Environmental Toxins Responsible for the Development of Chronic Kidney Disease of Unknown Etiology (CKDu) In Canacona Taluka, Goa

SYNOPSIS

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Chapter 1: Introduction and Review of literature

In this thesis we have worked with a rampantly progressing and strange form of Chronic kidney disease(CKD) that has been reported in a taluka of a developing country-India viz. Canacona taluka of south Goa since the past 25 years, which is believed to be of 'unknown etiology' as it is not linked to the traditional CKD risk factors (such as diabetes and hypertension).Owing to this disease's unidentifiable causative agents, it has been renamed as Chronic kidney disease of unknown etiology(CKDu).CKDu has been reported to affect various other developing countries like Central America, Sri Lanka, Tunisia and Egypt as well. Despite this disease affecting various geographical regions all over the world, it shares common demographic, clinical and histopathological features across all these regions. This disease is found to affect younger adults in the age group of 30-50 yrs. and is characterized by slow, progressive and asymptomatic development. It mainly targets economically backward rural communities that mainly rely on untreated groundwater (i.e. well water) for their drinking requirements. Histopathological analysis of CKDu has revealed chronic tubulointerstitial nephritis (CTN) to be the major pathological manifestation of this disease. CTN disrupts the structural and functional architecture of proximal tubular cells of the renal nephron which are intrinsically involved in toxin elimination and the interstitial cells surrounding proximal tubules, causing overall disruption of renal function that ultimately manifests in the development of chronic renal failure. This renal pathology is triggered by chronic exposure to various environmental nephrotoxins 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 resemblance of CKDu with CTN presentation, causative agents of CTN viz. environmental nephrotoxins are widely implicated as possible etiological agents for CKDu.

In this thesis, we have focused on the CKDu scenario in Canacona taluka of south Goa (India) as it is least publicized and understood as compared to the scenario noted in various other developing countries of the world. Despite the rising incidence of CKDu in this taluka very limited research has been conducted till date on the CKDu scenario in this region. A sole environmental cum biological monitoring study was conducted in 2005 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 headed by Dr.Saiyed to identify potential causals viz. environmental toxins involved in the development of CKDu in this region. This 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. and As) and ochratoxin and associated exposure to these toxins in affected individuals and healthy controls. The results from their study depicted that 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 this study had ruled

out contribution of these aforementioned nephrotoxins in CKDu development in taluka. In 2009, a preliminary medical screening of the pattern of genitourinary problems of 298 patients of Canacona was conducted by the Department of Urology, KLES (Karnataka) headed by Dr.Nerli. Herein parameters like blood glucose, hypertension, serum creatinine, serum urea and urinary uric acid were analysed. Results indicated elevated levels of blood and urinary parameters with normal blood pressure and glucose levels in 75% of the patients. This study only confirmed a high incidence (i.e.75%) of CKD cases in the taluka with unidentifiable causals like diabetes and hypertension supporting the unknown etiology of this disease. They also highlighted no gender bias in disease distribution and confirmed younger age groups (40-50 yrs.) to be affected**.** These aforementioned limited research resulted in the etiology of CKDu in Canacona being unidentified. Hence our group decided to take up the challenge in 2014 to decipher the etiology of CKDu in the taluka making this the core focus of the thesis.

Chapter 2: Demographic, epidemiological and biochemical analysis of the CKDu scenario in the Canacona taluka of Goa, India

This aforementioned limited research conducted on CKDu scenario in Canacona by Saiyed and coworkers in 2005 & Nerli and his team in 2009 suffered from major limitations. These studies failed to determine the prevalence statistics, geographical cum demographical distribution, potential risk factors and demographically stratified population groups that are most prone towards development of this disease. Moreover, the biochemical based pathological pattern of CKDu presentation that has already been described in other developing countries and are critical for identification of the type of renal pathology(glomerular or tubular) manifested has not been elucidated in Canacona preventing adoption of appropriate therapeutic measures to avert rise in CKDu incidences in this taluka. Hence this thesis addressed these limitations which have been described in Chapter 2.

