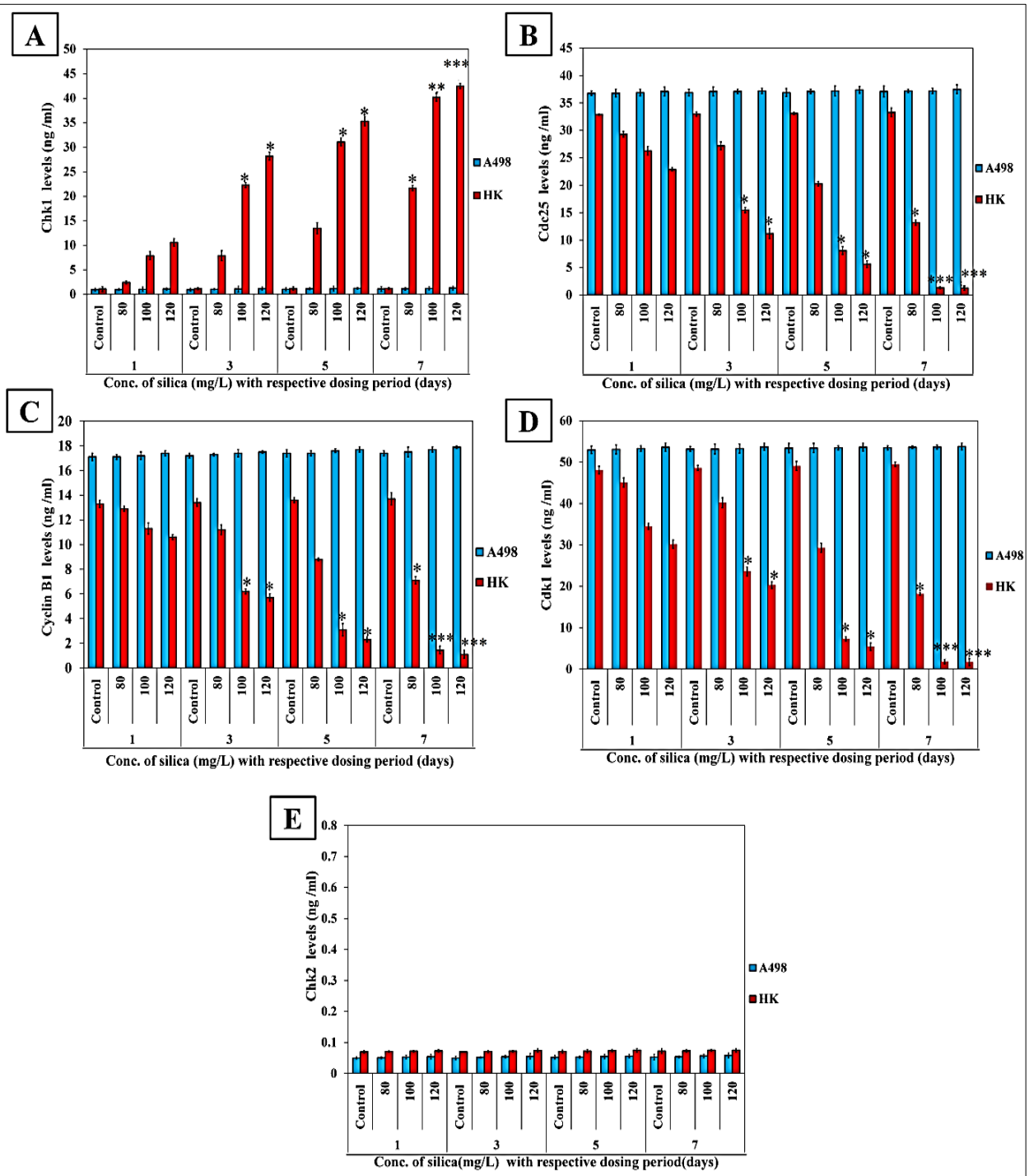


Figure 4.6 Silica induced DNA damage mediated G2/M checkpoint network activation in HK and A498 cells over a chronic dosing (1-7 days).

Recurrent and superfluous DNA injuries (single strand breakages) triggered by silica, elicited excessive G2/M cell-cycle arrest in HK cells for mitotic suppression, to prevent neoplastic alterations. This was apparent from the dose and time dependent increase in the major checkpoint effector viz. Chk1 (A) and diminution of the mitotic inducers that include Cdc-25 (B), Cyclin B1 (C) and Cdk1 (D). A significant ($p<0.05$) 34- and 35-fold increment in Chk1 and decrement of 25- and 26-fold in Cdc-25; 11- and 12-fold in cyclin B1 and 28- and 29-fold in Cdk 1 in comparison to untreated controls were observed in chronically exposed (7 days) sets of HK cells treated with 100 mg/L and 120 mg/L silica respectively. Moreover, a significant G2/M arrest was observed on long-term sub-toxic dosing (80 mg/L) of HK-cells as well. This was accredited to the activation of the enhanced repair for damage control of the insignificant DNA damage generated by the negligibly generated ROS. This heightened repair provides supportive evidence of the endogenous cellular-protection capacity remaining intact at such sub-toxic doses which prevents significant mortality (as indicated in the MTT results) on long-term dosing (>15 days). Additionally, double-stranded DNA damage triggered G1-checkpoint mediators viz. Chk2 (E) displayed simultaneous inactivation reflected from the unaltered levels. Therefore, signified single-stranded DNA damage to be the primary inducer of cycle-arrest. Absence of cell-division arrest in the malignant (A498) cells was evident from the enhanced activation of the mitotic stimulators viz. Cdc25, cyclin B1 and Cdk1.This was attributed to its intrinsic p53 mutation that failed to activate Chk1 and mitotic Cdk-inhibitor (p21) which resulted in uncontrolled mitotic cell-division. Data is indicated as mean ± S.E from three autonomous experiments. *$p < 0.05$;**$p < 0.01$; ***$p < 0.001$ in comparison with the untreated cells were considered significant. Error bars illustrate less than 5% deviation in values.

These findings of G2/M-arrest induced in HK-cells on toxic (100 mg/L) silica-dosing were consistent with G2/M proliferation-arrest mechanistic-route induced during heavy-metal nephrotoxicity in human proximal-tubular cells (Pari et al.,2014) & mice *(in-vivo)* (Lee et al.,2010). Thus confirming that toxic silica dose (≥100 mg/L) induced proximal-tubular toxicity is mediated by DNA-damage induced Chk1-dependent G2/M-checkpoint activation.

4.3.8 Silica triggered apoptotic pathway of cell death

The pathway of cell-death adopted viz. apoptosis or necrosis was evaluated in HK cells following prolonged silica exposure via flow-cytometric evaluation of their Annexin-V/ FITC-PI dually labeled DNA-content. The results (**Figure 4.7**) revealed a dose and time-dependent increase in the apoptotic-population, increasing from 4.8% (untreated cell-group) to 39.3%, 50.1% and 59.6% following chronic exposure (7 days) to 80,100 and 120 mg/L silica respectively. A significant-increase ($p=0.021$) in apoptotic-frequency was noted following chronic-dosing with ≥100mg/L silica, justifying the nephrotoxic-potential of this dosage by its ability to induce unwarranted cell-demise. Furthermore, no substantial apoptosis was observed on sub-toxic silica (80 mg/L) treatment for reduced exposure (1-5 days). This can be attributed to the absence of apoptosis inducing oxidative-DNA injuries **(Figure 4.5)** under these conditions owing to rapid toxin-clearance (possibly mediated by metallothioneins toxin efflux proteins) (Koizumi and Yamada, 2003). However, this apoptotic-frequency negligibly increased ($p=0.0523$) on prolonged (7 days) treatment with this sub-toxic dose (80 mg/L). This can be explained by the occurrence of mild oxidative DNA-insults by the minimal ROS generated from the negligibly accumulated residual silica-toxin (Isani et al., 2009). Nonetheless, this minimal levels of apoptosis induced was not adequate enough to trigger cell-demise (ultimate nephrotoxicity outcome) in the long-run. This was visible from the insignificant decrease in cell-viability **(Figure 4.1)** noted at this dose despite an extended exposure (15 days).This lack of significant cell-mortality can be attributed to the uninhibited cellular-repair capacity (primarily mediated by repair-enzymes and ROS scavengers like metallothioneins and antioxidants) at smaller-doses, that enabled the successful eradication of cell-death inducing intracellular-stressors (like DNA-damage) (Chargui et al., 2011). Contrarily, the malignant-counterpart (A498-cells) witnessed lack of significant apoptosis, accredited to the intrinsic p53-mutation that failed to activate the DNA-injury stimulated apoptotic-pathway (Seo et al., 2014).

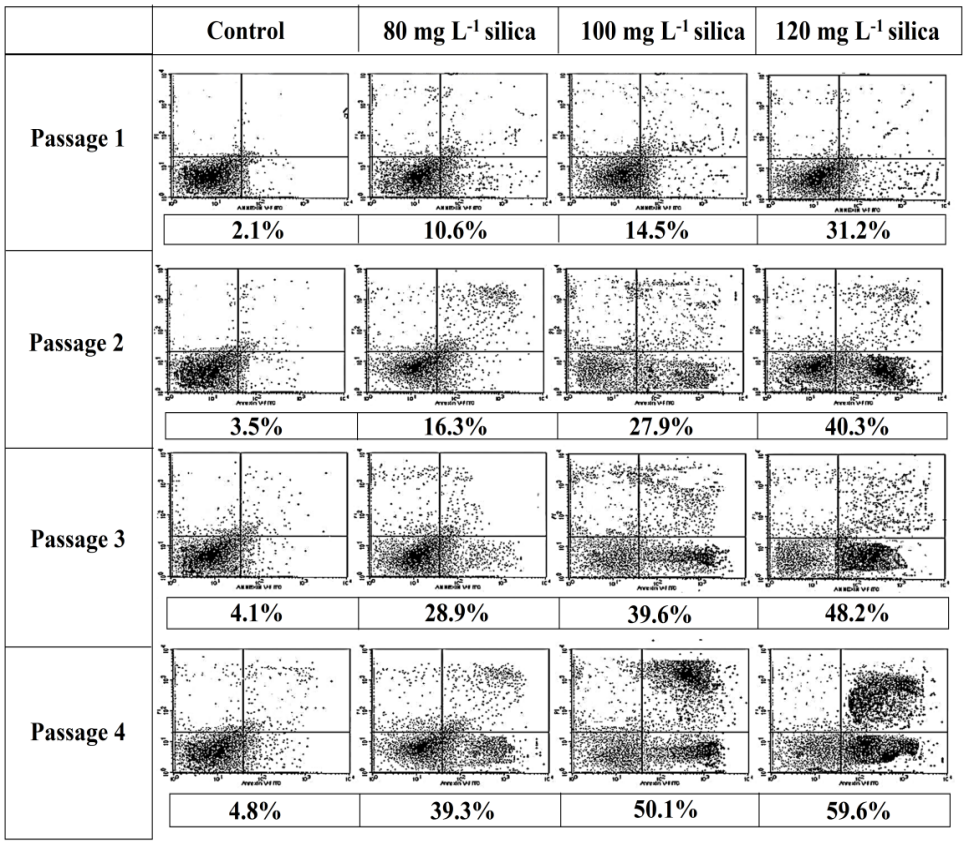


Figure 4.7. Dot plots of silica stressed HK cells on dual staining with Annexin V-FITC and PI.
Untreated culture and cells exposed to silica at three different concentrations (i.e. 80, 100 and 120 mg L^{-1}) for four passages were subjected to Annexin V-FITC/PI dual labelling. Annexin-V-FITC presents a high affinity to phosphatidylserine which undergoes externalization in early-apoptotic stages. The vital dye-PI differentiates between dead cells resulting from late-apoptosis or necrosis and early apoptotic cells. The dot plots are representative of three independent experiments depicting intact viable cells at the lower-left quadrant, FITC (-)/PI (-); necrotic cells at upper-left quadrant, FITC (-)/PI (+); early apoptotic cells at the lower-right quadrant, FITC (+)/PI (-) and late apoptotic cells at the upper-right quadrant, FITC (+)/PI (+). The percentage of apoptotic cells were quantified by Fluorescence-activated cell-sorter (Becton Dickinson FACS-Calibur, California, USA) based on the cell number and is indicated below each dot blot. The data indicated that apoptotic proportion increased significantly with increasing exposure period and dose .This was evident from the successive increase in scatter observed in the upper and lower right quadrants.

The increased apoptotic-rate in proximal-tubular cells (HK-cells) following prolonged exposure to high silica-dosages (≥100 mg/L) was reflected from the evidently enhanced phosphatidyl-serine(PS) externalization, a primary apoptosis characteristic detected by Annexin-V receptor.PS-externalization is a major implication of ROS induced membrane-damage. It is mainly manifested on selective-oxidation of the plasma-membrane's phospholipid-phosphatidyl-serine by enhanced ROS (generated by silica in this case).This phospholipid-oxidation inhibits the interaction with aminophospholipid-translocase (APT) enzyme which is critical for safeguarding plasma-membrane integrity. Moreover, the PS-externalization can be intensified by the silica generated caspase (apoptosis-effector) induced phosphatidyl-serine lysis, signifying adoption of apoptotic (and not necrotic) pathway of cell-demise during silica induced renal-toxicity(Segawa et al.,2015).Our findings of role of silica generated ROS in inducing PS-externalization on toxic-dosing (≥100 mg/L) are consistent with apoptotic cell-death mechanism expressed during heavy-metal elicited renal-toxicity in

human proximal-tubular cells (Verdugo et al.,2016) and mice (*in-vivo*) (Dkhil et al.,2016). Thus, indicating oxidative-stress to be a major apoptosis inducer during silica nephrotoxicity

Recently, emerging-reports have specified the contribution of nephrotoxin triggered cytochrome-c efflux in exaggerating PS-peroxidation and externalization during induced renal-toxicity. This PS-externalization was achieved by the catalytic redox-electrostatic interactions between the anionic-phospholipid and the cytosolically released cationic cytochrome-c enzyme during the mitochondrial-pathway of apoptosis (Alvarez-Paggi et al., 2017). Therefore, these observations stressed the need for further analysis of the apoptotic-route (mitochondrial or death receptor-based) adopted during silica induced nephrotoxicity.

4.3.9 Silica elicits mitochondrial route of apoptosis

The mitochondrial enrichment of proximal-tubular cells(of renal-nephron) predisposes them to nephrotoxin inflicted mitochondrial-injuries which on chronic accumulation, triggers incessant mitochondrial-routed apoptotic cell-death. This continued tubular cell-death ultimately manifests in tubular-atrophy and fibrosis resulting in diminution of the functional-nephrons that exaggerates into CKDu development (Gamboa et al.,2016).Intracellular-stressors (like ROS, DNA damage) are well-reported in triggering severe mitochondrial-damage and its associated apoptotic-pathway. This route is contrary to plasma-membrane localized death-receptor mediated apoptotic-route; stimulated by extracellular-stressors from adjacently stressed-cells (Che,2014). Our study has highlighted role of silica-toxin in generating various intracellular-stressors. This suggested the plausibility of adoption of mitochondrial-apoptotic molecular-pathway. To verify this theory, effectors of mitochondrial and death-receptor mediated (via caspase-8 activity) apoptotic-routes were assessed.

The pivotal step in the environmental-nephrotoxin triggered mitochondrial-apoptotic pathway is mitochondrial outer-membrane permeabilisation (MOMP). This MOMP is necessitated for the release of caspase activating inter-membranous-space protein like cytochrome-c, that ultimately induces proximal-tubular cell-death (Che et al., 2013).This process was replicated in our current-study as well, wherein a dose and time-dependent increase in the MOMP in HK-cells on silica exposure was noted, reflected from the elevations in green/red ratio **(Figure 4.8 A)**. This MOMP significantly increased ($p=0.010$) on chronic dosing with ≥100 mg/L highlighting the mitochondrial membrane-damage inflicting potency of this dose contributing to its nephrotoxic-potential. This increase in MOMP was further facilitated by the action of high-silica (≥100 mg/L) dose generated ROS and p53-activation. As described earlier, the ROS induced incessant lipid-peroxidation of the mitochondrial membrane-

phospholipids (Stepniewska et al., 2015) of the HK-cells that contributed to the membrane-permeabilisation. This permeabilisation was further aggravated by the silica induced enhanced p53-expression **(Figure 4.8 B),** which consequently stimulated the increased activation of apoptogenic mitochondrial-membrane porator (Bax-protein) **(Figure 4.8 C)** and inhibition of anti-apoptogenic Bcl-2 protein **(Figure 4.8 D)**. The p53-gene was majorly activated as a repair-response for the silica triggered oxidative DNA-damage to preserve the genomic-integrity. However, persistent DNA-injuries **(Figure 4.5)** in HK cells (on prolonged high-silica dosing) exceeded the cellular-repair capacity, which provoked the p53 to grant apoptotic death-verdicts via the simultaneous activation and inhibition of Bax and Bcl-2 respectively. This Bax-activation was majorly mediated by intracellular-stressor (DNA-damage) stimulated p53-triggered Bax-oligomerisation (facilitated by its trans-membrane integrating BH_3-domains interactions). This oligomerisation is rapidly antagonized under normal-metabolic situations by the anti-apoptogenic Bcl-2 protein by BH_3-domain sequestration that prevents Bax-activation (Martinou and Youle, 2011).This interplay of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) Bcl-2 family members are crucial for regulating the mitochondrial membrane-integrity. The MOMP was majorly worsened by the pro-apoptotic Bax **(Figure 4.8 C),** which permeated the outer-membrane by perpendicular insertion of amphipathic-helices on oligomerisation to create proteo-lipid pores. These pores enabled the leakage and cytosolic-translocation of the caspase activating mitochondrial-protein viz. cytochrome-c **(Figure 4.8 E) (**Zhan et al., 2013).

This entire series of apoptotic-events were replicated in HK-cells on silica exposure in a dose and time-dependent manner. Significant increase ($p<0.05$) in these apoptotic-effects were noted on chronic dosing with ≥100 mg/L silica. This was reflected from heightened p53-stimulated continual Bax-expression surpassing its anti-apoptotic counterpart-Bcl-2 which resulted in increased MOMP **(Figure 4.8A-D).** This high MOMP **(Figure 4.8A)** significantly enhanced cytosolic release of caspase stimulating inter-membranous protein viz. Cytochrome-c **(Figure 4.8 E)**.This incessantly released cytochrome-c significantly ($p<0.05$) activated the caspase-cascade **(Figure 4.9 A-B)** to induce apoptotic cell-death (**Figure 4.7**).