The results from the current study have depicted that CKDu prevalence in Canacona was restricted to two villages namely Chaudi and Ponsulem. On the demographic front, this disease was found to be unlinked to common CKD risk-factors viz. diabetes and hypertension (approving the etiology to be unknown), with no gender or occupational predisposition and targeted younger adults of 30-50yrs of age. Peculiarly, a high prevalence (55%) of skeletal ailments and chronic consumption of NSAIDs (a reported nephrotoxin) for pain alleviation were noted among the CKDu affected subjects suggesting the plausible role of NSAID's in augmentation of renal injuries noted in these subjects. Risk factors for CKDu development in the taluka were identified from statistical analysis of the demographic and lifestyle features of the study population via determination of significant risk-ratios(R.R).Results of which denoted prolonged consumption of untreated well water (R.R (total)- 13.78, 95% CI-7.34-25.85, p=<0.0001), prevalence of skeletal disorders (R.R(total)- 7.37, 95% CI-3.84-14.15, p=<0.0001),chronic intake of NSAID's for pain mitigation (R.R(total)-7.15, 95% CI-3.37-15.18,

p=<0.0001) and existence of an un-operational mine in the vicinity(R.R(total)-12.48, 95% CI- 2.72-19.12, p=0.0001) to be significant risk factors in CKDu development.

Each nephropathy has a distinct trend of expression(presentation) of specific biochemical markers that can be suitably measured in biological matrices like blood or urine which assists in the quick detection and identification of the type of renal pathology(i.e. glomerular or tubular) manifested so that necessary therapeutic and preventive measures can be taken to prevent progression to renal failure. Since the biochemical based pattern of disease presentation of CKDu in Canacona was not elucidated till date, this thesis described the same by analysis of biochemical markers specific of tubular and glomerular damage in the major biological matrices (i.e. blood and urine) of 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 specifically chronic tubulointerstitial nephritis(CTN) is represented by the significant elevation in urinary levels of markers such as uBCR (urinary b2M to creatinine ratio) and uNCR (urinary NAG to creatinine ratio) which denotes levels of low-molecular weight proteins (β2-microglobulin, b2M) and tubular cells localized enzyme (N-acetyl glucosaminidase, NAG) respectively, that considerably rise in the urine on tubular injury due to nephrotoxin induced disruption in the inherent tubular reabsorption of these proteins into plasma. Whereas glomerular damage is evident from considerable rise in urinary levels of markers such as 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 levels of proteins and its chief constituent i.e. albumin, that abnormally rises in the urine due to diabetes or hypertension triggered glomerular injury which under good-health conditions is not filtered through the glomerulus due to their higher molecular weight. Results from our biochemical analysis depicted that the CKDu affected subjects exhibited a tubular proteinuric pattern (consisting of increased excretion of tubular injury specific proteins viz. b2M and NAG in the urine) as opposed to normal levels of these markers being noted in the urine of healthy control subjects, signifying existence of tubular injury specifically CTN in CKDu affected subjects. These observations were further backed by existence of normal levels of glomerular injury markers in CKDu affected subjects with increased levels of the same being noted in general diabetes and hypertensive CKD affected individuals indicating CKDu and CKD in the corresponding endemic and non-endemic regions of Canacona to be of tubular and glomerular pathological origin respectively. Overall these results were in agreement with the demographic and biochemical findings of CKDu analyses in similarly affected regions of Sri Lanka, Andhra Pradesh (India), Central America and Egypt. Thus our results confirmed CKDu in Canacona to be of a tubular pathological manifestation specifically CTN that is mainly induced by chronic exposure to environmental nephrotoxins, hence contribution of these toxins in causation of CKDu in Canacona was explored in the following chapters.