The caspase-activation was initiated by the formation of a complex between leaked cytochrome-c, cytosolic-adaptor (Apaf-1, apoptotic protease-activating factor-1), inactive initiator-caspase (pro-caspase-9) and dATP to form an apoptosome. This apoptosome activated caspase-9 **(Figure 4.9 A)** further triggered excessive stimulation of executioner-caspases (caspase 3 and 6) **(Figure 4.9 B).**

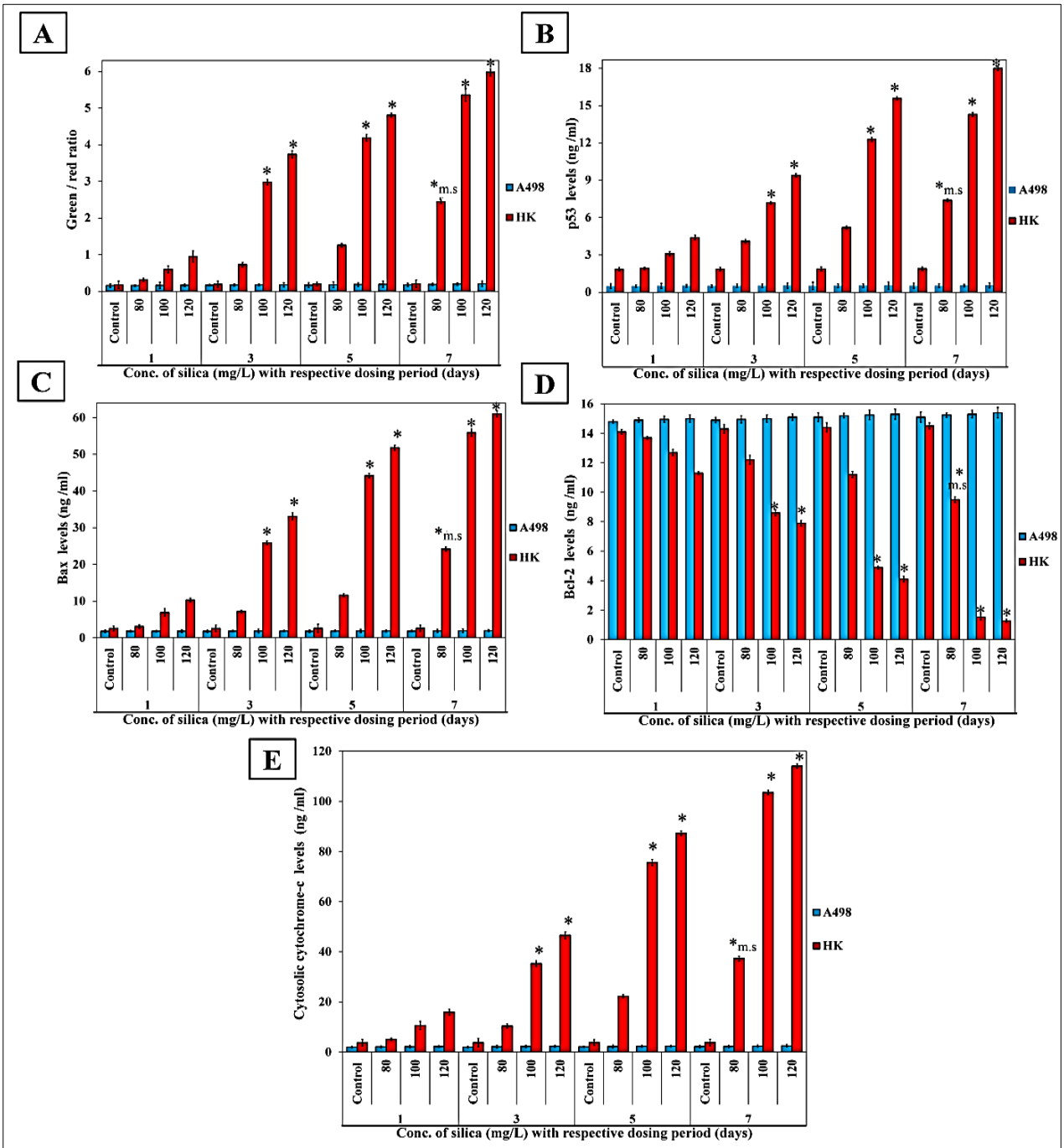


Figure 4.8. Mitochondrial membrane permeabilization triggered by silica in HK and A498 cells on long-term dosing (1-7 days)

Silica induced oxidative-stress (enhanced ROS) triggered the dose and time dependent increase in mitochondrial membrane permeabilisation in HK cells. This was reflected from the progressively elevated green/red fluorescence output ratio (A). This permeabilisation was further amplified by the pro-apoptotic mitochondrial porator activation viz. Bax (C) and simultaneous inhibition of the anti-apoptotic protein viz.Bcl-2 (D) by the DNA-damage responsive p53 gene (B). Persistent permeabilisation resulted in increased cytosolic efflux of inter-membranous apoptotic initiator i.e. Cytochrome-c (E), which consequently activated the caspase-cascade ensuing proximal-tubular cellular-mortality. A significant ($p<0.05$) fold decrease of 9 and 10 in Bcl-2 and an enhancement of 26 and 28 in green/red intensity ratio (equivalent to fold diminution in MMP),8 and 9 in p53 quantities, 22 and 23 in Bax levels and 29 and 30 in cytochrome-c intensities relative to untreated cell groups, were noted in chronically exposed groups of HK cells dosed with 100 and 120 mg/L silica respectively. Furthermore, marginally significant (p value approaching 0.05) increase in apoptotic inducers were noted on prolonged sub-toxic dosing(80 mg/L).This could be attributed to the damage control by the endogenous cellular-repair to eradicate the negligible number of damaged cells to prevent these cells from further inducing persistent apoptosis in a positive feedback mechanism. However, the negligible apoptosis did not cause cell-mortality in the long-run (evident from Fig.1A, Fig.S1) attributed to the elimination of the apoptosis inducer (DNA-damage) by the uninhibited repair at lower-doses. Simultaneously, lack of apoptosis in the carcinoma-equivalent (A498 cells) was accredited to the inherently amplified anti-oxidant protection and p53 mutation associated inactivation of the apoptotic initiators (Bax, Cytochrome c). Results are represented as mean ± S.E from three autonomous experiments.*$p < 0.05$ in comparison with the untreated cells was considered significant. *m.s =marginally significant with p values in the range of 0.051-0.059 i.e. approaching the alpha value of 0.05. Error bars illustrate less than 5% deviation in values.

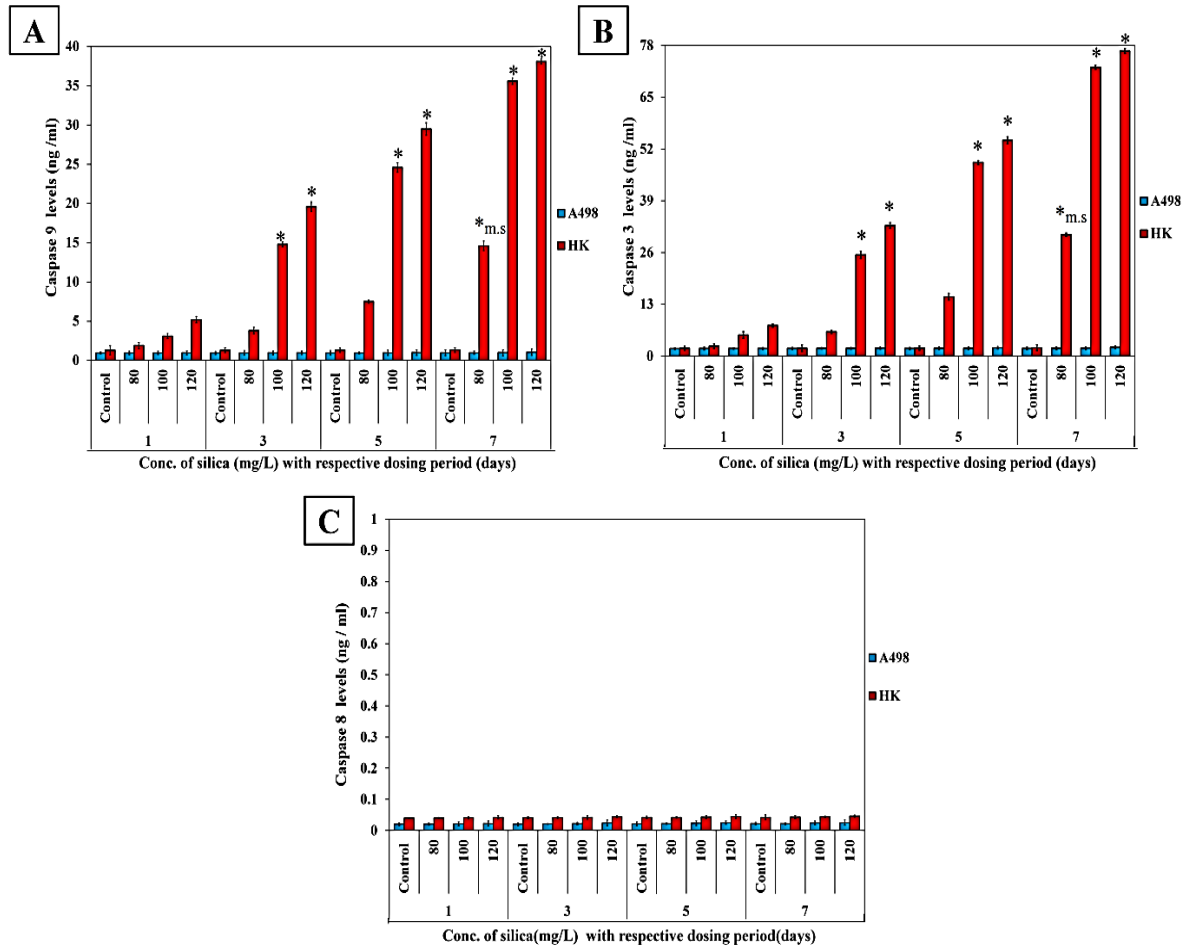


Figure 4.9. Silica incites the mitochondrial apoptotic pathway in HK and A498 cells on prolonged exposure (1-7 days).

Persistent oxidative-stress, DNA insults and immunotoxicity triggered by silica deleteriously targeted the intrinsically enriched mitochondria of the proximal tubular cells (HK cells). These incessantly elicited mitochondrial injuries surpassed the cellular-repair capacity, which consequently initiated the unwarranted apoptotic cellular-demise. This cellular-demise was majorly facilitated by the dose- and time-dependent activation of the caspase enzyme-cascade mediated by major apoptotic executioners viz. Caspase 9 (A) and Caspase 3 (B). The persistently released caspase activator (viz. cytochrome-c) into the cytosol primarily induced these cytosolic caspases (9 and 3) activation. This incessant proteolytic caspase-activation ultimately destroyed the cellular and nuclear structure (DNA-damage) that further amplified the apoptosis in a positive-feedback mechanism that resulted in uncontrolled cellular-death. This continuous tubular cell-death resulted in tubular atrophy which culminates into functional nephron loss that ultimately manifests as irreversible renal-damage (CKDu).A significant ($p<0.05$) fold surge of 27 and 28 in caspase 9 activities and 35 and 36 in caspase 3 levels relative to the untreated controls were observed in chronically exposed groups of HK cells treated with 100 and 120 mg/L silica respectively. Moreover, marginally significant (p value approaching 0.05) caspase (9 and 3) activation was noted on chronic sub-toxic (80 mg/L) silica dosing. This was attributed to the negligible ROS generated at lower-doses that stimulated the negligible mitochondrial permeabilisation associated release of the caspase activator (cytochrome-c). However, the negligible caspases activation could not induce significant cell-death in the long-run (evident from MTT-results).This was attributed to the elimination of the minimum number of damaged cells and caspases inducers viz. ROS and DNA-damage by the unaffected cellular-repair at lower-doses which prevented the persistent induction of apoptotic cell-death. Concurrently, an inactivation of the major executioner of the death-receptor apoptotic pathway viz. Caspase 8(C) was observed, which signified mitochondrial mediated apoptosis to be the major pathway of cell-death. Unaltered caspase activities noted in the malignant equivalent (A498) were attributed to the intrinsic p53 gene mutation related inactivation of the Bax protein that prevented the cytosolic efflux of the caspase-activator (viz.cytochrome-c). Results are denoted as mean ± S.E from three autonomous experiments. *$p < 0.05$ in comparison with the untreated cells was considered significant. *m.s =marginally significant with p values in the range of 0.051-0.059 i.e. approaching the alpha value of 0.05. Error bars illustrate less than 5% deviation in values.

This persistently activated caspases **(Figure 4.9 A-B)** induced severe sub-cellular damage (of DNA, membrane and protein) that elicited several stereotypical death-inducing effects like cell-shrinkage, cytoskeletal-collapse, DNA-fragmentation, membrane-blebbing and phosphatidylserine externalization. These death-effects (i.e. DNA and membrane-damage further activated caspases incessantly in a positive feed-back mechanism which resulted in an amplification of mitochondrial-mediated apoptotic cell-death (Jang and Padanilam, 2015).

Moreover, an inactivation of caspase-8 (**Figure 4.9 C)** (the effector of the death-receptor apoptotic pathway) was noted in HK-cells on chronic silica-exposure. This provided supporting-evidence to intrinsic-mitochondrial pathway to be the major apoptotic-effector mechanism underlying silica-induced nephrotoxicity (Che et al., 2013). Therefore, these findings of incessant mitochondrial-mediated apoptosis and associated cell-death inflicted on prolonged exposure of proximal-tubular cells (HK-cells) to higher silica-doses (≥100 mg/L) impeccably resonated with the apoptotic-mechanisms of heavy-metal induced nephrotoxicity in human proximal-tubular cells (Verdugo et al., 2016) and in rats (*in-vivo*) (Wongmekiat et al., 2018). Thus, confirming 100 mg/L silica to be the nephrotoxic-dose that is capable of inhibiting proximal-tubular cell-proliferation by inflicting continuous mitochondrial-mediated apoptotic tubular cell-demise. This persistent apoptotic cell-death ultimately manifests in the development of tubular-atrophy, the major pathological-manifestation of chronic tubulo-interstitial nephritis (CKDu). Tubular-atrophy contributes to the reduction of functional nephrons over-time which eventually culminates into irreversible renal-dysfunction that manifests as chronic kidney-damage (Havasi and Borkan, 2011).

Concurrently, the established role of ROS and associated DNA-damage in stimulating p53-mediated apoptosis explains the negligible activation [p(average of the apoptotic inducers)= 0.055] of the apoptotic pathway **(Figure 4.8, Figure 4.9)** in HK-cells on prolonged (7 days) dosing with the sub-toxic concentration (80 mg/L). This was attributed to the role of uninhibited endogenous cellular-repair in controlling the damage by completely eradicating the negligible amounts of genomically-damaged cells (at such doses) by apoptotic cell-death. This was done to prevent these damaged cells from further inducing incessant apoptosis in a positive-feedback mechanism (Chargui et al., 2011). Furthermore, this intact cellular-repair (assisted by repair-enzymes and ROS-scavengers like metallothioneins and anti-oxidants) could have been able to fix the negligible levels of caspases [p(average of the caspases)= 0.053] inflicted sub-cellular injuries (like DNA-damage) as well, that otherwise would have amplified the apoptotic-cascade (George et al., 2017). This response was validated from the absence of significant cell-mortality **(Figure 4.1)** despite a prolonged exposure (15 days). Thus indicating that these negligible levels of proximal-tubular apoptosis on chronic sub-toxic dosing could not suffice in overpowering the intrinsic cell-dividing cum repair capacity in the long-run, which prevented incessant tubular cell-death and associated nephrotoxicity (Havasi and Borkan, 2011). Overall, this lack of significant apoptotic outcomes on sub-toxic silica-dosing were in consensus with the tubular cell-responses inflicted on sub-toxic dosing with heavy-metals (Chargui et al., 2011).

Contrarily, a lack of apoptosis activation was noted in the carcinoma-equivalent (A498-cells) **(Figure 4.8, Figure 4.9)**. This was attributed to the intrinsic p53-mutation of the carcinoma cells that failed to activate the p53-mediated mitochondrial-apoptotic pathway and associated cell-death. Therefore, successfully negating the anti-tumorigenic potential of silica.

4.3.10 Mechanism of silica induced nephrotoxicity

The current-study depicted silica's ability in inducing nephrotoxicity at cellular & molecular-level in normal human renal proximal-tubular cells (HK-cells) as a function of dose & time.

Prolonged mitochondrial mediated apoptosis and incessant inflammation were proved to be major molecular-mechanisms underlying silica induced dose and time dependent nephrotoxicity **(Figure 4.10)**. Silica exhibited grave proximal-tubular-cytotoxicity (indicated by declining HK-cell-density) on long-term dosing (for 7 days) to high-concentrations (≥100 mg/L) **(Figure 4.1 A),** that was mediated by silica-toxin triggered mitochondrial-dysfunction. This mitochondrial-injury was an outcome of the inherent enrichment of proximal-tubular cells with mitochondria (owing to its high-energy demand for conducting diverse cellular functions) that renders these cells susceptible to toxin triggered mitochondrial damage (Gamboa et al.,2016).The high-silica-doses (≥100 mg/L) induced incessant mitochondrial injuries which resulted in continuous oxidative-stress (ROS >anti-oxidant defense) generation owing to the involvement of mitochondria in ROS-homeostasis. This chronic oxidative-stress (enhanced ROS) further inflicted severe DNA-damage (genotoxicity) that consequently elicited DNA-damage responsive p53 mediated damage repair coupled with G2/M cell-cycle arrest (Chk1-dependent). However, these persistently elicited DNA-injuries surpassed the cellular-repair potency, which provoked p53 to endlessly activate mitochondrial (intrinsic) series of apoptotic-events that resulted in incessant tubular (HK) cell-death **(Figure 4.10)**.

The amplification of this apoptotic-response in the renal-tubular cells was achieved by a p53 created cross-talk with the inflammatory cascade (the latter being primarily triggered by the ROS stimulated activation of the inflammatory transcription factor-NF-kβ). This cross-talk potentiated from silica generated intracellular-stressors (viz. ROS and DNA-injuries) stimulating the p53-gene which in-turn activated the inflammatory-regulator i.e. NF-kβ, owing to the presence of binding-sites in p53 for NF-kβ. This p53-bound NF-kβ complex simultaneously triggered the activation of the principal apoptotic-executioners (i.e. Bax and caspase-9, **Figure 4.8 C and Figure 4.9 A**) and pro-inflammatory cum fibrogenic-cytokines (IL-1β and TGF-β; **Figure 4.4 A, Figure 4.4 E**) (Kumar et al., 2015; Tucker et al., 2015); which collectively resulted in persistent tubular apoptotic cell-death and inflammation.

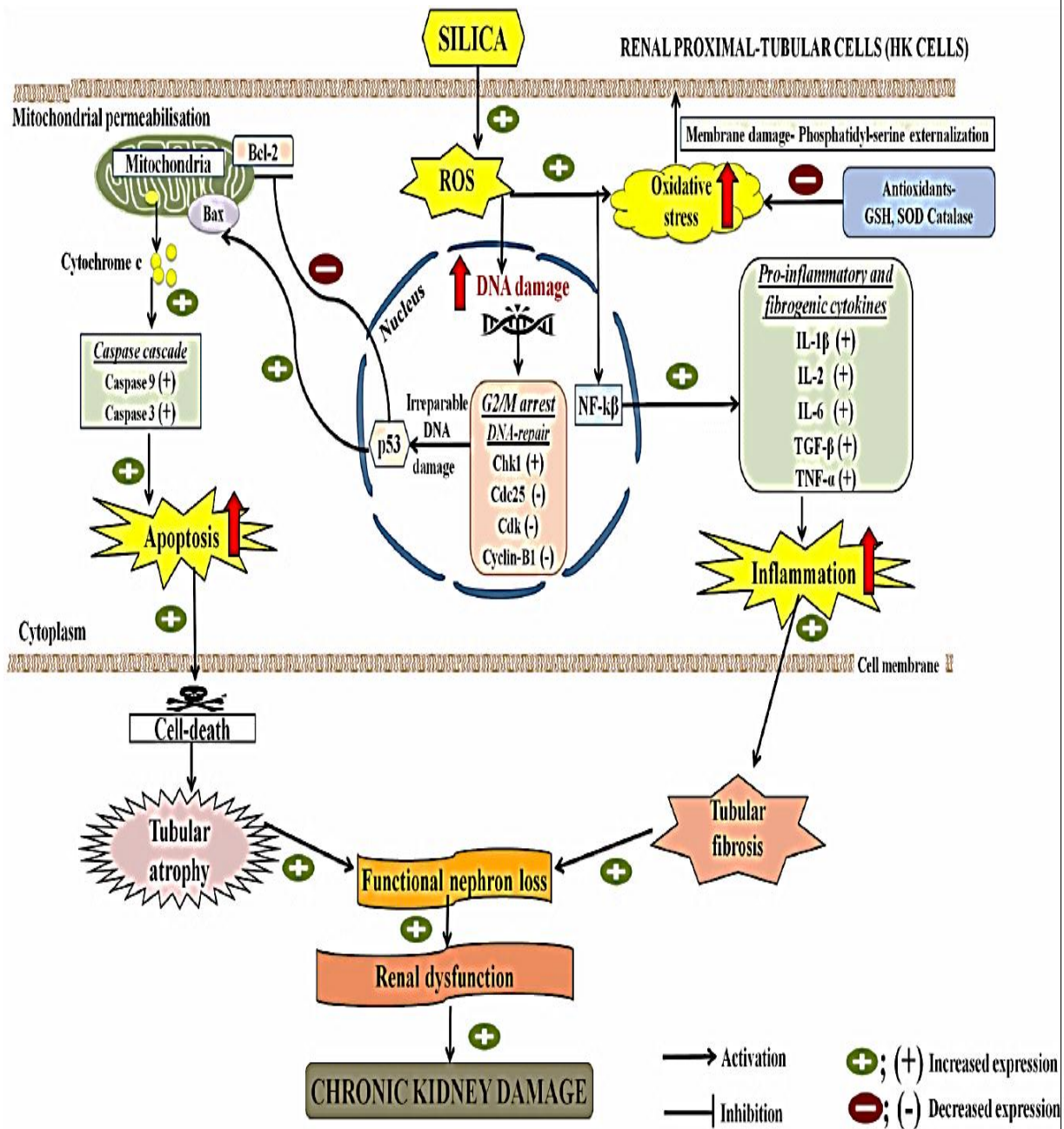


Figure 4.10. Cellular and molecular mechanisms of silica induced nephrotoxicity in renal proximal-tubular cells (HK-cells).