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 previously reported to be implicated in CKDu causation in similarly affected developing countries, their potential contribution in development of CKDu in Canacona was investigated as well. Two categories of nephrotoxins have been reported as CKDu causative agents which are naturally arising nephrotoxins (that arise from living organisms) like mycotoxin (i.e. ochratoxin) and phytotoxin (i.e. aristolochic acid) along with anthropogenically arising nephrotoxins (that emerges due to human activities) like heavy metals (such as lead, cadmium, arsenic and mercury).All of these abovesaid nephrotoxins exhibit a common cellular and molecular mechanism of nephrotoxicity i.e. they induce oxidative damage to renal proximal tubular cells that eventually manifests in unwarranted induction of apoptotic cell death and inflammation. This persistently induced cellular demise and inflammation amplifies into the development of tubular atrophy and fibrosis, the major hallmarks of CTN which is the pathological manifestation of CKDu. Recently, the nephrotoxic potency of a trace geogenic element viz. silica has been coming into light. Silica abundantly constitutes the earth's crust but possesses limited bioavailability under ideal environmental conditions hence categorized as a trace geogenic element. It's availability to humans when enhanced on anthropogenic disturbances of its major exposure matrix (i.e. groundwater) results in increased exposure that induces severe nephrotoxicity. Silica induced renal toxicity analyses in animal models and a few epidemiological studies in Andhra Pradesh (India) and Balkan region (Europe) have highlighted a causal association between chronic silica exposure (via intake of contaminated groundwater) and development of tubular injuries typical of CKDu. This contributory association was deduced from resemblance of silica induced renal histopathological alterations of tubular atrophy and fibrosis and elevated urinary expression of tubular injury biomarkers (viz.b2M & NAG) with that noted in CTN which is also the pathological hallmark of CKDu. Thus affirming the role of silica in development of tubular pathology, making it a potential nephrotoxin in CKDu causality.

These nephrotoxins viz. heavy metals, ochratoxin, aristolochic acid and silica ubiquitously occur in the environment with limited bioavailability under ideal environmental conditions. Bioavailability of these toxins for human exposure and consequent induction of renal toxicity can be greatly enhanced on human invasion of its exposure matrices i.e. groundwater (well-water) and food by activities like mining, industrial discharge etc. Groundwater exploited through infiltration wells is the primary drinking water source in CKDu targeted rural communities globally with Canacona taluka being no different. Since groundwater is entrapped in the earth's crust (that ubiquitously contains these nephrotoxins but is limitedly available for human exposure under ideal conditions due to inert complex formation with other organic and inorganic elements like metals and non-metals); it can serve as a hub for these toxins (mainly heavy metals and silica) when invaded by human activities like industrial effluent discharges or acid mine drainage(AMD) linked with mining which can cause

increased leaching out of these toxins from the aquifer's bedrock into the water table. Prolonged intake of this nephrotoxin polluted groundwater eventually can result in grave tubular injuries, deeming groundwater to be considered as the main exposure source to nephrotoxins in comparison to food in these CKDu affected regions. Alternatively, human invasion of terrestrial and aquatic ecosystems (like mining and industrialization) and inadequate farming techniques (like inappropriate food grains storage enabling the growth of ochratoxin producing fungal sp.-*Aspergillus ochraceus* & untimely clearing of aristolochic acid producing contaminating weed sp.-*Aristolochia indica*) can result in excessive contamination of food crops and animals with heavy metals, ochratoxin & aristolochic acid. Humans can be directly exposed to these toxins through food chain which on chronic exposure induces severe nephrotoxicity. Hence these abovementioned nephrotoxins were widely analysed in main exposure sources i.e. groundwater (well-water) & food consumed by Canacona's CKDu affected & unaffected regions, with results described in Chapter 3.

Our study's results depicted the groundwater of Canacona's CKDu affected region contained significantly elevated levels of silica nephrotoxin (i.e. 115.5 mg/L) which exceeded the levels of 90 mg/L that were previously reported to induce tubular toxicity on chronic intake(> 30 yrs.) (as obtained from animal and epidemiological silica toxicity analyses).Additionally the CKDu region's groundwater depicted borderline levels of lead (i.e. 9.98 µg/L) which were approaching the WHO permissible limit of 10 µg/Contrarily, the healthy control region's groundwater contained low silica concentration (i.e.13.5 mg/L) and WHO safe lead levels (i.e. 0.83 µg/L).The difference in these nephrotoxins levels in the CKDu affected and healthy regions were accredited to dissimilarities in the geological make-up of the aquifer's bedrock, pH and chemistry of the groundwater. CKDu affected region's groundwater contained elevated nephrotoxin levels possibly due to the close proximity of this region to a non-operational granite mine. As an outcome of which the consequential and inescapable acid mine drainage originating from the non-functional mine could have travelled to the neighboring CKDu hit region's aquifer that could have resulted in the increased groundwater acidity as noted(pH=5.6).This acidic groundwater ultimately caused the excessive leaching of these nephrotoxins (i.e. silica and lead) into the groundwater from the silica enriched (81% by weight) and lead laden(2.5 % by weight) granitic bedrock of the CKDu affected region aquifer [as geologically analysed by Fernandes and Widdowson,2009]; due to the tendency of toxin bioavailability to rise at an acidic pH. Another factor that contributed to the increased toxin availability was noted to be essential metal cation deficiency (viz. Ca, Mg, Al etc.) in the affected region's groundwater wherein these essential metal cations under optimal levels have been proved to restrict the toxins' availability by trapping them via formation of inert metal complexes with the toxins. This increased toxins' availability at the groundwater's acidic pH and essential metal cation scarcity in addition to aquifer's granitic composition illuminates the low toxins levels noted in the healthy region's groundwater. The low toxins levels in the unaffected region's groundwater was accounted to its neutral pH(pH=7) and metabasitic geological make-up of the aquifer's bedrock [that is silica poor(<40% silica), lead

poor(0.025%) and enriched in essential metal cations like Ca,Mg etc], hence do not pollute the groundwater with these toxins. Therefore, the considerably enhanced silica and lead levels in the CKDu affected region's groundwater ensued a significantly elevated daily intake of these toxins (which surpassed WHO set tolerable intake levels) and increased risk of developing severe nephrotoxicity (as noted from the target hazard quotients exceeding the WHO set safe threshold of 1) at the intake levels prevalent among CKDu affected subjects. Concurrently, no remarkable unsafe intake levels or associated renal-toxicity risk was observed for these nephrotoxins on exposure via the food route in the CKDu affected region due to occurrence of JCEFA set safe levels of these toxins in the food consumed in the taluka. Parallely, WHO and JCEFA set safe intake levels and no related nephrotoxicity risk was noted on food and groundwater ingestion in the taluka's healthy region, accredited to the presence of safe nephrotoxin levels in this region's food and groundwater. Therefore our results highlighted groundwater to be the major exposure source of these nephrotoxins viz. silica and lead in Canacona's CKDu affected region which was consistent with a similar trend observed in other CKDu hit regions such as Balkan region(Europe), Uddanam region of Andhra Pradesh(India) and Central America.

In order to provide 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, we conducted the biological monitoring of the toxins. This was achieved by analysis of the levels of biomarker of nephrotoxin exposure i.e. measurements of toxin levels in blood (that reflects the toxin's internal dose) and correlation(via Pearson's correlation coefficient calculation) of these internal exposure levels with induction of tubular dysfunction evident from 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 causal nephrotoxins(i.e. silica and lead) in triggering CKDu at the prevalent internal exposure levels. The levels of nephrotoxins exposure were also measured in healthy unaffected controls to rule out any existing and future risk of CKDu development in these subjects. Moreover levels of other nephrotoxins (i.e.Cd,As,Hg,ochratoxin and aristolochic acid) in blood were analysed and levels were correlated with tubular injury markers in CKDu affected subjects to rule out causative contribution(if any) of these nephrotoxins in taluka's CKDu manifestation. From our results, it was noted that significantly elevated levels of silica (100.2 mg/L) and lead(317.8 µg/L) were noted in the blood of CKDu affected subjects that was found to exceedingly surpass their respective WHO permissible limits and levels of 90mg/L(for silica) and 60 mg/L(for lead) which are well reported in inducing severe proximal tubular toxicity. These significantly high levels of the nephrotoxins in the blood of these affected individuals possibly stemmed from the chronic consumption of groundwater containing remarkably high levels of the same which was backed by significantly($p<0.05$) higher Pearson's correlations between the levels in the groundwater and blood. These remarkably elevated internal exposure levels of silica and lead in the blood of CKDu affected subjects exhibited a significantly($p<0.05$) strong and higher dose