Prolonged exposure to high-silica doses (≥ 100 mg/L) induced the formation of excessive ROS which surpassed the anti-oxidant defense mechanisms (mediated by GSH, catalase and SOD), that culminated in the generation of oxidative-stress. This incessantly generated ROS subsequently triggered severe oxidative-damage to sub-cellular components (like DNA, lipids etc.) which manifested in the form of grave DNA-injuries and membrane-lipid peroxidation. This persistently triggered DNA-damage was attempted to be repaired by the intrinsic cellular repair mechanisms via stimulation of the endogenous Chk1 checkpoint. This activated Chk1 further prevented the advancement of genomically damaged cells into mitosis by inhibiting the mitotic-inducers (viz. Cdc25, Cdk and Cyclin-B) that resulted in the arrest of cell-proliferation at the G2/M phase. However, these silica elicited DNA injuries were so severe that it exceeded the repair-capacity, which eventually provoked the cell to grant death-verdicts. These death verdicts were potentiated by the activation of the p53 gene by the Chk1-checkpoint, which ultimately activated the mitochondrial series of apoptotic events. This incessantly activated p53 stimulated the enhanced expression of the major mitochondrial porator viz. Bax protein that caused the persistent cytosolic release of the caspase stimulating protein viz. cytochrome c. This continuously released cytochrome c further caused the incessant activation of the caspase-cascade (caspase 9 and 3) which eventually resulted in enhanced proximal tubular apoptotic cell-death. Concurrently, this unceasingly generated ROS also stimulated the simultaneous activation of the redox-sensitive inflammatory transcription-factor (NF-kβ) .This activated inflammatory regulator (NF-kβ) consequently induced the development of incessant inflammation and associated fibrosis via enhanced activation of pro-inflammatory and fibrogenic cytokines (IL-1β, TNF-α, TGF-β, IL-2 and IL-6). Therefore, persistent mitochondrial mediated apoptosis and enhanced inflammation on chronic dosing to high silica concentrations (≥100 mg/L) were established to be the major molecular mechanism of silica-induced nephrotoxicity in proximal tubular cells. This incessant tubular apoptotic cell-death and inflammation eventually culminates in the development of tubular-atrophy and fibrosis respectively at the organ-level. This prolonged tubular-atrophy and fibrosis eventually reduces the functional efficiency of the nephrons (owing to proximal-tubule majorly constituting the nephron), which ultimately culminates into irreparable renal-dysfunction that clinically manifests as CKDu.

This silica induced incessant tubular cell-death and inflammation eventually culminates in the development of tubular-atrophy and fibrosis (at the organ-level), which is characteristics of chronic tubulointerstitial nephritis, the major hallmark of CKDu pathogenesis **(Figure 4.10)**. This prolonged tubular-atrophy and fibrosis collectively reduces the nephron's functional efficiency (owing to proximal-tubule majorly constituting the nephron) over-time, which manifests into irreparable renal-dysfunction or renal failure (Nakagawa et al.,2015).These *in-vitro* mechanisms of silica induced nephrotoxicity (viz. incessant mitochondrial-mediated apoptotic cell-death and inflammation that culminates into tubular-atrophy and fibrosis) were consistent with major renal histo-pathological alterations i.e. tubular-atrophy and fibrosis that were noted previously during *in-vivo* silica toxicity animal studies (Dobbie and Smith,1982;Markovic and Arambasic, 1971), thus confirming continual mitochondrial apoptosis and inflammation triggered by intracellular-stressors (i.e. enhanced ROS and DNA-damage) being generated on elevated silica dosing (≥100 mg/L) to be the major molecular-mechanisms of silica induced nephrotoxicity. Moreover these silica induced nephrotoxicity mechanisms were consistent with those inflicted by well-known renal-toxins (i.e. heavy-metals); thus justifying nephrotoxic potential of silica in causation of CKDu (Erboga et al.,2016;Lentini et al., 2017).

4.4 Conclusion

This study elucidated the cellular and molecular mechanistic-aspects of dose and time-dependent nephrotoxicity inflicted by silica for the first-time using ideal *in-vitro* nephrotoxic assessment cell-model viz. normal human renal proximal-tubular cells (HK-cells).

Persistent mitochondrial-mediated apoptosis and inflammation induced by intracellular-stressors (viz. unwarranted ROS and DNA-damage) generated on high-silica dosing (≥100 mg/L) were established to be the major molecular-mechanisms of silica induced nephrotoxicity. This silica triggered prolonged apoptosis and inflammation *in-vitro* subsequently resulted in persistent proximal-tubular cell-death and fibrosis which on extrapolation to the organ-level (*in-vivo*) manifests in the development of tubular-atrophy and fibrosis (the major histo-pathological features of environmental nephrotoxin induced CKDu). These silica induced nephrotoxicity-mechanisms were found to be in consensus with those inflicted by well-known nephrotoxins (like heavy-metals) (Erboga et al., 2016; Lentini et al., 2017), thereby validating the potential of silica as a renal-toxin. Furthermore, our study also confirmed ≥100 mg/L to be the nephrotoxic silica-dose, which was capable of inducing severe renal proximal-tubular (HK-cells) toxicity and possibly CKDu development on long-

term exposure. This dosage's CKDu-inducing potential was established from its resemblance with the silica-levels in the drinking-groundwater (115.5 mg/L, please refer Table 3.1.5 of section 1, Chapter 3) and blood (100.2 mg/L, please refer Table 3.3.1 of section 3, Chapter 3) of the affected individuals that were responsible for the development of CKDu in an Indian taluka viz. Canacona taluka of south Goa (described in sections 1 and 3 of chapter 3 of this thesis and in our published work viz. Mascarenhas et al., 2017). Moreover, this same nephrotoxin exhibited a reversal of toxicity on lower sub-toxic dosing. This was justified from the absence of CKDu-incidence in other non-endemic regions of the Canacona taluka of Goa containing sub-toxic silica concentrations (below 100 mg/L) i.e. 13.5 mg/L in the potable-groundwater (please refer Table 3.1.5 of section 1, Chapter 3) and 30.7 mg/L in the blood (please refer Table 3.3.1 of section 3, Chapter 3) of the residing non-affected individuals (Mascarenhas et al., 2017). This non-toxicity could be possibly attributed to the faster toxin-clearing capacity (possibly mediated by metallothioneins) in conjunction with its uninhibited cellular-repair potency of these proximal-tubular cells at lower-doses that prevented the induction of nephrotoxicity. Thus, provided supporting evidence to the potential of this nephrotoxic silica-dose (100 mg/L) in renal-damage induction and associated CKDu development on chronic exposure. Overall, our findings suggest that the nephrotoxic potential of silica can be a primary considerant when deciphering the etiology of the environmentally-triggered CKDu problem in developing-countries, which are susceptible to anthropogenic influence. This investigation also highlights the need for adoption of relevant pre-emptive measures to reduce the silica nephrotoxin dosing and its triggered toxic-effects. It simultaneously encourages the need for designing appropriate therapeutic-cum-protective strategies (like anti-oxidant treatment) for guarding silica's sub-cellular target (viz. mitochondria, nucleus) (Rinaldi et al., 2017) to prevent the future rise in silica inflicted CKDu-occurrences; which could decrease the global burden of CKD.

Chapter 5

Development of a 'fluorimetric chemosensor' for 'detection of silica bioaccumulation' in human renal proximal tubular cells

5.1 Introduction

It has been well reported that silica possesses limited bioavailability owing to its high affinity for oxygen which categorizes it as a trace geogenic compound (Cornelis and Delvaux, 2016). The health perils such as pulmonary disorders (like silicosis and bronchitis) and chronic kidney disease specifically chronic tubulo-interstitial nephritis (CTN) (Vupputuri et al., 2012; Pollard, 2016) arises when silica emanates in the air and water as an outcome of various anthropogenic activities like granite mining, sand mining, acid mine drainage etc. Our previously reported comprehensive analysis of various environmental nephrotoxins in the groundwater (i.e. the major source of drinking water among the residents of the Canacona taluka) (described in sections 1 and 3 of Chapter 3) had established chronic silica exposure routed through contaminated groundwater consumption to be a potential causal factor responsible for the high incidence of CKDu in this region (Mascarenhas et al., 2017). A similar case scenario was noted in the Nellore district of Andhra Pradesh, India (Uddanam region) specifically the Uchapally village, India (Khandare et al., 2015) and Balkan region as well (Markovic; 1968; Markovic and Lebedev, 1965; Markovic and Arambasic. 1971; Radovanovic et al., 1991; Stiborova et al., 2016). Moreover limited epidemiological silica exposed case-control studies (Ghahramani, 2010; Millerick-May et al., 2015; Ricco et al., 2016) and in-vivo renal toxicity analyses in guinea pigs, mice and beagle dogs (Dobbie and Smith, 1982; Kawanabe et al., 1992; Markovic and Arambasic, 1971; Newberne & Wilson, 1970) had also stated that prolonged silica administration through drinking water significantly induces development of CTN in exposed subjects, the major hallmark of CKDu.

Hence the reported evidence had prompted us to scrutinize the existing literature for the form of silica that could potentially be responsible for this health hazard. On extensive literature search it was noted that silica possesses limited solubility in water with only the monomeric form of silica that is orthosilicic acid [H_4SiO_4 or $Si(OH)_4$] being water-soluble. This form of silica is commonly present in aqueous environments like natural water sources, groundwater, oceans and surface water when the water flows over the earth's crust, restricting its availability to below 20 mg/L of concentration. Orthosilicic acid being a monomeric form of silica was reported to be highly reactive and possesses the propensity for generation of cytotoxic H^+ ions when taken up by living systems such as diatoms. However the levels of these H^+ ions can be easily regulated under ideal cellular repair conditions. (Nishizono et al., 2004; Kucki and Fuhrmann-Lieker, 2012).In addition to being reactive, orthosilicic acid has also exhibited tremendous bioaccmulative capacity in these diatoms. However, on

anthropogenically induced increased and prolonged bioavailability of orthosilicic acid and subsequent uptake by these diatoms, this excessively accumulated orthosilicic acid can quickly turn from a boon into a curse. The reason being that these high levels of accumulated orthosilicic acid surpasses the super saturation point of polymerized silica gel formation and prefers to remain in the unpolymerised form. These large levels of undissociated orthosilicic acid are then capable of incessant generation of toxic H^+ ions and free radicals on prolonged exposure which exceeds the oxidative repair capacity of these diatoms which ultimately manifests in fatality (Nishizono et al., 2004; Kucki and Fuhrmann-Lieker, 2012).

This reported toxicity of excessively accumulated orthosilicic acid to diatoms over a long term exposure to high silica levels in their aqueous environments had prompted us to hypothesize that it could be this bioaacumulative water-soluble monomeric moiety of silica (i.e. orthosilicic acid) which could be responsible for the induction of severe nephrotoxicity, on exposure via the contaminated water route. This hypothesis was additionally backed by the findings from our *in-vitro* nephrotoxicity analyses of silica on proximal tubular HK cells (elucidated in Chapter 4), which had established the intrinsic mitochondrial apoptotic route to be the major pathway of proximal tubular cell-death adopted. It is well reported that this apoptotic route is primarily mediated by intracellular stressors like ROS, DNA damage etc. as evidenced in our *in-vitro* silica nephrotoxicity analyses (described in Chapter 4 of this thesis) as well, which is mainly generated from the intra-cellularly accumulated toxin (in this case-silica).This intrinsic apoptotic approach is contradictory to the extrinsic apoptotic pathway (also known as the plasma membrane localized death-receptor mediated apoptotic route) that is potentiated by extracellular stressors triggered from the external toxin or adjacent toxin-stressed cells (Che, 2014).Thus in order to provide supporting evidence to our discovery of mitochondrial apoptotic pathway (described in Chapter 4) triggered by intracellular toxin accumulation to be the principal pathway responsible for induction of proximal tubular cytotoxicity (Mascarenhas et al., 2018); we attempted to develop a biocompatible chemosensor, a prime requirement for toxicity analyses, for the quick detection of silica accumulation specifically orthosilicic acid within mammalian cells like human renal proximal tubular cells (HK cells) and possibly red blood cells. This sensor was developed in collaboration with Dr.Amrita Chatterjee's and Dr. Narendra Nath Ghosh's labs wherein the formerly mentioned labs assisted us in the partial synthesis and spectral cum fluorimetric characterization of the probe. Moreover since this thesis (sections 1 and 3 of Chapter 3) has established high silica exposure routed through contaminated groundwater consumption to be a potential etiological factor responsible for the high incidence of CKDu in Canacona taluka

(Mascarenhas et al.,2017), this sensor could possibly help in quick detection and of silica induced CKDu in this region.Thereby, allowing adoption of necessary treatment &preventive measure to reduce silica exposure possibly averting future rise in taluka's CKDu incidences.

In spite of silica's diverse toxicological potential, studies analysing cellular penetration and accumulation of silica in targeted mammalian cells (specifically human cells) on prolonged exposure, which is an essential prerequisite for toxicity assessments, are unavailable till date. Few reports have utilised labour intensive and expensive instrumentation like SEM coupled with X-ray analysis (Kumar et al.,2017b), X-ray fluorescence spectrometer for silica deposit visualisation in grasses (McLarnon et al.,2017) and diatoms (Tesson and Hildebrand, 2010). However, these techniques suffer from major limitations of longer data acquisition time, necessity of sophisticated and expensive instrumentation, requirement of trained personnel, tedious sample preparation(such as solubilisation or in-situ charring of cells to estimate the silica content),thereby negating the possibility of its application in live human cell imaging of silica species. Owing to its trace amounts, influences of coexisting substances in real samples, severe deleterious effects induced by bioaccumulated silica species and limitations of analytical techniques, development of sensitive, rapid on-site detection of toxic silica sp. (especially Orthosilicic acid) and imaging of silica contaminated live mammalian cells (specifically human renal proximal tubular cells or red blood cells) are necessary.

Fluorimetric techniques involving small organic molecules as chemo-reactants or chemo-dosimeters are well-validated methods for the detection of toxic analytes or even imaging of biological species due to high selectivity and sensitivity, instant signal transduction, low-cost instrumentation etc. (Ueno and Nagano, 2011; Terai and Nagano, 2013). In this context, rhodamine with high quantum yield is a vastly used fluorophore for molecular sensing of various analytes (Beija et al., 2009; Chan et al., 2012), which often sense the analytes by the opening of its non-fluorescent spirolactam ring to a highly fluorescent and coloured form (Kim et al., 2008). Some rhodamine derivatives have successfully utilized this principle in developing fluorimetric and colourimetric probes for the determination of pH of a solution (Best et al., 2010; Tian et al., 2012; Liu et al., 2014a); present method is based on the same principle. Relevant to this, a couple of rhodamine derivatives have also been used as simple dyes for the detection of silica deposition in diatoms based on the formation of orthosilicic acid via dissolution of silica in the aqueous intra-cellular environment (Brzezinski and Conley, 1994; Kucki and Fuhrmann-Lieker, 2012). However, use of some amount of organic solvents is inevitable to dissolve these organic dyes in the working solution. This is a serious concern for biotechnological applications as organic solvents can exaggerate severe toxicity

to biological systems restricting their application in live cells or any other living species. One of the possible ways to overcome this difficulty is the use of water-dispersible nanomaterials on which a chemodosimeter can be grafted by physical or chemical modifications.

Nanomaterials, many of which possess excellent water dispersibility, enormous surface area/ volume ratio, large adsorption capacity, great mechanical strength and surface reactivity, intrinsic optical/ electrochemical/ magnetic/ spectroscopic properties, have proved their efficacy in recent years as suitable candidates for development of material based sensors by selective interactions of their surface (pre-functionalized with signal transduction unit) with the analytes (Su et al.,2012; Rowland et al,2016).Often nanomaterial-based chemosensors outshine traditional detection techniques with decreased sampling time, quicker analytical response, high sensitivity and low cytotoxicity (Kumar et al., 2017a; Walekar et al., 2017). Some of the nanomaterials comprehensively used for biological and environmental assessment of lethal analytes include metal nanoparticles (e.g. Au, Ag nanoparticles), carbon materials (carbon nanotubes, and graphene), magnetic nanoparticles (Fe, Ni,Co), quantum dots (like ZnS, CdTe, CdSe) & metal-oxide nanomaterials (viz.TiO_2, CuO, ZnO) (Liu et al., 2014b; Yao et al.,2014). Of our interest,TiO_2 nanoparticles are good candidates to be used as matrix for sensing purpose owing to its unique properties including enhanced adsorptive surface area (Schneider et al.,2014), resistance to photochemical erosion (Chen et al.,2012), chemical & biological inertness (Rajh et al.,2014), low-cost synthesis, monodispersity (Keller et al.,2010), and nontoxicity(Ji et al.,2010). Moreover, excellent biocompatibility (Zhao et al.,2014) and easy cellular uptake on dispersion in aqueous media (Taurozzi et al.,2013) extends its potential application for live-cell imaging of intracellular toxic silica sp. deposits.

Herein, we report, a water-dispersible ‘‘turn-on’’ fluorimetric sensing material comprising rhodamine-based chemodosimeter adsorbed on TiO_2 nanoparticles for the detection and imaging of toxic silica species (viz. orthosilicic acid) bioaccumulation in an ideal in-vitro nephrotoxicity cellular-model viz. human renal proximal tubular HK-cells. We chose rhodamine hydrazide (Rh1) as the chemodosimeter unit, which was first reported by Dujols et al (1997) as a sensor for Cu (II) ions in water and later used by others (Yang et al.,2002; Xiang et al.,2007; Zhang et al.,2011; Asano et al., 2014; Kang et al.,2015) for various sensing and imaging studies. It was presumed that acidic environment generated inside an intrinsically neutral cell due to incessant release of H^+ ions from largely accumulated orthosilicic acid (on chronic exposure to high silica concentrations in the water) would induce spirolactam ring-opening and transduce fluorescence (Kim et al., 2008; Zhang et al., 2011). To demonstrate our concept silica affected nephrotoxic kidney cells were chosen.

5.2 Material and Methods

5.2.1 Chemicals

Rhodamine B. HCl and Hydrazine hydrate were purchased from Sigma Aldrich (India) and used as received. $TiCl_3$ was purchased from Spectrochem Pvt. Ltd. Mumbai (India). Ethylene diamine tetra acetic acid (EDTA) was purchased from Merck, India. All solvents and other chemicals were of AR grade and were procured from different commercial suppliers and used without further purification. Millipore water (18 MΩ) was used for all spectroscopic studies.

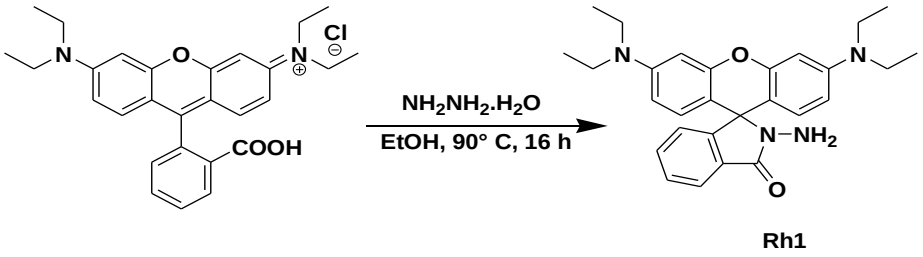
5.2.2 Instruments and Measurements

NMR spectra were recorded on Bruker Avance (400 MHz) NMR spectrometer. Mass spectra were obtained from Agilent technologies 6460 triple quard LC-MS (ESI). Fluorescence spectra were measured on a JASCO FP-6300 spectrofluorometer, the slit width was 2.5 nm for both excitation and emission. Absorption spectra were recorded on a JASCO V570 UV/ Vis/NIR spectrophotometer. Infra-Red spectra were taken on IR Affinity-1 FTIR spectrophotometer, Shimadzu. Elemental analysis was carried out on Vario elementar CHNS analyzer. Thermogravimetric analysis (TGA) data were recorded using a DTG-60, Shimadzu. Field Emission Scanning Electron Microscopy (FESEM) images of the TiO_2 nanoparticles were obtained on FESEM Quanta 250 FEG (FEI) with 10 kV and resolution 2 nm. The powder X-ray diffraction (XRD) patterns were obtained from powder X-Ray diffractometer (Mini Flex II, Rigaku, Japan).

5.2.3 Synthesis of materials

5.2.3.1 Preparation of Rhodamine hydrazide (Rh1)

The chemodosimeter, Rh1 was prepared according to a reported method (Yang et al., 2002; Wang et al., 2012) **(Scheme 5.1)**. In a 100 ml round bottom flask, Rhodamine B (1.0 g, 2.08 mM) was dissolved in 15 ml absolute alcohol, to which excess of Hydrazine hydrate (98%, 2.08 g, 4.16 mM) was added under stirring at room temperature. The reaction mixture was then refluxed for 16 h at 110 °C. After completion of the reaction, 10 ml water was added and the residue was filtered, washed with water (3 × 10 mL), and then air-dried. The crude product was recrystallized from the ethanol-water mixture to obtain the sufficiently pure product, Rh1 (650 mg, 67.5 % yield).



Scheme 5.1. Synthesis of Rhodamine B hydrazide (Rh1).

5.2.3.2 Preparation of TiO_2 nanoparticles

TiO_2 nanoparticles were prepared using an EDTA precursor based method, which was developed by Ghosh and coworkers (Naik et al., 2013). In a 250 mL glass beaker, 21.1 mL of $TiCl_3$ (192.6 mM) was taken, and then an aqueous solution of HNO_3 was added to it dropwise to oxidized Ti^{3+} to Ti^{4+}. Separately, in a beaker aqueous solution of EDTA (10% w/v) was prepared by dissolving EDTA in hot water with dropwise addition of dilute NH_4OH solution. After complete dissolution of EDTA, the solution was boiled to remove excess NH_3. This EDTA solution was then added to a solution containing Ti^{4+} ions (keeping Ti^{4+} : EDTA molar ratio 1:1) with constant stirring. The reaction mixture was evaporated to dryness over a hot plate at 125 °C to obtain the precursor powder for TiO_2. Finally, the precursor was calcined at 550 °C for 3 h to obtain TiO_2 nanoparticles with an average diameter of ~ 35 nm.

5.2.3.3 Preparation of the sensing material, Rh1@TiO_2

For the grafting of Rh1 onto the TiO_2 nanoparticles, 100 mg of sonicated TiO_2 nanoparticles was added to Rh1 (10 mg, 0.022 mM) in dry acetonitrile solution (100 mL) and magnetically stirred in the dark for 60 min to ensure it reaches adsorption-desorption equilibrium (Spada et al., 2017). The Rh1 adsorbed TiO_2 nanoparticles (Rh1@TiO_2) were collected by centrifugation at 2500 rpm for 5 min followed by washing the residue with acetonitrile (2 x 25 mL) and dried in a vacuum desiccator (in the dark) for 4 h to obtain a white solid (110 mg, 100% yield) as the final sensing material.

5.2.4 The general procedure of sample preparation for fluorescence measurement

10 mg of the probe Rh1@TiO_2 was dispersed in 10 mL of deionized water (MilliQ, 18 MΩ) to get a 1 g/L of the stock solution as and when required and diluted further as per requirement. 1g/L (10 mM) stock solution of orthosilicic acid was prepared in deionized water and diluted further for the preparation of working solutions. To analyse the effect of different metal ions stock solutions (10 mM, 5 mL) were prepared by dissolving their respective nitrates/ chlorides in deionized water. All analyte solutions were subjected to 0.22

μm syringe filtration to avoid any interference by particulate matter in fluorescence measurement. After analyte addition, each solution was incubated for 10 min before recording their respective fluorescence spectrum at an excitation wavelength of 525 nm and the emission was recorded from 526 to 650 nm. All the experiments were executed at room temperature. For the equivalence study, orthosilicic acid doses were altered from 0–100 μL of 10 mM (or 0.0-33.3 mg/L) for fluorescence measurement.

5.2.5 General procedure for fluorescence study

The fluorescence study was carried out by addition of 30 μL of the sensing material, Rh1@TiO_2 (1g/L stock solution) in deionized water (3 mL) in a cuvette. To this solution, increasing amount of Orthosilicic acid (1g/L stock solution) were added and fluorescence response was measured after every 10 min, respectively. For equivalent study concentrations of Orthosilicic acid were changed from 0- 33.3 mg/L (i.e. 0-105 μL from stock solution). For each reaction mixture fluorescence response was recorded. The same procedure was followed for comparative study with other metal ions.

5.2.6 Procedure for pH dependency study

For preparation of acidic pH solutions, sodium acetate in the acetic acid buffer and for basic pH solutions phosphate buffer was used. pH dependency study was carried out by addition of 30 μL of Rh1@TiO_2 sensing material (from 1g/L stock) in 3 mL of each individual pH buffer (ranging from 3- 10) in a cuvette and fluorescence response was recorded. This was followed by addition of 30 μL of Orthosilicic acid (1g/L) to each individual pH buffer solution containing Rh1@TiO_2 sensor &fluorescence intensity was recorded after10min incubation.

5.2.7 Cytotoxicity study

Cytotoxicity of Rh1@TiO_2 was tested using the colorimetric MTT assay (Adan et al., 2016). HK cells (Human Kidney cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C and humidified 5% CO_2. 5 x 10^4cells were plated per well in a 24-well plate. Rh1@TiO_2 (1 g/L) was dissolved in aqueous PBS (pH 7) to make a stock solution. Following 24 h incubation, the cells were dosed with increasing concentrations of the sensing material, Rh1@TiO_2 (1, 10, 50, 100, and 200 mg/L) formulated by serial dilution in DMEM. Unexposed controls were maintained. After additional 24 h incubation, 50 μL of MTT (5 mg/mL in PBS) was added per well and incubated for 4 h. Following which the media was aspirated from the wells, 0.5 mL of DMSO

was added. Absorbance was recorded at 570 nm and cell viability was expressed as a percentage of relative absorbance of the sample vs. unexposed control cells for every probe concentration. Each set of experiments were triplicated and the average results are presented.

5.2.8 Fluorescence imaging of toxic silica species in living cells

For fluorescence imaging of silica (i.e.orthosilicic acid) accumulation in living cells, HK cells were chosen. HK cells were plated in 6-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. The cells were then incubated with increasing silica doses (0.1, 1, 10 and 100 mg/L) for 3 different time points (1 h, 24 h, and 8 days), followed by treatment with the sensing material (100 µL of 1 g/L of stock solution was added to 2 mL culture medium) at 37 °C and 5% CO_2 for 30 min. Fluorescence images were captured by a fluorescence microscope (Olympus IX51) under a TRITC filter and reported. Each experimental study was triplicated ensuring data reproducibility.

5.3 Results and Discussion

5.3.1 Characterizations of the synthesized Rh1, TiO_2 nanoparticles, and Rh1@TiO_2

The synthesized materials were characterized by using several instrumental techniques, such as ^{1}H NMR, ^{13}C NMR, ESI-MS, IR, CHN, XRD, TGA, and FESEM. Rh1 was characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS, IR and CHN. The ^{1}H and ^{13}C NMR spectra of the Rh1 are shown in **Figure 5.1 and Figure 5.2,** respectively and obtained data are in well-agreement with reported values of Rh1 (Dujols et al.,1997; Wang et al.,2012). Mass spectroscopy analysis (*m/z*= 457 $[M + H]^+$) and CHN analysis (Calculated C,H and N amount for $C_{28}H_{32}N_4O_2$: C:73.66; H: 7.06; N: 12.27 and data obtained from CHN analysis: C: 73.59; H: 6.99; N: 12.19) of the product also confirmed the formation of Rh1 (molecular formula $C_{28}H_{32}N_4O_2$). The crystalline phase and crystallite size of the synthesized TiO_2 were determined by using XRD. XRD pattern of the synthesized TiO_2 nanoparticles **(Figure 5.3)** showed the presence of peaks at 2θ = 25.3°, 37.8°, 48.1°, 53.9°, and 55.06°, which are corresponding to the (101), (004), (200), (105) and (211) planes of anatase phase [JCPDS Card no. 21-1272], and confirmed the formation of pure anastase phase of TiO_2. The crystallite size of the synthesized TiO_2 nanoparticles (calculated by using Scherrer equation) was found to be ~38 nm.

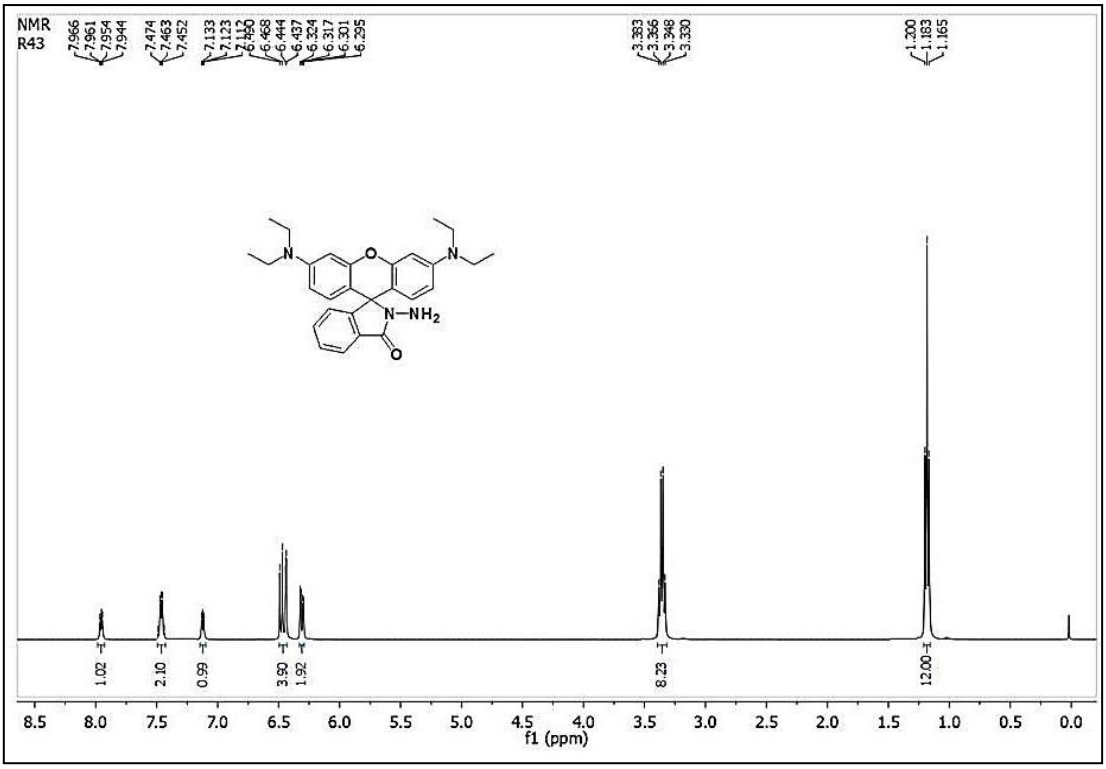


Figure 5.1. ^{1}H NMR spectra of Rh1 (^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.18 (12 H, t, *J* = 6.8 Hz), 3.36 (8 H, q, *J* = 7.2 Hz), 6.32 (2 H, dd, J_1 = 2.8 Hz, J_2 = 8.8 Hz), 6.44-6.49 (4 H, m), 7.11-7.13 (1 H, m), 7.44-7.49 (2 H, m), 7.93-7.98 (1 H, m)).

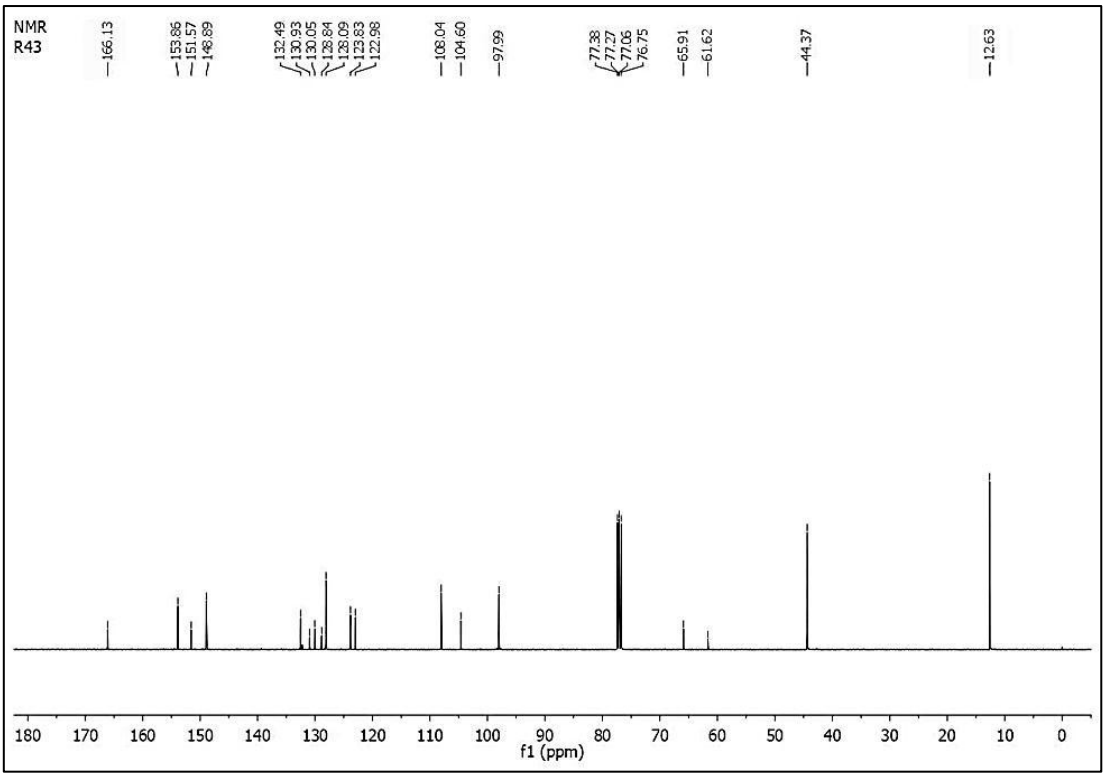


Figure 5.2. ^{13}C NMR spectra of Rh1 (^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 12.62, 44.37, 65.91, 97.98, 104.62, 108.03, 122.27, 123.83, 128.08, 128.84, 130.05, 130.93, 132.49, 148.88, 151.56, 153.85, 166.13)

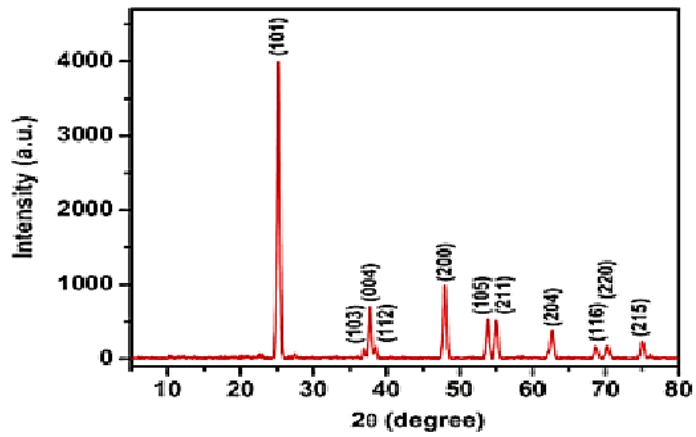


Figure 5.3. XRD spectra of the synthesized TiO_2 nanoparticles.

The sensing material, Rh1@TiO_2 was prepared by grafting TiO_2 nanoparticles with Rh1. The thermogravimetric analysis (TGA) was utilized to determine the amount of Rh1 in Rh1@TiO_2 (**Figure 5.4**). TGA thermograms showed that pure Rh1 was fully decomposed within temperature range of 600 °C, whereas TiO_2 nanoparticles (NP) were quite stable in this temperature range. In case of Rh1@TiO_2, 8% weight loss was observed when sample was heated to 600 °C, which indicated that Rh1@TiO_2 is composed of 8 wt% Rh1& 92 wt% of TiO_2.This is in agreement with ratio of probe, Rh1& TiO_2 used during synthesis (1:10), thus concluding that approx.90% of Rh1 successfully adhered onto TiO_2 NP surface.

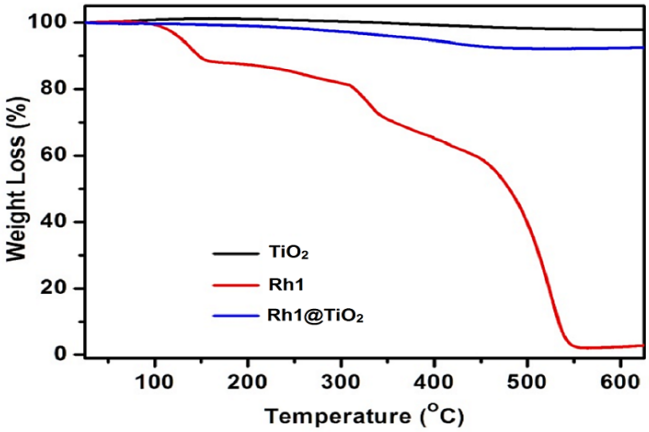


Figure 5.4. TGA curves of TiO_2 nanoparticles (black line), probe Rh1 (red line) and sensing material Rh1@TiO_2 (blue line).

In the IR spectra of Rh1@TiO_2 (**Figure 5.5**) the presence of the signature peaks of Rh1 (IR bands at 3340, 3082, 2969, 1774, 1618, 1519, 1272, 1115 cm^{-1}) also indicated the physisorption of Rh1 on TiO_2.SEM micrographs revealed that the diameter of TiO_2 nanoparticles was in the range of 25- 6 nm, with an average of ~ 35 nm (**Figure 5.6 A**). SEM images of Rh1@TiO_2 also indicated that TiO_2 nanoparticles retain their original surface morphology after modification with fluorophore unit (Rh1) (**Figure 5.6 C**).

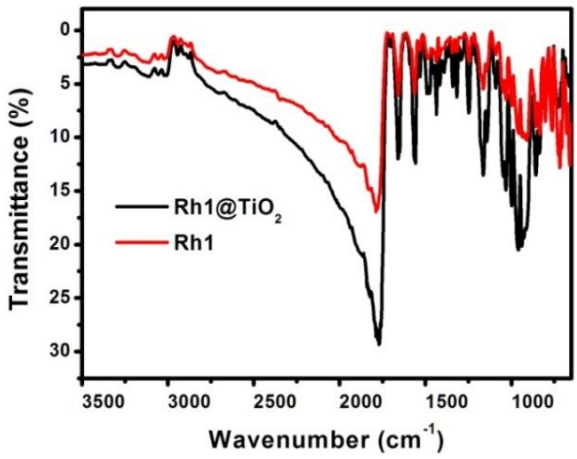


Figure 5.5. IR spectra of Rh1 and the sensing material, Rh1@TiO_2

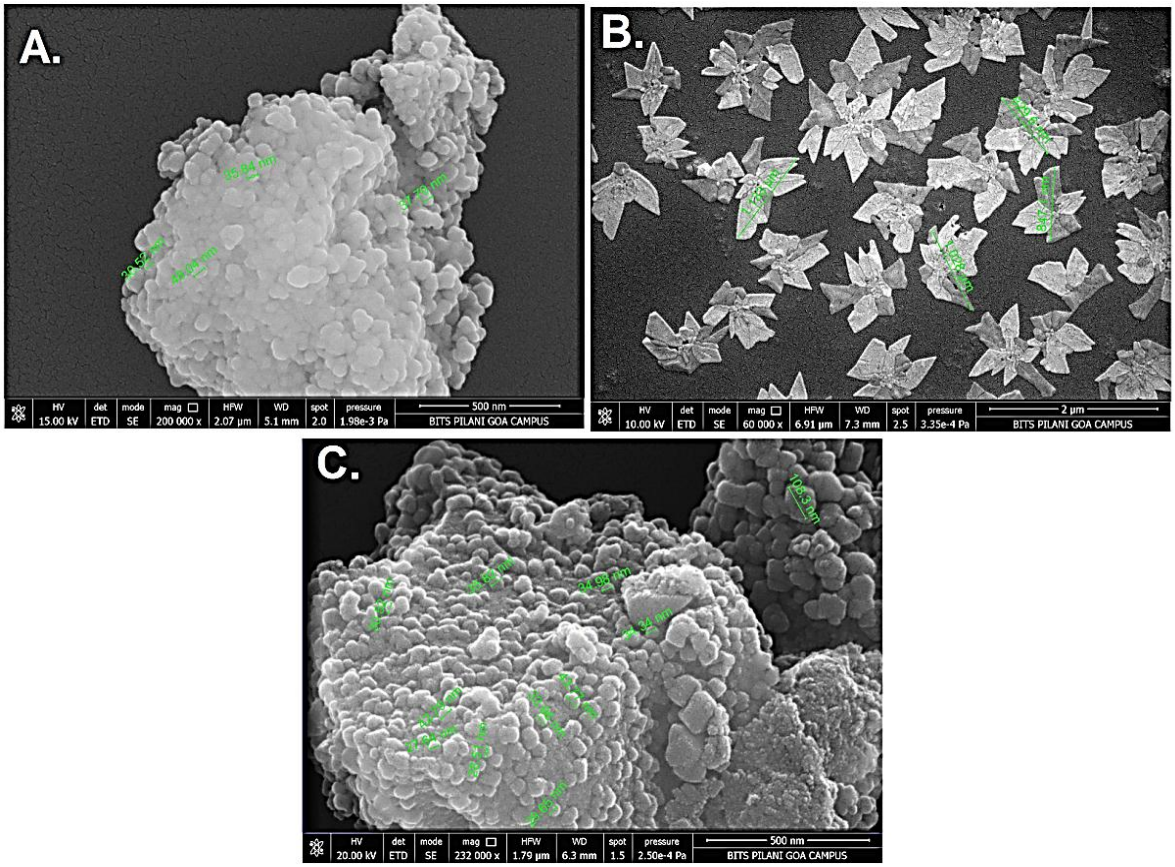


Figure 5.6. FESEM images of (A) anatase TiO_2 nanoparticles; (B) free Rh1 and (C) the sensing material, Rh1@TiO_2.

5.3.2 Effect of pH on the fluorescence response of the sensing material (Rh1@TiO_2)

It was conceived that the fluorophore, Rh1, would respond to acidic pH by going to its ring-opened form. The response of Rh1 at different pH was carried out in CH_3CN-H_2O and the fluorescence response was noted. As the sensing material can be dispersed in water only, the pH dependency study of the sensing material (Rh1@TiO_2) was carried out in water and buffer solutions, and pH was maintained by the addition of acid or base. In the present study,

the chosen pH range was 3.0- 10.0.The fluorimetric response was negligible at neutral and basic pH, but soared up in the acidic pH. The highest response was seen at pH 5(**Figure 5.7**).