response association in the induction of tubular pathology typical of CKDu which was evident from tubular injury markers(i.e. b2M & NAG) rising in strong association(evident from higher Pearson correlation coefficients) with increasing levels of these toxins in the blood. Overall, the occurrence of significant dose-response associations between the high blood lead and silica levels and elevated tubular nephropathic markers as noted in the Canacona's CKDu affected subjects were in agreement with similar levels of dose-response associations noted for these toxins in induction of tubular damage typical of CKDu in Central America and Balkan region (Europe) respectively. Thus our results is the 1st ever report that supported the role of silica and lead nephrotoxins at the high internal exposure levels in blood that are prevalent in the affected subjects (from chronic intake of contaminated groundwater) in the development of CKDu in Canacona. These observations were backed by the occurrence of non-toxic levels of silica (30.7 mg/L) and lead (6.3µg/L) in the blood of the unaffected healthy individuals that exhibited no significant associations with the low and normal levels of tubular injury markers in these subjects denoting absence of toxin induced tubular damage in these subjects as expected. This silica and lead induced renal toxicity in Canacona's CKDu affected subjects was further aggravated by significantly lowered essential metals ions levels (like zinc, manganese, copper, iron, selenium) in the blood of CKDu affected subjects as these metals ions under optimal levels retards intestinal absorption of heavy metals by causing competitive exclusion of heavy metals in the urine by preventing the binding of these heavy metals to the common metal transporter. Essential metals also confers anti-oxidative defense from heavy metal triggered oxidative renal damage as these ions serve as metal cofactors for anti-oxidant enzymes like superoxide dismutase and glutathione peroxidase. Hence reduced levels of these essential cations in CKDu patients' blood failed to restrict the toxin availability and related accumulation and also enhanced the vulnerability to lead induced oxidative tubular damage. This toxin limiting potency and anti-oxidative capacity of essential cations explains the lack of renal damage in the healthy study group attributed to the their blood containing optimal essential cations levels and WHO set safe silica and lead levels.

Therefore, overall our results strongly confirmed the etiological role of higher and prolonged exposure to two nephrotoxins viz. lead and silica via chronic consumption of untreated nephrotoxin polluted groundwater in the induction of severe tubular injuries associated with the pathogenesis of CKDu in Canacona.

Chapter 4: Elucidation of the cellular and molecular renal toxicity mechanisms of an 'emerging environmental nephrotoxin - Silica' by 'in-vitro' cytotoxicity studies

Previous biochemical based analyses of the CKDu cases in the Balkan region(Europe) and histopathological assessments of silica induced renal toxicity in animal models(in-vivo) have established chronic tubulointerstitial nephritis(CTN) to be the main histopathological manifestation of silica triggered nephrotoxicity. But these investigations failed to illuminate the mechanisms of silica induced renal-toxicity. Hence this PhD research provided the 1st ever report of the cellular and

molecular toxicity mechanism of silica induced nephrotoxicity 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. Herein the proximal-tubular cytotoxicity outcomes following chronic exposure (for 7 days) of HK-cells to rising silica doses (80-120 mg/L) were examined via a panel of assessments consisting of cell-viability, oxidative-injuries, inflammatory responses, genomic-damage, cell-cycle arrest, mitochondrial-integrity and apoptotic-pathway regulation. Moreover, our research provided supporting evidence to the causal involvement of silica in CKDu development in Canacona. The results of this study are described in Chapter 4.

From our study, it was observed that silica exhibited nephrotoxicity as function of dose and time wherein severe proximal-tubular cytotoxicity (evident from decreasing HK-cell-density) was noted only on prolonged exposure (for 7 days) to increasing-doses (≥100 mg/L), that was mediated by silica-toxin triggered mitochondrial-dysfunction. Silica interrupted the structural and functional architecture (i.e. cellular respiration) of the mitochondria owing to the inherent enrichment of the proximal tubular cells with mitochondria due to the high energy requirements for reabsorption and filtration functions. Prolonged exposure to high-silica doses (≥100 mg/L) triggered incessant mitochondrial-injuries that 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 owing to the role of mitochondria in ROS homeostatic maintenance. 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 triggered the Chk1-checkpoint kinase of the cell to grant death verdicts by activating the p53 gene which ultimately triggered 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 continual activation of pro-inflammatory and fibrogenic cytokines (IL-1β, TNF-α, TGF-β, IL-2 and IL-6) which resulted in unwarranted inflammation that ultimately could have manifested in the development of fibrosis. Therefore, persistent mitochondrial mediated apoptosis and enhanced inflammation triggered by intracellular-stressors (i.e. enhanced ROS and