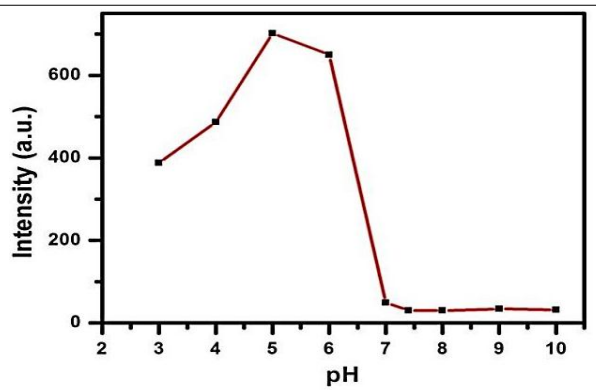


Figure 5.7. The effect of pH on fluorescence response of Rh1 probe at different pH in 20% CH_3CN-H_2O

The response of Rh1@TiO_2 towards Orthosilicic acid was also measured at the same pH range. Strongly alkaline pH was avoided as basic metal silicates precipitate out at this condition (Hermosilla et al., 2012; Milne et al., 2014). The strong acidic condition was also avoided as cells cannot bear such harsh condition. **Figure 5.8** shows fluorescence responses of Rh1@TiO_2 as a function of pH in presence and absence of Orthosilicic acid. As expected, Rh1@TiO_2 started emitting strong fluorescence signals as the pH of the solution went below 7. The fluorescence output showed steady rise up to pH 3 (excited at 525 nm). This was well-expected, considering the possibility of protonation of the spirolactam ring of Rh1 which leads to the formation of the fluorescent ring-open state in Rh1 under acidic pH. However, the sensing material, Rh1@TiO_2 showed very weakly to negligible fluorescence response at pH ≥ 7, suggesting that the spirocyclic form of the probe molecule remained intact at this pH condition. The pH-controlled emission measurements established that Rh1 and Rh1@TiO_2 could be applied for the detection of the incessant release of H^+ from a hydrated form of silica (viz. Orthosilicic acid). Considering the fact that the detection of silica species bioaccumulation *in-vitro* or *in-vivo* would require cellular pH, the media for fluorometric detection of Orthosilicic acid was set at pH 7.0 for further studies.

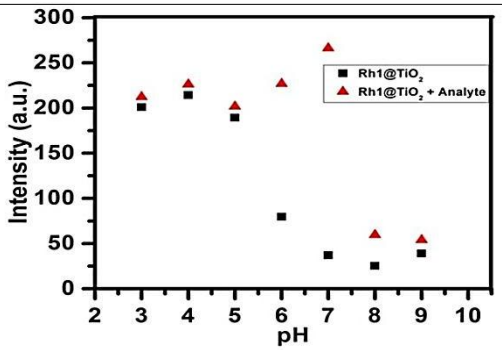


Figure 5.8. The fluorescence response of Rh1@TiO_2 sensing system (10 mg/L) after 10 min with &without silicic acid(10 mg/L, 1:1 ratio) in different pH buffers (pH 3.0–10.0) at room temperature (λ_{ex} = 525 nm).

5.3.3 Spectrofluorometric & spectrophotometric titrations of Orthosilicic acid by Rh1@TiO_2

To comprehend the toxic silica species (viz. Orthosilicic acid) sensing abilities of Rh1@TiO_2, fluorescence response of an aqueous dispersion of sensing material upon steady addition of 0– 100 µL of 10 mM (0.0-33.3 mg/L) of Orthosilicic acid was measured. The fluorescence output of fluorophore unit (Rh1) at λ_{max}=585 nm was intensified with incremental addition of Orthosilicic acid, up to 12 fold, demonstrating quick recognition efficacy (**Figure 5.9**). This is an upshot of transformation of non-fluorescent spirolactam form of Rh1 in the sensor to its ring-opened fluorescent state on reacting with H^+ ions released by Orthosilicic acid. A time-dependent fluorescence measurement of an equimolar mixture of Rh1@TiO_2 & Orthosilicic acid (both 10 mg/L) revealed that 10 min of incubation obtained highest fluorescence response suggesting a quick conversion to spirolactam form in the presence of H^+ ions.

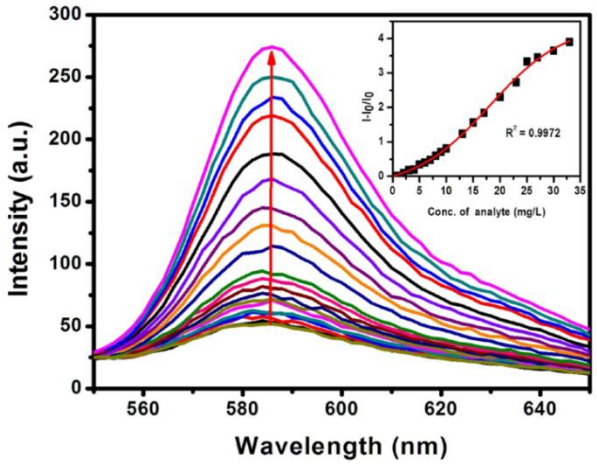


Figure 5.9. Fluorescence response of Rh1@TiO_2 sensing system (10 mg/L) upon addition of Orthosilicic acid (0.0-33.3 mg/L) in deionized water at room temp. after 10 min [excitation at λ_{max} = 525 nm]. Inset: plot of increment in emission against the concentration of the analyte.

Lines represent a unit increase in fluorescence intensity of Rh1@TiO2 sensor (10 mg/L) upon unit concentration addition (i.e.1.6 mg/L) of orthosilicic acid (from 0 to 33.3 mg/L)

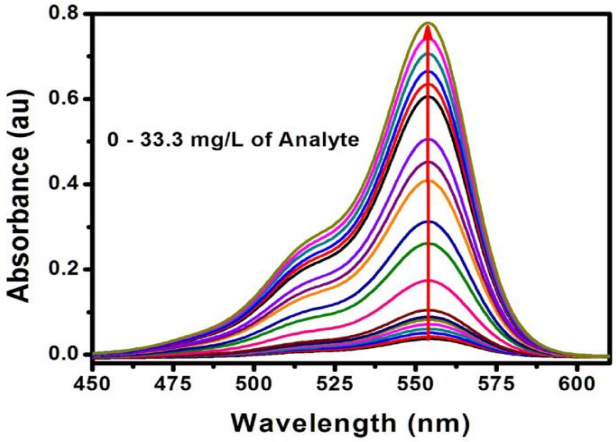


Figure 5.10. Absorbance response of the Rh1@TiO_2 sensing system (10 mg/L) after 10 mins of the addition of Orthosilicic acid (0.0-33.3 mg/L) in deionized water at room temperature.

Lines represent a unit increase in absorbance of Rh1@TiO2 sensor (10 mg/L) upon unit concentration addition (i.e.1.6 mg/L) of orthosilicic acid (from 0 to 33.3 mg/L)

In a similar study, the chromogenic response of the Rh1@TiO_2 towards Orthosilicic acid was investigated by the absorbance measurements of an aqueous probe solution on steady addition of 0–100 µL of 10 mM solution of Orthosilicic acid (or 0.0-33.3 mg/L). The absorbance signal peak, at λ_{max}= 558 nm, was found to be intensified with increasing Orthosilicic acid doses in solution (**Figure 5.10**). Both the investigations strongly portrayed the efficacy of the probe in H^+ ion detection (from Orthosilicic acid) in aqueous solutions.

5.3.4 Mechanistic aspects of silica species sensing by Rh1@TiO_2 and spectral features

The spirolactam form of Rhodamine derivatives (viz. Rh1 and sensing material, Rh1@TiO_2) is non-fluorescent and colourless at neutral pH.However, binding with H^+ ions (from Orthosilicic acid) induces a strong orange fluorescence peak and an intense pink colour due to spontaneous conversion of the spirolactam form of Rhodamine residue to its ring-opened form, Rh2 (**Scheme 5.2)** (Ki et al., 2008;Pandurangappa and Kumar, 2011). This phenomenon is justified by appearance of an anticipated peak of the ring-opened form of Rhodamine B hydrazide at 586 nm in the emission spectra.The formation of ring-opened form (Rh2) was confirmed by carrying out a reaction of Rh1 in presence of Orthosilicic acid at a larger scale and taking IR and NMR of the isolated product and matching with reported data (Pandurangappa and Kumar, 2011).

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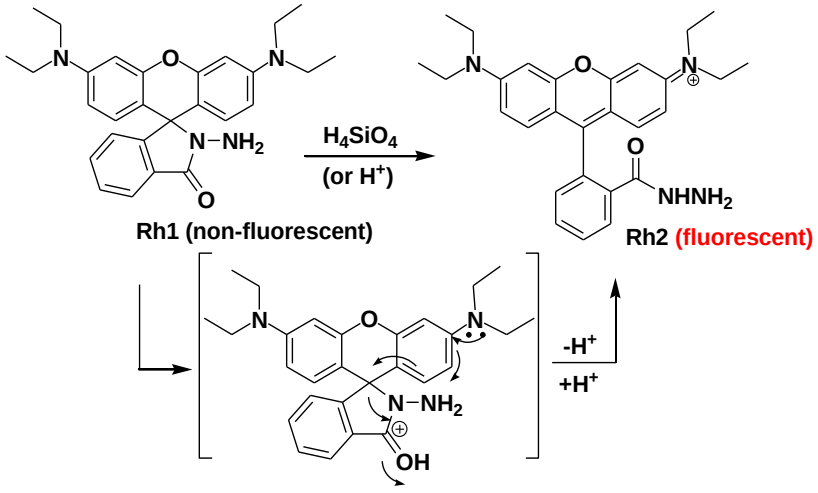

Scheme 5.2. A plausible mechanism of the H^+ mediated ring opening of Rh1 in the process of detecting orthosilicic acid.

5.3.5 Fluorescence response at different time intervals

The probe Rh1 was previously reported by Czarnik (Dujols et al., 1997) for Cu^{2+} and Chang (Kim et al., 2008) for Hg^{2+} detection. However, we observed that the sensing material ($Rh1@TiO_2$) hardly shows any fluorimetric response in the presence of Cu^{2+} and Hg^{2+} ions under the sensing condition for Orthosilicic acid (refer to **Figure 5.14**).

These observations insisted us to carry out a time-dependent study for both Cu^{2+} and Hg^{2+} ions in water. In a separate study, we have repeated the studies of Czarnik (Dujols et al.,1997) and Chang (Kim et al., 2008).In the present study, free probe, i.e.Rh1, was separately treated with Cu^{2+}, Hg^{2+}, Fe^{3+},Zn^{2+} and H_4SiO_4 in 20% CH_3CN-HEPES buffer (at pH 7) and 10% CH_3OH-phosphate buffer (at pH 7) and the fluorescence response was noted after 30 min, 2 h, and 6 h. It was observed that Rh1 is a very effective sensor for Cu^{2+} in 20% CH_3CN-HEPES buffer with the highest response after 30 min (**Figure 5.11 A**).However, Hg^{2+} started to show a response after 2 h and surpasses Cu^{2+} after 6 h (**Figure 5.11 B and C**).

In 10% CH_3OH-phosphate buffer Hg^{2+} showed a strong response, whereas other metal ions showed negligible response (**Figure 5.12**). In both cases, H_4SiO_4 did not show any response as H^+ availability was restricted by buffer medium.

In a separate study, the sensing material, $Rh1@TiO_2$ was separately treated with Cu^{2+}, Hg^{2+}, and H_4SiO_4 in water and the fluorescence response was noted after 10 min, 30 min, and 6 h (**Figure 5.13**). While H_4SiO_4 started to show strong response immediately after 10 min, the fluorimetric response of Hg^{2+} picked up only after 6 h of exposure (**Figure 5.13C**). Presumably, approach of Hg^{2+} ions was somewhat restricted inside the core of sensing material ($Rh1@TiO_2$) and thus, the chemodosimeter (Rh1) does not get quick access to these cations to react with. This resulted in a very low fluorometric response in water after 10 min.

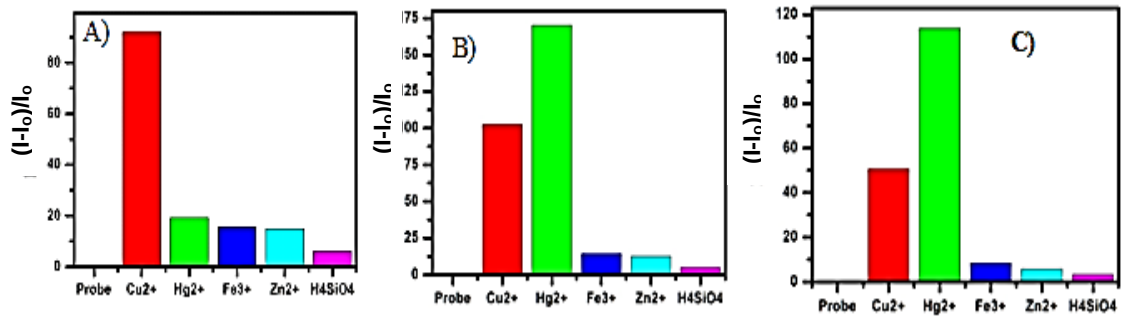

Figure 5.11. Time-dependent study of Cu^{2+}, Hg^{2+}, H_4SiO_4 and other metal ions against Rh1 after (A) 30 min, (B) 2h and (C) 6 h in 20% CH_3CN-HEPES buffer (at pH 7).

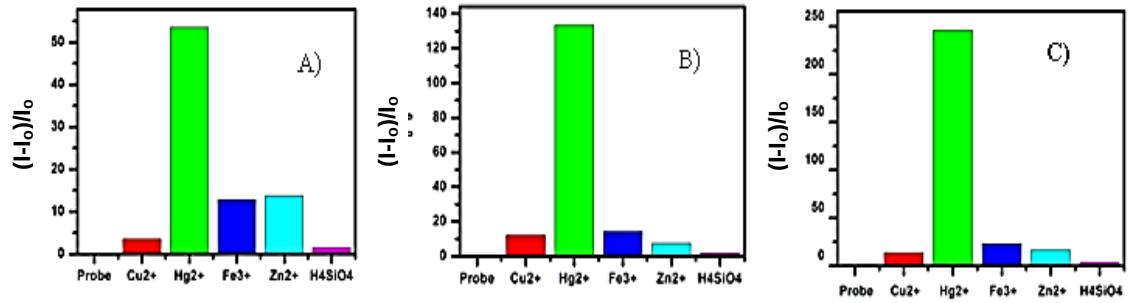

Figure 5.12. Time-dependent study of Cu^{2+}, Hg^{2+}, H_4SiO_4 and other metal ions against Rh1 after (A) 30 min, (B) 2h and (C) 6 h in 10% CH_3OH-Phosphate buffer (at pH 7).

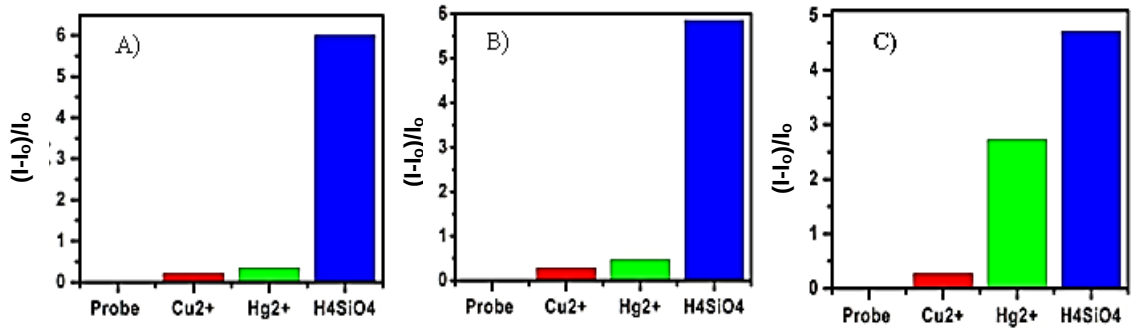

Figure 5.13. Time-dependent study of Cu^{2+}, Hg^{2+} and H_4SiO_4 and other metal ions against Rh1@TiO_2 after (A) 10 min, (B) 30 min, (C) 6 h in water.

5.3.6 Selectivity study with interfering metal ions

Kidneys, specifically the proximal tubular cells are actively involved in maintaining a balance of major elements in the body viz. Na^+, K^+, Mg^{2+} and trace elements, like Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+} ions, and thereby inherently contain these cationic species which are essential for various cellular functions, such as heart and muscle contractions, nerve signaling, hormone production, anti-oxidative enzyme action, hemoglobin formation, glucose homeostasis, and reno-protective effects (Agarwal et al., 2011; Gupta and Gupta et al., 2014). These organs additionally serve as the primary target of metal toxicity owing to its high reabsorptive and accumulative properties. Some of the metal species are well-reported to inflict severe nephrotoxicity when surpassing the permissible levels of Cd^{2+}, Hg^{2+}, Pb^{2+} (Orr and Bridges.,

2017; Wilk et al., 2017). The specificity of Rh1@TiO_2 sensing material for a toxic hydrated form of silica (viz. orthosilicic acid) in an aqueous solution was examined analogously in the presence of various competing toxic and inherent renal metal ions under comparable conditions.For this purpose, fluorescence responses of Rh1@TiO_2 after addition of equal mixture (10 mg/L) of each metal ion (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{+2}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Na^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Sr^{3+}, Zn^{2+}) and orthosilicic acid (H_4SiO_4 which acts as a source of H^{+}) in the aqueous dispersion of sensing material(10 mg/L) were recorded(**Figure 5.14**).

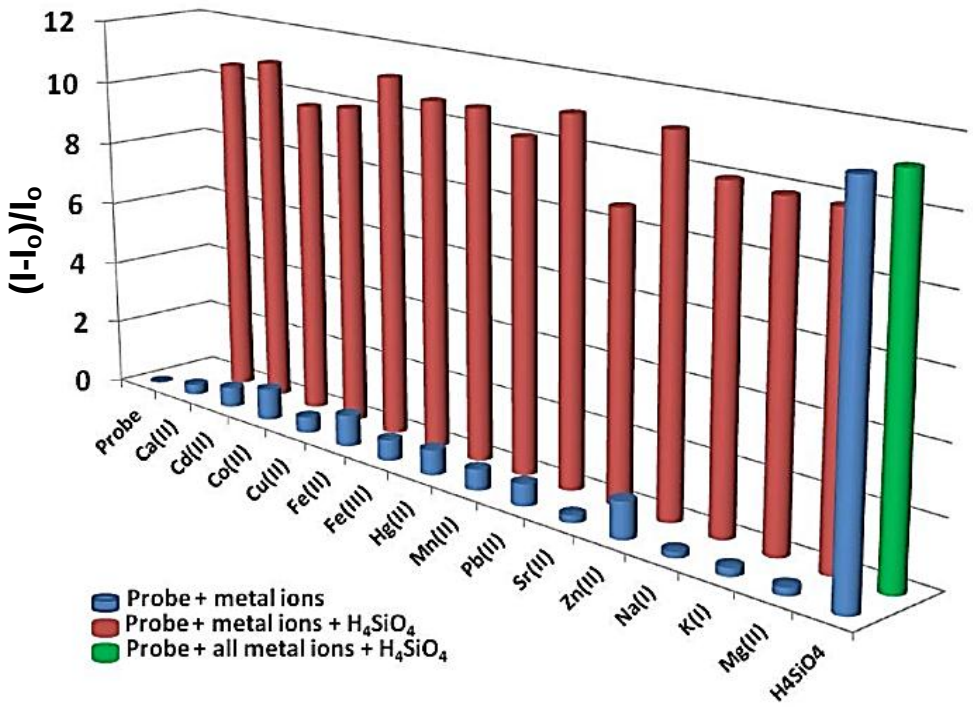

Figure 5.14. Maximum fluorescence responses of Rh1@TiO_2 (10 mg/L), which were recorded after 10 min of the addition of equal amount of various analytes (metal ions), in deionized water at room temperature.

As represented in **Figure 5.14**, the sensing material, Rh1@TiO_2 exhibited negligible to no response for most of the potential rival metal ions and generated a strong fluorescence response upon interaction with orthosilicic acid at the working pH. Notably, low response aroused out of interaction with Hg^{2+}, Zn^{2+}, Fe^{2+} or Co^{2+} under the sensing condition. This is in sheer contrast with the reported results by Czarnik (Dujols et al., 1997) for Cu^{2+} and Chang (Kim et al., 2008) for Hg^{2+} with Rh1. We repeated their studies and got similar results as per their reports for Rh1 when it is free. However, in a separate study, it was observed that Rh1@TiO_2 responds to Hg^{2+}after long exposure in water (**Figure 5.13**). Presumably, the approach of larger metal ions is somewhat restricted inside the core of the sensing material (Rh1@TiO_2) and therefore, the chemodosimeter (Rh1) does not get quick access to these cations to react with. This results in very low fluorometric response at pH 7 after 10 min. The

fluorescence output in the presence of these cations is still very nominal under the sensing condition making the probe practically unperturbed by the presence of the interfering metal ions. This fact is further established through competition experiments conducted in the presence of large excess (5 times of orthosilicic acid) of all metal ions and orthosilicic acid (**Figure 5.14**). As anticipated, the orthosilicic acid induced fluorescence response of the sensing material, Rh1@TiO_2 was unaltered by the existence of the competing metal ions, suggesting that the presence of H^+ ions amidst several other cations is univocally identified by the sensing material with no loss in fluorescence output. The response of the sensing material towards orthosilicic acid amongst diverse nephrotoxic and inherent renal metallic species in an aqueous solution extends its application for selective *in-vitro* detection of toxic silica species accumulation in affected kidney cells or elsewhere.

5.3.7 Determination of the detection limit

The limit of detection (LOD) is an essential property of a sensing system, assessed to gauge its utility for real sample analysis. To elucidate the LOD, the fluorescence output of Rh1@TiO_2 at lower concentrations of orthosilicic acid in deionised water was plotted (**Figure 5.15**).Under prevailing conditions, sensing material,Rh1@TiO_2 responds linearly to varying concentration of orthosilicic acid (0.1-1mg/L or 0.3–3.0µL in 3 mL) with $R^2 = 0.9973$ and from this, detection limit was calculated as 8.4 ppb or 8.4 x10^{-8}M of orthosilicic acid.

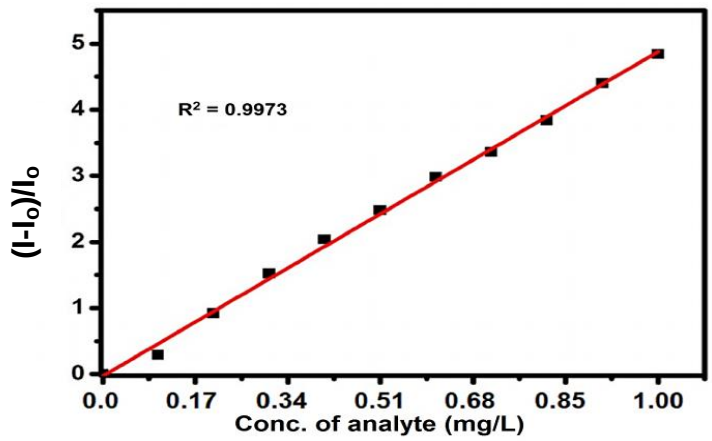

Figure 5.15. A plot of relative fluorescence intensity vs. concentration of analyte obtained from a solution of sensing material Rh1@TiO_2 (10 mg/L) and lower concentration range of orthosilicic acid (0.1-1 mg/L) after 10 min of interaction.

5.3.8 Determination of Cytotoxicity of Rh1@TiO_2 and fluorescence imaging of toxic silica species in live cells

If a sensing system is intended to apply to live biological species, it is essential to check its biocompatibility and cytotoxicity. As an ideal in-vitro nephrotoxic cellular model system,

human kidney proximal tubular cells viz. HK cells (Human Kidney cells) (Li et al., 2017) was chosen for cytotoxicity study and detection of the toxic silica sp. (specifically orthosilicic acid) accumulation via cellular imaging. Accordingly, the cytotoxicity of different concentrations of Rh1@TiO_2 to HK cells was assessed using MTT assay (Adan et al., 2016) to determine cell sustainability at an ideal probe concentration. For this, 70% confluent HK cells were exposed to enhancing Rh1@TiO_2 sensing system concentrations, serially diluted in DMEM (i.e. 1, 10, 50, 100, and 200 mg/L) in a 24 well plate, and incubated for 24 h. Unexposed control was simultaneously maintained. The percentages of viable cells relative to untreated controls were determined and plotted (**Figure 5.16**). The cell viability was ascertained to be greater than 90% on 24 h exposure to the highest Rh1@TiO_2 concentration (200 mg/L). This suggests that even high Rh1@TiO_2 concentrations are non-toxic to the living human cells and can be employed for detection of toxic silica species viz. orthosilicic acid bioaccumulation in living organisms.

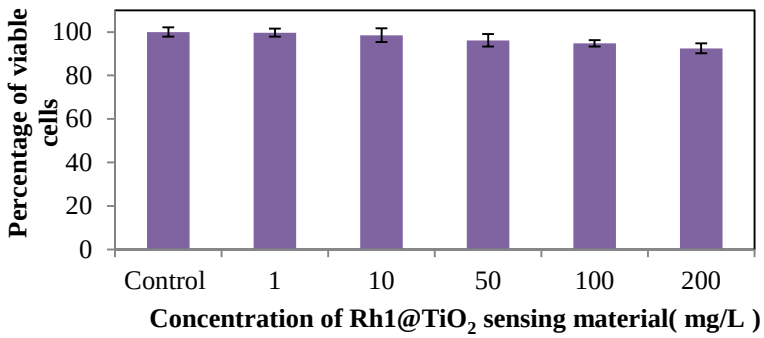

Figure 5.16. Cytotoxicity study of the sensing material, Rh1@TiO_2 on HK cells at varying doses for 24h.

Since no cytotoxic effect of Rh1@TiO_2 was detected, a median concentration of Rh1@TiO_2 (i.e., 50 mg/L) was employed for fluorescence monitoring of orthosilicic acid deposition in HK cells, which were exposed to increasing doses of orthosilicic acid. The orthosilicic acid deposition over the 1st time point was monitored by pre-treating four sets of HK cells with increasing doses of orthosilicic acid (0.1 mg/L (**Figure 5.17**), 1 mg/L, 10 mg/L and 100 mg/L) for 1 h, followed by PBS washing to remove the non-accumulated species. This was followed by incubation with 50 mg/L of the Rh1@TiO_2 for 30 min at 37 °C and 5% CO_2 succeeded by a triple PBS wash (to remove excess sensing-system) and fluorescence images were captured (**Figure 5.18**).These steps were replicated for the separate sets of cells pre-incubated with orthosilicic acid for 24 h and 8 days, respectively. At each time point, set of HK cells were separately incubated with solely 50 mg/L of Rh1@TiO_2 in absence of orthosilicic acid for 30 min to serve as probe controls negating possibility of intrinsic

fluorescence of the Rh1@TiO_2 sensing material, justified by the absence of fluorescence as recorded by fluorescence microscope (**Figure 5.18**).

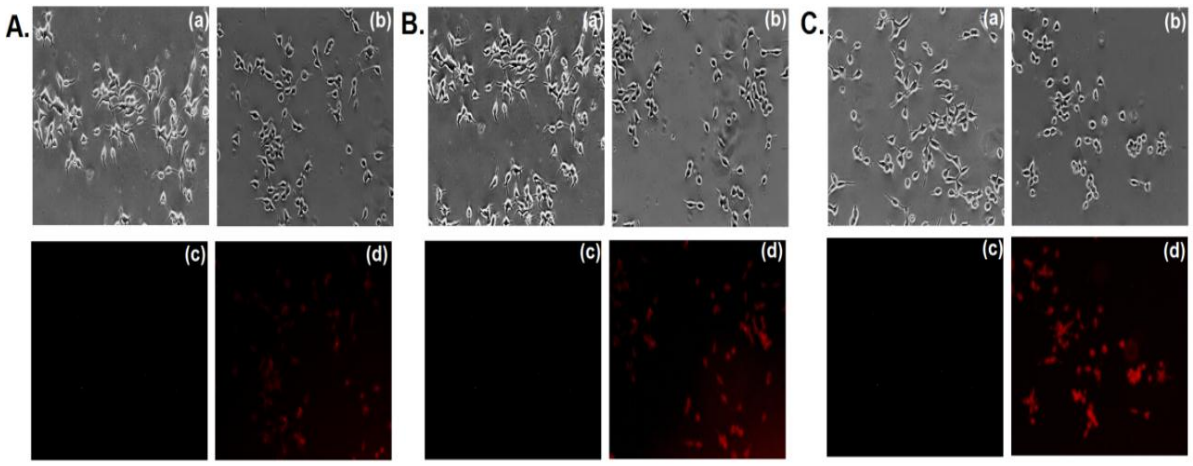

Figure 5.17. Time-dependent fluorescence detection of toxic silica species (specifically orthosilicic acid) deposition in HK cells treated with low orthosilicic acid (0.1 mg/L) utilizing sensing material,Rh1@TiO_2.
HK cells incubated with 0.1 mg/L orthosilicic acid for three different exposure periods viz. 30 min (A), 24 h (B) and 8 days(C) followed by treatment with50 mg/L of Rh1@TiO_2 sensing material (for 30 min) after each dosing period.Images recorded after sensing material exposure in the absence(a,c)and presence(b,d) of orthosilicic acid at each temporal point. Scale bars are 200µm.

As depicted in **Figure 5.18**, significant orange fluorescence was observed from the intracellular region of nephrotoxic HK cells contaminated with orthosilicic acid and consequently treated with the probe Rh1@TiO_2. It can be considered that the nanosize of the probe helps to smoothly cross the cell membrane and subsequent interact with the accumulated silica (in the form of orthosilicic acid) to get converted to its ring-opened fluorescent form of Rh1. The fluorescence intensity was apparently a function of the dose and time of orthosilicic acid exposure manifested by an enhanced response on exposure to the highest orthosilicic acid dose (100 mg/L) for the longest period (8 days) due to more and more interaction with intercellular H^+ ions leading to fluorescent ring-opened state of the probe. However, the cell density diminishes on exposure to higher doses of silica species (orthosilicic acid) over a longer period of time highlighting dose and time-dependent toxicity. Notably, the sensitivity of this analytical tool was found to be very high as the accumulation of low concentration orthosilicic acid (0.1 mg/L) in HK cells is good enough to generate sufficiently strong fluorescence signals from the intra-cellular region (**Figure 5.17**). This study provides substantial evidence on the ability of this sensing material to detect silica species accumulation in human kidney cells specifically proximal tubular cells on chronic exposure to increasing dosages that inflicts severe nephrotoxicity ultimately manifesting as Chronic Kidney Disease. A low-level detection and imaging of silica in affected HK cells may lead to the conclusion that the sensing material, Rh1@TiO_2 can eventually be employed for *in-vitro* monitoring of toxic silica species bioaccumulation in any biological samples.

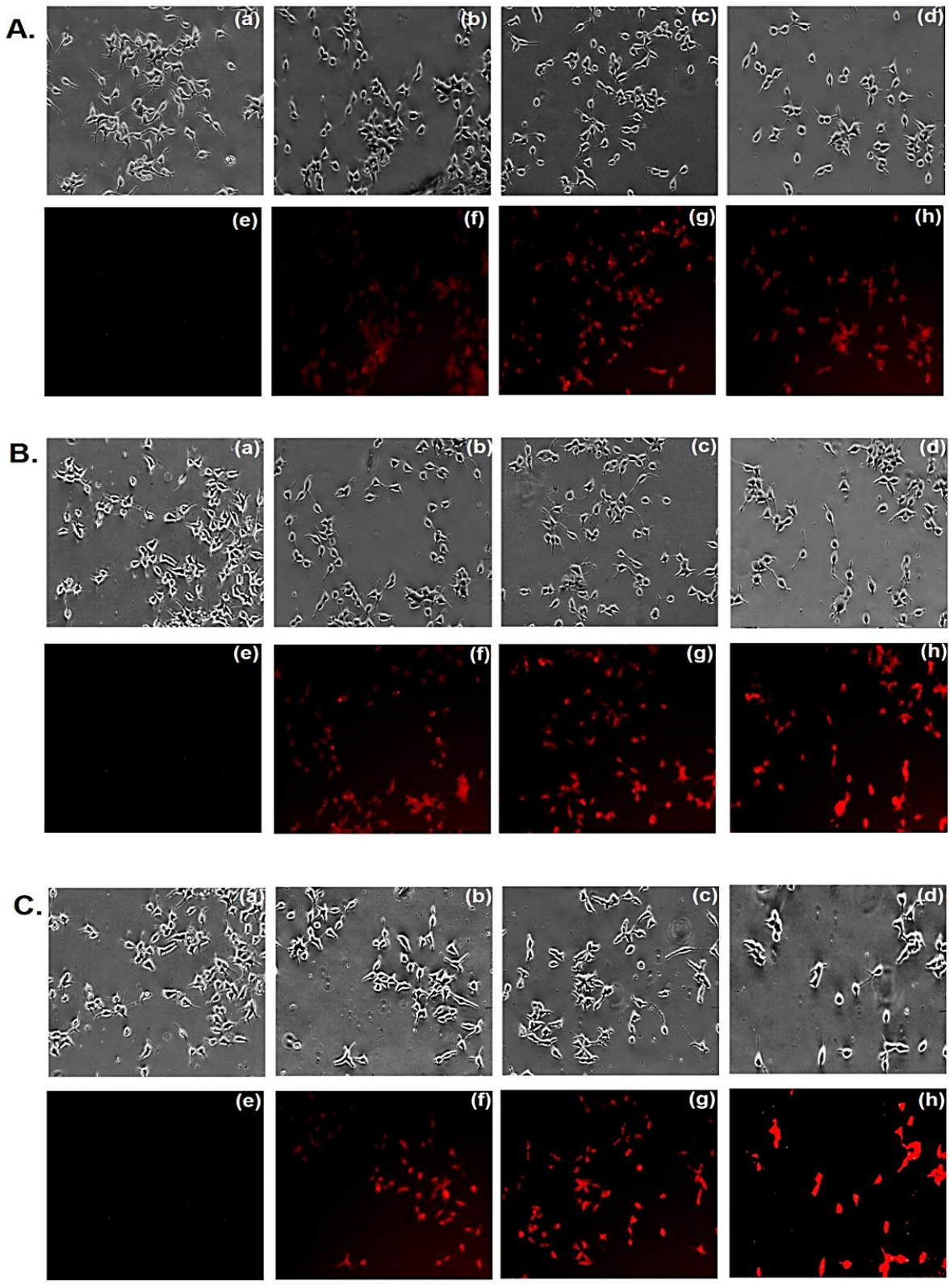

Figure 5.18. Dose and time-dependent monitoring of toxic silica species (viz. Orthosilicic acid) accumulation in live HK cells with the sensing material, Rh1@TiO_2.

Phase contrast (b-d) and fluorescence images (f-h) of HK cells on treatment with 1 mg/L (b,f), 10 mg/L (c,g) and 100 mg/L (d,h) of Orthosilicic acid for three different exposure time points viz. 1 h (A), 24 h (B) and 8 days (C) followed by 30 min incubation with Rh1@TiO_2 (50 mg/L) at each temporal point. Images of HK cells (a,e) were captured at each time point in the absence of orthosilicic acid and presence of Rh1@TiO_2 to serve as controls. Gradual escalation in fluorescence intensity noted with increasing Orthosilicic acid doses and exposure period signifying progressive intracellular accumulation of toxic silica species inflicting chronic nephrotoxicity. Scale bars are 200µm.

5.4 Conclusion

In summary, the present work demonstrates an effective strategy to detect and image the toxic silica species (orthosilicic acid) accumulation in the live cells. The sensing material Rh1@TiO_2 was prepared by simple physisorption of a Rhodamine derivative, which acts as a chemodosimeter based pH sensor, onto the biocompatible, non-toxic TiO_2 nanoparticles. This newly developed water-dispersible fluorescent nanoprobe has demonstrated its capability to detect orthosilicic acid in aqueous media, and in biological systems via interaction with the incessantly released H^+ ions. The H^+ ions generate the fluorescent ring-opened form of the fluorophore and resulted in a strong orange fluorescence output.

The studies were carried out on human kidney cells at biological pH (pH 7). The cell imaging studies confirmed that this probe is biocompatible and can easily cross the cellular surface of nephrotoxic HK cells interacting with bioaccumulated orthosilicic acid produced under silica stress conditions.

The synthesized sensing material Rh1@TiO_2 offers several advantages like good water dispersibility, the absence of organic solvents usage during fluorimetric studies, quick turn-on type signal transduction, low-level imaging. This sensing material has the potential to be used as a medical tool for early identification of silica-induced nephrotoxicity with effective detection and imaging of toxic silica species deposition in the susceptible human renal proximal tubular cells. Therefore allowing the health professionals and medical experts to recommend necessary prophylactic measures which may enable to reduce the overall burden and risk of developing chronic kidney disease.

Chapter 6

Summary of results and future prospects

Chapter 1: Introduction and Review of literature

In this thesis we have worked with a strange form of Chronic kidney disease (CKD) that has been reported since the 1990s with no identifiable cause whatsoever and is known to be endemic in various developing countries of the world like India, Central America, Sri Lanka, Egypt. This mysterious form of CKD has been rechristened as Chronic kidney disease of 'unknown etiology' (CKDu) as its causation is unlinked to traditional causal agents like diabetes and hypertension. Reported research on CKDu till date has stated that this disease is characterized by a progressive, slow and asymptomatic development which is known to affect younger adults in the 3rd to 5th decade of life. This disease also mainly targets rural communities especially with poor socio-economic status that mainly rely on groundwater for their drinking requirements. Histopathological studies of CKDu till date have shown the major pathological manifestation of this disease to be chronic tubulointerstitial nephritis (CTN). CTN is major pathological condition known to affect proximal tubular cells of the renal nephron which are functionally involved in toxin elimination and the interstitial cells surrounding proximal tubules, which ultimately progresses in chronic renal failure development. This nephropathy is mainly induced by exposure to nephrotoxins present in the environment like heavy metals (Pb, Cd, Hg and As), mycotoxin (i.e. ochratoxin), phytotoxin (i.e. aristolochic acid) and more recently trace geogenic element viz. silica. Owing to the histopathological similarities in CKDu and CTN presentation, environmental arising nephrotoxins have been widely considered as potential etiological agents for CKDu.

This wave of CKDu has managed to engulf the most famous tourist destination of the world that is the state of western India viz. Goa specifically the Canacona taluka of South Goa district. Although this strange form of endemic nephropathy has managed to affect the Canacona taluka of south Goa, this disease is the least publicised and understood as compared to other regional nephropathies reported in various other developing countries of the world. The electronic media, print and the Directorate of Health Services, Goa have constantly reported the rising incidence of CKDu for the past two decades (since 1990s) in a few villages of the Canacona taluka. But the exact prevalence statistics and geographical distribution of this disease in taluka is unavailable till date due to lack of maintenance of appropriate registries. Troubled by this epidemic curse, a sole environmental cum biological monitoring study was conducted by a joint team from NIOH (National Institute of Occupational Health), ICMR (Indian Council of Medical Research) and Department of Preventive and social medicine of Goa medical hospital, in an attempt to resolve the etiology of this endemic nephropathy in 2005. In this study, this team had tested for the potential role

of three environmental nephrotoxins i.e. cadmium, arsenic and ochratoxin in CKDu causation via exposure through food, water and blood routes in the affected region of the taluka. However the levels of these nephrotoxins in the limited number of samples tested were found to be well below the WHO established permissible limits and were comparable in the CKDu affected and the control regions (Saiyed et al., 2005). Hence this study had potentially ruled out the contribution of these aforementioned nephrotoxins in CKDu development in the Canacona taluka. Unfortunately, no follow up studies were conducted by this research group or any other groups till date to assess for the contribution of various other environmental nephrotoxins like heavy metals (lead, cadmium, arsenic and mercury), ochratoxin, aristolochic acid and silica in the causation of the high incidence of CKDu disease in the taluka. This aforementioned sole environmental monitoring study resulted in the etiology being unidentified, until our group decided to take up the challenge in 2014 of attempting to decode the etiology of this rapidly progressing mysterious form of CKDu disease noted in the Canacona taluka of south Goa. Hence was established as the core focus and aim of this thesis.

Chapter 2: Demographic, epidemiological and biochemical analysis of the CKDu scenario in the Canacona taluka of Goa, India

This only environmental monitoring study conducted by Saiyed and his team in 2005 suffered from chief limitations. Firstly, they failed to establish the detailed prevalence statistics along with the geographical cum demographical distribution of CKDu in the taluka. Secondly, prospective risk factors and demographically stratified population groups that were most susceptible towards the development of the disease were not identified. Thirdly the biochemical based pathological pattern of CKDu presentation that has been appropriately described in various other regional nephropathies noted in other developing countries and are crucial for identification of the type of renal pathology and presentation of the disease manifested has not been accurately described for the CKDu disease witnessed in the Canacona taluka of Goa. Hence this thesis attempted to address these basic limitations which have been extensively described in Chapter 2.

The results from the current study have shown that the CKDu disease in the taluka was mainly pervasive to two villages namely Chaudi and Ponsulem. On the demographic front, the disease was found to exhibit a complete absence of the prevalence of common causals of CKD viz. diabetes and hypertension (confirming the etiology to be unknown), lack of gender or occupational predisposition and was remarkably more pronounced in adults in their third to fifth decade of life. Strangely, an increased prevalence (of approximately 55%) of skeletal

disorders and prolonged consumption of NSAIDs (a reported nephrotoxin) for pain mitigation were noted among the CKDu affected subjects. This finding pointed out the possible contributory role of NSAID's in the aggravation of the renal damage noted in the affected population of the taluka. Additionally various risk factors/predictors of CKDu were identified by statistical analysis of the assessed demographic, anthropometric and lifestyle characteristics of the study population by determination of significant risk-ratios. From this study it was noted that chronic consumption of untreated well water (R.R (total)- 13.78, 95% CI-7.34-25.85, p=<0.0001), occurrence of skeletal discomfort among the CKDu population (R.R(total)- 7.37, 95% CI-3.84-14.15, p=<0.0001), previous history of NSAIDs consumption (R.R(total)-6.31, 95% CI- 3.39-11.74, p=<0.0001), prolonged intake of NSAID's for pain alleviation (R.R(total)-7.15, 95% CI-3.37-15.18, p=<0.0001), presence of a non-operational mine in the proximity(R.R(total)-12.48, 95% CI- 2.72-19.12, p=0.0001) were identified as prominent and significant risk factors in the development of CKDu associated with chronic renal failure manifestation in the Canacona taluka.

Each disease has a distinguishing trend of presentation of various biochemical markers that can be appropriately measured in the biological matrices like blood or urine which can help in accurate identification of the disease pathology without employment of high health-risk invasive biopsy procedures and associated histopathological diagnosis. Since the biochemical pattern of disease presentation of CKDu in the Canacona taluka was not discovered till date, this thesis illuminated the same by measurement of biochemical markers distinctive of two varied origins of renal pathological damage viz. tubular and glomerular origin in the chief biological matrices (i.e. blood and urine) of the CKDu affected, general diabetes or hypertensive CKD affected and healthy control study populations, for comparison of the disease presentation pattern in CKDu and general CKD pathology. Tubular damage is mainly assessed by measurement of tubular dysfunction specific biochemical markers which include uBCR (urinary b2M to creatinine ratio) and uNCR (urinary NAG to creatinine ratio) which denote the levels of low-molecular weight proteins (β2-microglobulin, b2M) and tubular cells localized enzyme (N-acetyl glucosaminidase, NAG) respectively, that significantly rise in the urine on tubular damage due to the nephrotoxin induced compromise in the intrinsic tubular reabsorption of these proteins into the plasma. Whereas glomerular damage is analysed by measurement of glomerular dysfunction biomarkers that include uPCR (urinary protein to creatinine ratio), uACR (urinary albumin to creatinine ratio), uAPR (urinary albumin to protein ratio) and uAlb/b2M ratio (urinary albumin to b2M ratio) which denotes the levels of proteins and its major component i.e. albumin, that remarkably increases in the urine owing

to diabetes or hypertension induced glomerular damage which under good-health conditions normally precludes glomerular filtration of these proteins into the urine due to their higher molecular weight. Reported evidence on the biochemical presentation till date have reported that since the pathology of CKDu was observed to be of tubular origin (i.e. chronic tubulointerstitial nephritis) owing to the tendency of nephrotoxins to characteristically target proximal tubular cells, a significant trend of increase in the tubular dysfunction biochemical markers will be obtained. In line with the aforementioned existing evidence, a tubular proteinuric pattern (comprising of increased excretion of tubular specific proteins viz. b2M and NAG in the urine along with increased elimination of electrolytes like bicarbonates and phosphates (that are characteristically reabsorbed by the proximal tubules) in the urine were noted in the CKDu pathological presentation of the taluka as well. These observations were further supported by the occurrence of normal levels of glomerular dysfunction markers in the CKDu affected subjects with enhanced levels of the same being noted in the general diabetes and hypertensive CKD affected individuals signifying CKDu and CKD in the corresponding endemic and non-endemic regions of the taluka to be of tubular and glomerular pathological origin respectively. Overall these results were consistent with the demographic and biochemical findings of the CKDu investigations in homologously affected regions like Sri Lanka, Uddanam region of Andhra Pradesh (India), Central America and Egypt. Hence our results provided justifying evidence to CKDu in the taluka to be of tubular pathological manifestation which strongly emphasized the etiological contribution of environmental nephrotoxins in disease development, therefore was extensively explored in the following chapters of this thesis. Thus by elucidation of the CKDu disease pattern in Canacona could help in quick detection and diagnosis, which can possibly assist in adoption of necessary preventive and treatment measures that can avert the progression to renal failure.

Chapter 3: Environmental and biological monitoring of the role of various environmental based nephrotoxins in the causation of CKDu in the Canacona taluka

Since environmental nephrotoxins have been reported to be associated with CKDu causality in homologously affected developing countries, their prospective contribution in the etiology of CKDu manifestation in the Canacona taluka was expansively analysed as well. The environmental nephrotoxins that have been described to be involved in CKDu causation include naturally arising nephrotoxins (that emerge from living organisms) like mycotoxin (such as ochratoxin) and phytotoxin (such as aristolochic acid) along with anthropogenically emerging nephrotoxins (that arises as a result of human activities) like heavy metals (such as

lead, cadmium, arsenic and mercury). All of these aforesaid nephrotoxins share a similar molecular and pathological mechanism of nephrotoxicity i.e. induction of oxidative renal tubular damage which ultimately triggers the excessive induction of apoptotic cell death and inflammation. This incessantly triggered cellular death and inflammation exaggerates into the development of tubular atrophy and fibrosis, the pathological characteristics of CTN which is also the hallmark of CKDu. In recent times, growing evidence has arisen for the nephrotoxic potential of a trace geogenic element viz. silica. Silica abundantly comprises the earth's crust but holds limited bioavailability hence considered as a trace geogenic element. Its availability to human beings can be rapidly heightened on anthropogenic disturbances of its major exposure matrices (i.e. air and more recently groundwater) that can result in elevated exposure which ultimately induces grave renal injuries. Animal based histopathological examinations of silica induced renal toxicity and a few epidemiological studies in the Balkan region (Europe) have emphasized a causal association between prolonged consumption of silica contaminated groundwater and development of chronic tubulointerstitial nephritis which is also the CKDu pathological manifestation. This contributory association was deduced from the observance of renal histopathological alterations of tubular atrophy and fibrosis and enhanced expression of tubular dysfunction biomarkers in the urine on chronic silica exposure in animal and epidemiological studies respectively that distinctively signified the role of silica in induction of tubular pathology, deeming it to be a potential nephrotoxic player in CKDu causation. These nephrotoxic candidates viz. heavy metals, aristolochic acid, ochratoxin and silica although being universally present in the environment are limited in its bioavailability under normal environmental conditions. However their bioavailability can be rapidly heightened on anthropogenic disruptions of its chief exposure matrices viz. groundwater and food by activities like mining, industrial discharge etc. that can subsequently inflict severe renal injuries on chronic and elevated exposure levels. Groundwater exploited through infiltration wells are the major source of the drinking requirements in CKDu targeted rural communities all over the world with Canacona taluka being no different. Since groundwater is entrapped in the earth's crust (that is ubiquitously enriched in these nephrotoxins bound to various other organic and inorganic elements (metals and non-metals) that under ideal conditions restrict its bioavailability to human exposure); it can serve as a hub for these environmental nephrotoxins (specifically heavy metals and silica) when disturbed by anthropogenic activities like acid mine drainage associated with mining or industrial effluent discharges, which can subsequently cause the extensive leaching out of these nephrotoxins from the aquifer's bedrock into the water table. Chronic consumption of

this nephrotoxin contaminated groundwater can ultimately result in severe tubular dysfunction, deeming groundwater to be significantly considered as the major exposure source to various nephrotoxins as compared to the food matrix in these CKDu affected region. On the other hand anthropogenic invasion of the terrestrial and aquatic ecosystem and improper farming practices (such as untimely cutting of the aristolochic acid generating contaminating *Aristolochia* weed sp. along with improper storage of food grains supporting the growth of ochratoxin generating *Aspergillus* fungal sp.) can result in severe nephrotoxin contamination of the food crops and animals with heavy metals, aristolochic acid and ochratoxin. These nephrotoxins can gain direct entry into the humans through the food chain causing significant induction of renal toxicity. Hence these aforesaid nephrotoxins were lengthily analysed in chief exposure sources i.e. groundwater and food consumed by Canacona's CKDu affected and unaffected region, whose findings are described in Chapter 3. This study's results demonstrated significantly elevated levels of silica (i.e. 115.5 mg/L) which exceeded the established limit of 90 mg/L (determined from animal and a few epidemiological silica toxicity studies) along with borderline levels of lead (i.e. 9.98 µg/L) which were approaching the WHO allowable limit of 10 µg/L were noted in the groundwater of the CKDu affected region of the Canacona taluka. Perversely, lowered levels of silica (i.e.13.5 mg/L) and below WHO allowable limits of lead (i.e. 0.83 µg/L) were observed in the healthy control region's groundwater. The variations in both of these nephrotoxins concentrations in the CKDu affected and unaffected regions were attributed to the differences in the geological constitution of the aquifer's bedrock, pH and chemistry of the groundwater. The higher concentrations of the nephrotoxins in the groundwater of the CKDu affected region was principally associated with the close proximity of the affected region to an unoperational granite mine. As a result of which the consequential and unavoidable acid mine drainage initiating from the abandoned mine could have made its way to the neighboring affected region's aquifer that could have caused the enhanced acidity of the groundwater which manifested in the unwarranted leaching of these nephrotoxins (i.e. silica and lead) from the aquifer's bedrock into the groundwater owing to the ability of an acidic pH to solubilize and enhance these nephrotoxins bioavailability. Additionally, the silica (81% by weight) and lead (2.5% by weight) loaded granitic constitution of the aquifer's bedrock in the CKDu affected region as geologically analysed by Fernandes and Widdowson (2009); could have further contributed to the increased nephrotoxin enrichment in the groundwater attributed to acidic groundwater augmented rock-water interactions. An additional causative factor to this increased availability was observed to be essential metal cation deficiency (viz.

Ca, Mg, Al etc.) in the affected region's groundwater which under optimal levels have been proved to limit the availability of these nephrotoxins by binding and trapping them through the formation of inert metal complexes. This metal cation deficiency was accredited to the aquifer's granitic make-up which was reported by Fernandes and Widdowson (2009) to contain negligible essential metal deposits. This enhanced nephrotoxins availability at the groundwater's acidic pH and essential metal cation deficiency along with the aquifer's granitic constitution clarifies the lowered levels of these nephrotoxins reported in the groundwater of the taluka's healthy unaffected region. The decreased nephrotoxins levels in the healthy region's groundwater was attributed to its neutral pH and metabasitic geological constitution of the aquifer's bedrock (which is innately silica deficient-comprising <40% silica, lead poor containing 0.025% lead and overly enhanced in essential metal cations like Ca, Mg etc), hence do not heighten the nephrotoxin contamination in the groundwater. Therefore, the elevated levels of silica and lead nephrotoxins in the groundwater of the CKDu affected region resulted in a significantly heightened daily intake of these nephrotoxins (exceeding the WHO set allowable intake levels) and a considerably enhanced risk of developing grave renal-toxicity (as obvious from the target hazard quotients surpassing the WHO set nontoxic threshold of 1) at the predominant exposure levels in the taluka's affected subjects. Concomitantly, no noteworthy dangerous intake levels or related renal-toxicity risk was noted for these nephrotoxins on exposure via the food route in the CKDu affected region due to the presence of JCEFA recommended safe levels of these nephrotoxins in the food ingested in the taluka. Whereas on the other hand, WHO and JCEFA set non-toxic intake levels and no linked renal toxicity risk was observed on groundwater and food ingestion in the taluka's healthy region, attributed to the occurrence of non-toxic nephrotoxin levels in the region's groundwater and food. Thus these results emphasized groundwater to be the chief source of exposure to these nephrotoxic candidates viz. silica and lead in the taluka's CKDu affected region which resembled the trend noted in homologous CKDu targeted regions such as Balkan region (Europe), Uddanam region of Andhra Pradesh (India) and Central America.

It has been well reported that despite the presence of high levels of nephrotoxins in the exposure sources, it does not guarantee that the targeted population will be exposed to such nephrotoxins that will subsequently lead to induction of grave renal damage. Therefore in order to accurately gauge the level of nephrotoxin exposure and whether the exposure level will induce renal tubular damage, it becomes mandatory to monitor for various biomarkers of exposure (i.e. the nephrotoxin itself) and biomarkers of effect (i.e. tubular dysfunction) in the biological matrix of the human being viz. blood through a process called biomonitoring.

Hence was carried out in this thesis whose results are listed in Chapter 3. From the results of the current study, it was noted that elevated levels of silica and lead in the groundwater exposure source directly translated into significantly increased concentrations of these nephrotoxins in the blood (i.e. silica in blood=100.2 mg/L and Pb in blood =317.8 µg/L) of the CKDu affected subjects of Canacona, which was strongly backed by high correlations between the concentrations in the groundwater and blood. Perversely, reduced and WHO set non-toxic levels (i.e. below the permissible boundaries of 90 mg/L for silica and 50 µg/L for lead) of these nephrotoxins (viz. silica in blood=30.7 mg/L and Pb in blood=6.3µg/L) were observed in the healthy individuals blood accredited to reduced nephrotoxin intake from the region's groundwater. These increased silica and lead exposure concentrations (internal doses) observed in the CKDu affected subjects blood signified higher nephrotoxic potential in triggering grave renal tubular injuries at such levels on long-term exposure. This silica and lead elicited renal toxicity was aggravated by significantly reduced blood essential metals ions concentrations (like zinc, manganese, copper, iron, selenium) in CKDu affected subjects as these metals ions under ideal levels elicits heavy metal clearing by increased metal excretion protein synthesis (i.e. metallothionein proteins) and confers anti-oxidative defense from heavy metal triggered oxidative renal injuries as these ions serve as metal cofactors for anti-oxidant enzymes like superoxide dismutase and glutathione peroxidase. Hence decreased levels of these aforementioned essential cations in the CKDu affected subjects blood enhanced the vulnerability to lead induced oxidative damage and associated tubular injury. These essential cations anti-oxidative defense propensity clarifies the nonexistence of renal injuries in the taluka's healthy study population attributed to the occurrence of normal essential cations concentrations and WHO set non-toxic silica and lead levels observed in the healthy controls blood. Additionally the significantly elevated levels of silica and lead in the blood of CKDu affected subjects exhibited a heightened and significant ($p<0.05$) dose-effect response association with tubular injury biomarkers viz. uBCR and uNCR, wherein the concentrations of these tubular proteinuric markers consistently amplified with rising blood silica and lead nephrotoxin concentrations (internal doses).These observations were backed by lack of relationships of blood silica or lead concentrations with tubular damage markers in healthy subjects, indicating absence of nephrotoxin triggered tubular damage in these subjects as expected. Overall, the occurrence of significant dose-response associations between blood lead and silica levels and tubular nephropathic markers as noted in the Canacona's CKDu affected subjects were in agreement with results of autonomous dose-tubular damage response analyses of these nephrotoxins that are reported in CKDu causation in Central

America and Balkan region (Europe) respectively. Therefore, our results is the first ever report that strongly proved the etiological role of higher and chronic exposure to these two nephrotoxins viz. lead and silica via prolonged ingestion of untreated nephrotoxin contaminated groundwater in the induction of grave tubular injuries associated with the pathogenesis of CKDu in the Canacona taluka.

Chapter 4: Elucidation of the cellular and molecular renal toxicity mechanisms of an 'emerging environmental nephrotoxin - Silica' by 'in-vitro' cytotoxicity studies

Previous *in-vivo* animal based histopathological assessments and a few epidemiological originated biochemical cum clinical analyses in the Balkan region of Europe have determined chronic tubulointerstitial nephritis to be the primary histopathological demonstration of silica elicited nephrotoxicity. But these studies were unsuccessful in describing the cellular and molecular toxicity mechanisms of silica triggered renal-toxicity till the present time. Hence this study attempted to elucidate the toxicological mechanisms of silica induced nephrotoxicity at the cellular and molecular level, as a function of dose and time using the kidney's nephrotoxin targeted cells viz. normal human renal proximal-tubular cells (HK-cells) as an *in-vitro* model. To achieve this, the proximal-tubular cytotoxicity effects following long-term exposure (for 7 days) of HK-cells to enhancing silica doses (80-120 mg/L) were investigated via a panel of assessments consisting of cell-viability, oxidative-injuries, inflammatory responses, genomic-damage, cell-cycle arrest, mitochondrial-integrity and apoptotic-pathway regulation. Additionally, this study tried to provide supporting contributory evidence to the involvement of silica in CKDu manifestation in the Canacona taluka. The results of which are detailed in Chapter 4. From the current study's results, it was observed that silica demonstrated severe proximal-tubular cytotoxicity (evident from decreasing HK-cell-density) on chronic dosing (for 7 days) to increasing-concentrations (≥100 mg/L), that was facilitated by the silica-toxin elicited mitochondrial-dysfunction. Silica was found to mainly disrupt the structural and functional architecture (i.e. cellular respiration) of the mitochondria owing to the innate enrichment of the proximal tubular cells with mitochondria due to the high energy demand for filtration and reabsorption functions. The elevated silica-doses (≥100 mg/L) incited continual mitochondrial-injuries resulted in the generation of incessant oxidative-stress (i.e. excessively produced ROS surpassed the anti-oxidant defense), owing to the role of mitochondria in ROS homeostatic maintenance. This persistent oxidative-stress (heightened ROS) further imposed grave protein, membrane and DNA-injuries (genotoxicity) which subsequently stimulated the DNA-damage responsive p53

gene facilitated damage repair in conjunction with the G2/M cell-cycle arrest (Chk1-dependent). However, these incessantly induced DNA-injuries exceeded the cellular-repair capacity, which triggered p53 to continuously stimulate the mitochondrial (intrinsic) series of apoptotic-events that terminated in the continual proximal-tubular (HK) cell-demise. The intensification of this renal tubular apoptosis activation was achieved by a p53 produced cross-talk with the inflammatory pathway (i.e. the latter being predominantly incited by ROS triggered activation of the inflammatory transcription-factor NF-kβ). This cross-talk potentiated from silica induced intracellular-stressors (viz. ROS and DNA-damage) activating the p53-gene which in-turn stimulated the inflammatory-regulator i.e. NF-kβ expression, accredited to the presence of binding-sites for NF-kβ in the p53 gene. This p53-bound NF-kβ complex concurrently induced the heightened activation of principal apoptotic-executioners (i.e. Bax, cytochrome c, caspase-9 and caspase 3) and pro-inflammatory cum fibrogenic-cytokines (IL-1β, IL-2, IL-6, TGF-β and TNF-α) which cumulatively manifested in incessant tubular apoptotic cellular-demise and inflammation. Hence continual mitochondrial-mediated apoptosis and inflammation triggered by intracellular-stressors (i.e. enhanced ROS and DNA-damage) being generated on elevated silica dosing (≥100 mg/L) were determined to be the major molecular-mechanisms of silica induced nephrotoxicity. This silica triggered continuous tubular cellular-demise and inflammation ultimately results in the development of tubular-atrophy and fibrosis, which are distinctive histopathological characteristics of chronic tubulointerstitial nephritis, the major hallmark of CKDu pathogenesis. These silica triggered renal toxicological mechanisms were consistent with those incited by well-known nephrotoxins (like heavy-metals); therefore validating the renal tubular toxicity inducing potential of silica in CKDu development. Additionally, our study also justified ≥100 mg/L to be the nephrotoxic silica-concentration, which could trigger severe renal proximal-tubular (HK-cells) toxicity and related CKDu development on long-term exposure. This dose's CKDu-inducing potency was further backed by its resemblance with the CKDu inducing silica concentrations noted in the drinking-groundwater (115.5 mg/L) and blood (100.2 mg/L) of the affected individuals of the Canacona taluka. Additionally, this same nephrotoxin exhibited a reversal of nephrotoxicity on lower sub-toxic dosing. This was evident from the lack of CKDu prevalence in other non-endemic regions of the taluka comprising of sub-toxic silica concentrations (below 100 mg/L) i.e. 13.5 mg/L in the potable-groundwater and 30.7 mg/L in the blood of the healthy individuals. This non-toxicity could be possibly accredited to the quicker toxin-excretion capacity (probably facilitated by metallothioneins) in union with uncompromised cellular-repair potency of these proximal-tubular cells at lower-doses

which could have prevented nephrotoxicity infliction at sub-toxic dosages. Thus, our results provided contributory evidence to the causal role of this nephrotoxic silica-concentration (100 mg/L) [prevalent in the blood of the affected individuals of the taluka], in significantly inciting renal tubular damage and related CKDu development on prolonged exposure in the Canacona taluka. Our results strongly suggests that the nephrotoxic potential of silica can be a prime considerant when decoding the etiology of the environmentally-triggered CKDu problem witnessed in developing countries that are susceptible to anthropogenic influence.

Chapter 5: Development of a 'fluorimetric chemosensor' for 'detection of silica bioaccumulation' in human renal proximal tubular cells

Our previous studies, have strongly established chronic and higher levels of silica exposure through long-term untreated contaminated groundwater consumption to be strongly implicated in the development of chronic tubulointerstitial nephritis, the major hallmark of CKDu. A few of the previously reported epidemiological studies in the Balkan region (Europe) have established this aforementioned nephrotoxic potency to be mediated by the water soluble, monomeric, and highly reactive form of silica viz. orthosilicic acid (H_4SiO_4). This silica form (i.e. orthosilicic acid) has the tendency to produce H^+ ions, the levels of which are well controlled under lower silica exposure conditions by the inherent cellular repair capacity. However, due to the bioaccumulative tendency of orthosilicic acid it can overly accumulate on anthropogenically triggered increased and chronic bioavailability of orthosilicic acid (arising from enhanced silica exposure), which permits it to exceed the super saturation point of polymerized silica formation causing orthosilicic acid to be retained in its unpolymerised state. This unpolymerised form on long term and increased levels of exposure continuously produces toxic H^+ ions and free radicals which on surpassing the innate cellular oxidative repair capacity results in the manifestation of severe nephrotoxicity as described earlier(in Chapter 4). Regardless of the grave nephrotoxic potency displayed by silica, probes or sensor detecting accumulation of its toxic form viz. orthosilicic acid in targeted human cells like proximal tubular cells, which is a basic prerequisite for toxicity analysis, are not available. Hence, we developed a water-dispersible and biocompatible ''turn-on'' type fluorimetric sensing material (nanoprobe) consisting of a rhodamine-based chemodosimeter adsorbed onto TiO_2 nanoparticles for the detection and imaging of the toxic silica species (viz. orthosilicic acid) bioaccumulation in an ideal nephrotoxicity assessment cellular-model viz. human renal proximal tubular HK-cells, in-vitro. The sensing material (Rh1@TiO_2) was formulated by simple physisorption of a Rhodamine derivative (i.e. rhodamine hydrazide,

Rh1), which acts as a chemodosimeter based H^+ sensor, onto the biocompatible and non-toxic TiO_2 nanoparticles. Rh1 was chosen as the chemodosimeter unit, based on the presumption that the acidic environment generated inside the inherently neutral proximal tubular cell due to the incessant release of H^+ ions from the excessively accumulated orthosilicic acid (on chronic exposure to elevated silica concentrations) would induce the chemodosimeter's spirolactam ring-opening, which would transduce a strong orange fluorescence output owing to the formation of rhodamine. This synthesized silica sensor demonstrated various merits such as absence of intrinsic interference from the probe at an acidic pH, good water dispersibility, the complete absence of organic solvents usage during fluorimetric and spectrophotometric studies, quick 'turn-on' type of signal transduction and detection cum imaging of silica accumulation even at reduced levels of accumulated orthosilicic acid (i.e. on longer exposure to 0.1 mg/L of silica for 8 days). The probe suitably proved its biocompatible efficiency (the biocompatibility being conferred due to the adsorption of the rhodamine derivative onto non-toxic TiO_2 nanoparticles) by effortlessly penetrating the cellular membrane (possibly by endocytosis) and rapidly detecting silica (specifically orthosilicic acid) accumulation as a function of dose and time. Herein the fluorescence intensities significantly and proportionally ($p<0.05$) elevated with increasing time-period of exposure (i.e. for 8 days) to the highest silica concentration (i.e. 100 mg/L), which was an outcome of the increased orthosilicic acid accumulation under such conditions resulting in enhanced fluorescence output. Therefore, this sensing material exhibited a promising potency for the quick detection and diagnosis of silica accumulation in susceptible renal proximal tubular cells and its associated nephrotoxicity concomitant with CKDu development in the taluka.

Conclusion and future scope

This PhD research managed to describe the demographic stratification of CKDu witnessed in the Canacona taluka wherein it was found to affect younger adults in the age group of 30-50yrs belonging to rural communities and displayed no occurrence of traditional causals like diabetes and hypertension. Moreover this endemic nephropathy displayed no occupational bias and targeted populations residing in the affected region for more than 15 years i.e. for approximately 38 years stating an involvement of an environmental factor in the CKDu causation. Additionally the risk factors for CKDu development were identified to be chronic consumption of untreated well water, occurrence of skeletal discomfort among the CKDu population, prolonged intake of NSAID's for pain alleviation, presence of a non-operational

mine in the vicinity. Thus by knowledge of known risk factors it can help in prediction of the nephrotoxicity risk and by suitable intervention and modification of the recognized CKDu risk factors in the early stages could be useful in the prevention of progression of this endemic nephropathy to End Stage Renal Disease (ESRD). This thesis also established chronic tubulointerstitial nephritis (CTN) to be the major pathological manifestation of CKDu in the Canacona taluka for the first time by analysis of the biochemical based pattern of disease presentation via suitable assessment of associated biomarkers (Chapter 2). Thus by illumination of the biochemical based CKDu disease pattern in Canacona could help in quick and early screening, detection and diagnosis of CKDu in susceptible populations permitting the adoption of suitable preventive or treatment measures to inhibit advancement towards renal failure, therefore improving patient safety, clinical outcomes and morbidity alleviation.

Since CTN was the major pathological presentation of CKDu, exposure to environmental nephrotoxins were implicated in CKDu causation in the taluka. From this PhD research, we have managed to confirm prolonged exposure to high levels of silica and lead routed through untreated nephrotoxin contaminated groundwater consumption to be the major environmental nephrotoxins that are responsible for the etiological development of CKDu in the Canacona taluka. This is the first ever report to establish the potential etiological agents for CKDu in the taluka since the past two decades (Chapter 3). With the knowledge of these nephrotoxic players suitable preventive or remediative measures can be taken to reduce the impending exposure of high risk population groups to these nephrotoxins, thus decreasing the future rise in occurrences of CKDu in the taluka. In line with previously reported remediation techniques, the following measures could be incorporated in the near future for the appropriate reduction of nephrotoxins exposure which are stated as follows. Since direct intake of untreated groundwater enriched with nephrotoxins was demonstrated to be the major route of nephrotoxin exposure among the CKDu affected subjects in this taluka, thus remediation or treatment of this exposure source could help in lessening the nephrotoxin exposure and related induction of renal tubular damage (i.e. CKDu development) in this endemic region. Hence, we recommend that the consumption of filtered municipality supplied surface-water (from the Galgibagh-river that should be pretreated) in the taluka could help in attaining the aforesaid purpose. This alternative water source for drinking has been proposed for two reasons: Firstly, it inherently comprises of lower silica and lead levels (as estimated in our study described in Chapter 3), due to the reduced rock-water interactions because of the lowered residence-time with the river's metabasitic-bedrock (that is deficient in reserves of the nephrotoxins- silica-and lead as geologically analysed by Fernandes and

Widdowson, 2009), hence eventually can result in reduced toxin exposure. Secondly, environmental-nephrotoxins (like silica and heavy-metals such as lead) can be additionally and majorly reduced through water-treatment techniques like membrane-filtration (reverse-osmosis or ultrafiltration) and chemical-coagulation (addition of alum to co-precipitate silica and lead by formation of inert and insoluble metal-complexes) by the municipality prior to distribution, and can be further expedited by household water-purifiers equipped with reverse-osmosis/ultrafiltration technology. These aforementioned techniques have been suggested as they have been well-reported to appreciably reduce these nephrotoxins in the drinking water (Greenlee et al., 2009; Najm and Trussell, 1999; Oner et al.,2011).

Previous animal based histopathological studies and epidemiological studies (including our study) have stated CTN to be the major pathological manifestation of silica induced nephrotoxicity but have failed to establish the cellular and molecular toxicity mechanism of silica incited renal toxicity. This PhD research elucidated the cellular and molecular mechanistic-aspects of dose and time-dependent nephrotoxicity inflicted by silica for the first-time using an ideal *in-vitro* nephrotoxic assessment cell-model viz. normal human renal proximal-tubular cells (HK-cells) (Chapter 4).The toxicity mechanism was mainly mediated by dose and time reliant silica triggered mitochondrial mediated oxidative damage in proximal tubular cells that resulted in incessant induction of apoptosis and inflammation, which clinically exaggerated into the development of tubular atrophy and fibrosis, the major hallmarks of CTN. These nephrotoxicity mechanisms were homologous to that of other well-reported nephrotoxins like lead confirming the renal proximal tubular toxicity inducing potential of silica. Furthermore, through our *in-vitro* cytotoxicity studies, we have elucidated the sub-cellular targets of silica induced nephrotoxicity to be mitochondria and nucleus which were homologous to the targets of other well-reported nephrotoxins like lead. Thus in line with our findings, necessary prophylactic, preventive and therapeutic modalities(such as anti-oxidant therapy) can be suitably implemented to protect the aforesaid cellular targets from the toxic effects of these nephrotoxin, thereby averting the induction of tubular nephrotoxicity which could help in deterring the future increase in CKDu prevalences in this taluka. Owing to the positive silica triggered proximal tubular toxicological response obtained in our *in-vitro* investigations (Chapter 4), a follow up *in-vivo* study (using the previously studied silica toxicity animal model viz. guinea pigs or mice) would help to corroborate our findings and further understand the established cellular and molecular toxicological mechanisms of silica induced nephrotoxicity (Chapter 4) at the organ-level (*in-vivo*).

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Appendix A

Ethical clearances

A1. Institutional ethical clearance

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI, RAJASTHAN
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Ref. No: IHEC/15 M/1 Date: 27/09/2016

To
Dr. Anasuya Ganguly
Assistant Prof. Dept. of Bio.Science
BITS Pilani, K. K Birla- Goa Campus

Subject: Approval of submitted research proposal

This is to inform that your proposal submitted to the Institutional Human Ethics Committee has been approved as mentioned below, at the 15th meeting of the committee held on Tuesday, 27th September 2016 at 3.50 pm in the office (2146-H) of Prof. S.K Verma, IHEC-Chairperson, Pilani

Name of PI	Dr. Anasuya Ganguly
Proposal No.	T-IHEC-R- 39/16-1
Approval No.	IHEC-39 /16-1
Title	Role of Environmental Toxins in Kidney Related Diseases of Canacona Taluka, Goa
Date of Approval	27/09/16
Date of Expiry	26/09/17

You are hereby directed to
* Quote the above approval No. for all further correspondence with the committee.
* Also maintain, and submit the records of subject requirement and subject data sheet of the human participants in the study.
* Ensure that all the studies are done under the supervision of the person (s) whose name have been approved under the proposal submitted.
* Reports any unusual events, accidents or emergencies encountered during the course of the study to the committee.
* PIs have to submit an interim progress report on a six month, or yearly basis and project completion report should be submitted within one month from the end of the project period.

Yours truly,

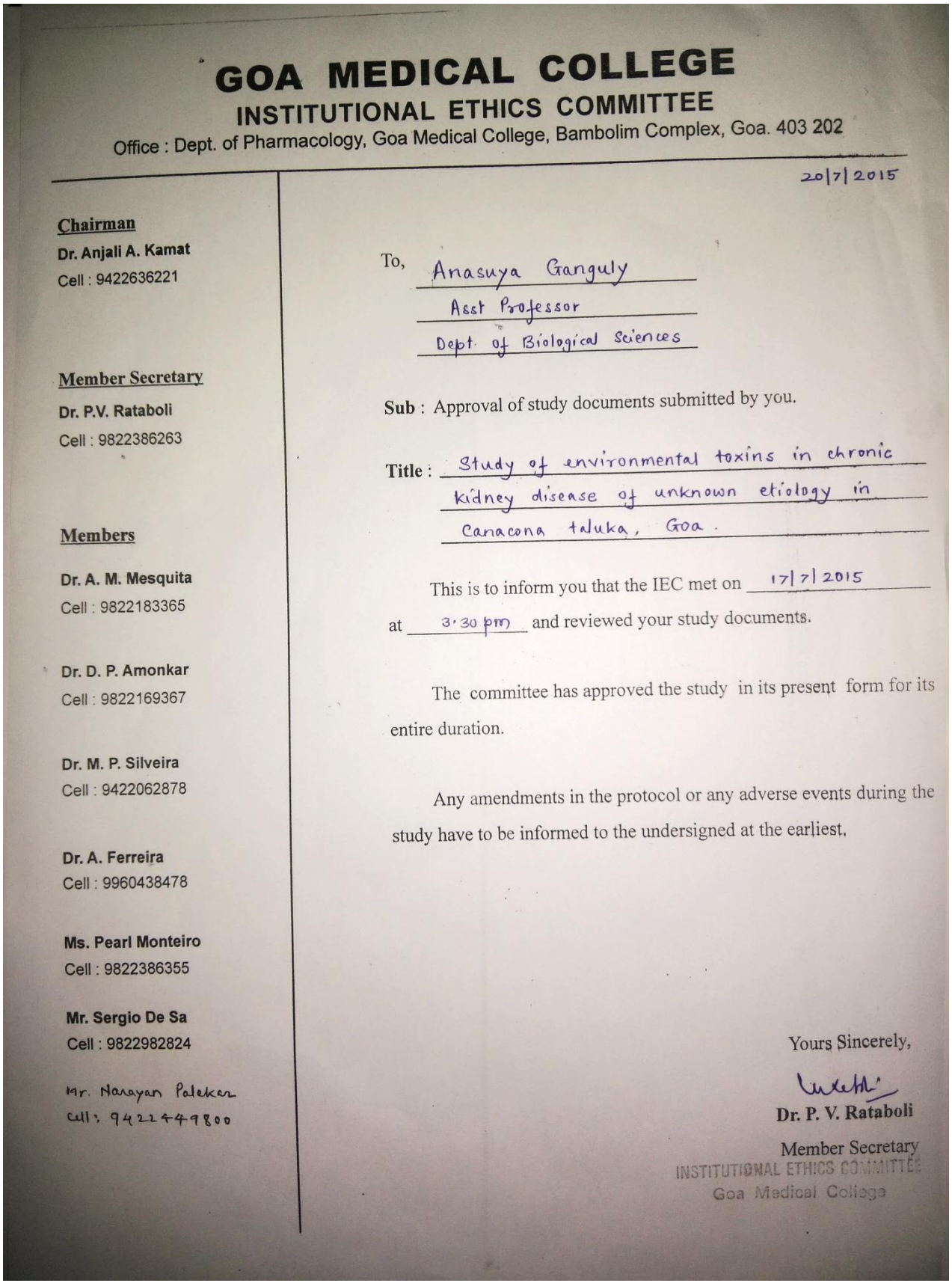
Dr. Rajeev Taliyan
(Member Secretary, IHEC)
BITS-Pilani, Rajasthan

Prof. S.K Verma
(Chairman, IHEC)
BITS-Pilani, Rajasthan

Phone: +91 159645073/74: Fax: + 9159644183: e-mail: rajeev.taliyan@pilani.bits-pilani.ac.in

Non-compliance of IHEC guidelines may lead to cancellation of an approval

A2. Ethical clearance from Goa medical college.



GOA MEDICAL COLLEGE

INSTITUTIONAL ETHICS COMMITTEE

Office : Dept. of Pharmacology, Goa Medical College, Bambolim Complex, Goa. 403 202

20/7/2015

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Member Secretary
Dr. P.V. Rataboli
Cell : 9822386263

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Ms. Pearl Monteiro
Cell : 9822386355

Mr. Sergio De Sa
Cell : 9822982824

Mr. Narayan Paleker
Cell: 9422449800

To, Anasuya Ganguly
Asst Professor
Dept. of Biological Sciences

Sub : Approval of study documents submitted by you.

Title : Study of environmental toxins in chronic kidney disease of unknown etiology in Canacona taluka, Goa.

This is to inform you that the IEC met on 17/7/2015 at 3.30 pm and reviewed your study documents.

The committee has approved the study in its present form for its entire duration.

Any amendments in the protocol or any adverse events during the study have to be informed to the undersigned at the earliest.

Yours Sincerely,

Dr. P. V. Rataboli
Member Secretary
INSTITUTIONAL ETHICS COMMITTEE
Goa Medical College

Appendix B

List of publications and presentations

B1. List of publications related to the thesis

1. **Mascarenhas, S**., Mutnuri, S. and Ganguly, A., 2017. Deleterious role of trace elements–Silica and lead in the development of chronic kidney disease. ***Chemosphere* (I.F=5.1)**, *177*, pp.239-249.

2. **Mascarenhas, S.,** Mutnuri, S. and Ganguly, A., 2018. Silica-A trace geogenic element with emerging nephrotoxic potential. ***Science of The Total Environment* (I.F=5.7)**, *645*, pp.297-317.

3. **Mascarenhas, S**., Gawas, R.U., Ghosh, B.K., Banerjee, M., Ganguly, A., Chatterjee, A. and Ghosh, N.N., 2018. Water-Dispersible Rhodamine B Hydrazide Loaded TiO2 Nanoparticles for "Turn On" Fluorimetric Detection and Imaging of Orthosilicic Acid Accumulation In-Vitro in Nephrotoxic Kidney Cells. ***Journal of nanoscience and nanotechnology* (I.F=1.35)**, *18*(12), pp.8142-8154.

B2. Other publications

1. Chatterjee, A., Banerjee, M., Khandare, D.G., Gawas, R.U., **Mascarenhas, S.C**., Ganguly, A., Gupta, R. and Joshi, H., 2017. Aggregation-Induced Emission-Based Chemodosimeter Approach for Selective Sensing and Imaging of Hg (II) and Methylmercury Species. ***Analytical chemistry* (I.F=6.35)**, *89*(23), pp.12698-12704.

2. Hiremath S.D.,Chatterjee, Gawas, R.U., **Mascarenhas, S.C**., Ganguly, A., Banerjee, M., Chatterjee, A.,2019. A water soluble AIE-gen for organic solvent free detection and wash free imaging of Al^{3+} ions and subsequent sensing of F^- ions and DNA tracking. ***New Journal of Chemistry* (I.F=3.1)**, *43*(13), pp.5219-5227.

B3. Conferences and workshops attended

- Poster presentation on the topic entitled "Silica-A trace geogenic element with emerging nephrotoxic potential" at the 3rd International conference on emerging contaminants (emcon forum 2017) held at Kaohsiung, Taiwan, Republic of China in the year 2017.
- Fullbright specialist program on "The importance of Engineering-Economics-Entrepreneurship (3-Es) in engineering education and nation building" conducted at BITS Pilani K K Birla Goa campus in the year 2018.
- BIRAC workshop on "Bio-entrepreneurship, Grant writing and Intellectual property management" conducted at BITS Pilani K K Birla Goa campus in the year 2018.

B4. Scholarships/ Grants-in-aid

- Worked as a junior research fellow on a CSIR project entitled "Role of environmental toxins in kidney related diseases of Canacona taluka, Goa (Scheme number:-27 (0284) /13/ EMR-II)" for the period of October 2013 to June 2016.
- Awarded the senior research fellowship by the Indian council of medical research (ICMR-SRF) [Fellowship file no: 3/ 1/ 2 (6)/ Nephro/ 18-NCD-II] for the period from July 2018 to July 2019.

Appendix C

Brief Biography of the Candidate

Starlaine Mascarenhas was born and raised in Goa, India. She received her Bachelor of Science (B.Sc) degree in Biotechnology from Dhempe College of Arts and Science, Goa University, India with a distinction in 2011. For her Bachelors' dissertation, she worked with Prof. Sonali Borges on the topic "Isolation of pathogens from commonly available food samples and its analysis on Differential Blood Agar with effective antibiotics against the pathogens." Starlaine then obtained her Master of Science (M.Sc) degree in Biotechnology from St. Aloysius College, Mangalore University, India with a distinction in 2013. She cleared the national GATE competitive exam in the year 2013 in the discipline of Biotechnology. For her Masters dissertation, she worked under the guidance of Dr. Cathrine Sumathi (Scientist grade 'C') on "Spectrophotometric determination of dye decolorization by fungal isolates obtained from corn-cob" at the CSIR-National institute of Oceanography, Dona Paula, Goa. Starlaine then enrolled herself in the Ph.D. program of the Department of Biological Sciences, BITS Pilani KK Birla Goa campus. During this time, she worked as a project fellow (October 2013-June 2016) on a CSIR project entitled "Role of environmental toxins in kidney related diseases of Canacona taluka, Goa (Scheme number:-27 (0284)/ 13/ EMR-II)". She later received the senior research fellowship from the Indian Council of Medical research (ICMR) for completion of her PhD research work.

Starlaine has co-authored 5 international publications in journals of high impact factor and has presented her work at 1 international conference so far in her career.

Apart from science, Starlaine like to travel, read and enjoy a few sports like badminton and tennecoit.

Appendix D

Brief Biography of the Supervisor

Dr. Anasuya Ganguly is an Associate Professor in the Department of Biological Sciences, BITS Pilani KK Birla Goa campus and has been employed by the institute since 2005. Dr. Ganguly received her Master of Science degree in Zoology with a specialization in Advanced Cytology and Genetics from the University of Calcutta in 1996. She received her Ph.D. degree from Jadavpur University in 2003. Her Ph.D. thesis entitled ”Regulation of Cell Division in Entamoeba” was carried out under the supervision of Prof. Anuradha Lohia, Bose Institute, Calcutta, India. Dr. Ganguly was subsequently employed as a post-doctoral fellow at the University of Texas Health Science Center, Cellular and Structural Biology, Texas, USA for three years where she worked extensively with cell and tissue culture techniques with an emphasis on cellular stress and aging. Dr. Ganguly has diverse research interests ranging from stem cells and tissue engineering to environmental toxicology and biodiversity studies.

Dr. Ganguly has been a principal investigator for 3 projects and co-investigator in 4 extramural projects so far. She has co-authored 24 publications so far in her career. Dr. Ganguly is also the co-Director of the company “Bactreat Environmental Solutions LLP” along with Prof. Srikanth Mutnuri since April 2015.

Other than science, Dr. Ganguly is interested in everything but politics and economics.