DNA-damage) being generated 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 silica induced incessant tubular apoptotic cell-death and inflammation eventually culminates in the development of tubular-atrophy and fibrosis (at the organ-level), the typical features of chronic tubulointerstitial nephritis which is the major hallmark of CKDu pathogenesis. 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. These silica triggered nephrotoxicity mechanisms were in agreement with those inflicted by nephrotoxins (like heavy-metals) that have been previously implicated in CKDu causation; thus validating the renal tubular toxicity inducing potency of silica in CKDu development. Furthermore, our study also highlighted ≥100 mg/L to be the nephrotoxic silica-dose that induced severe renal proximal-tubular (HK-cells) toxicity and linked CKDu development on chronic exposure. This dose's CKDu-inducing potential was supported by its resemblance with the CKDu inducing silica levels noted in the blood (100.2 mg/L) of the affected individuals of Canacona. Moreover silica exhibited reversal of nephrotoxicity on lower sub-toxic dosing which was apparent from the lack of CKDu development in healthy control subjects containing sub-toxic silica levels(30.7 mg/L) in the blood. This non-toxicity could be accredited to the faster toxin-clearing by metallothioneins due to lowered detoxification demand at such doses in conjunction with the uninhibited cellular-repair potency at lower-doses which prevented toxin accumulation & induction of nephrotoxicity. Therefore, our results provided supporting evidence to the causal role of this nephrotoxic silica-dose (100 mg/L) [prevalent in the blood of the affected individuals of Canacona], in significantly inducing renal tubular damage and linked CKDu development on chronic exposure in Canacona. Thus suggesting that the nephrotoxic potency of silica can be a major considerant when deciphering the etiology of CKDu witnessed in developing countries that are prone to anthropogenic influence.

Chapter 5: Development of a 'fluorimetric chemosensor' for 'detection of silica bioaccumulation' in human renal proximal tubular cells

Since silica was established to the major causal nephrotoxin responsible for the development of CKDu in Canacona we decided to develop a probe for the quick detection of accumulated silica in susceptible human renal proximal tubular cells(HK-cells). Previously reported epidemiological studies in the Balkan region (Europe) have established the nephrotoxicity of silica to be exhibited by its water soluble, monomeric, and highly reactive form viz. orthosilicic acid (H_4SiO_4) that has the capacity to produce H^+ ions, the levels of which are well regulated under lower silica exposure conditions by the intrinsic cellular repair capacity.

However, due to the bioaccumulative potency of orthosilicic acid it can excessively accumulate in the cells on prolonged and higher silica exposure that allows it to exceed the super saturation point of

polymerized silica formation resulting in orthosilicic acid being retained in its unpolymerised state. This overly accumulated unpolymerised form can continuously generate toxic H^+ ions and free radicals which are beyond neutralization by the innate oxidative repair capacity of the cells resulting in severe induction of tubular toxicity. Hence we hypothesized that by detection of H^+ ions in the targeted cells (i.e. renal proximal tubular cells) on silica exposure will give us an indirect measure of the accumulation of silica specifically orthosilicic acid inside these cells that could possibly assist in the early diagnosis of silica induced nephrotoxicity. Thus, 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 toxic silica species (viz. orthosilicic acid) bioaccumulation in nephrotoxin targeted cells viz. human renal proximal tubular-HK-cells, in-vitro. The sensor (Rh1@TiO_2) was produced 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 due to its ability to detect the H^+ ions that will be continuously released from the overly accumulated orthosilicic acid (on prolonged exposure to high silica concentrations) that would induce the chemodosimeter’s spirolactam ring-opening, generating a strong orange fluorescence output due to rhodamine formationhis synthesized silica sensor displayed various merits such as good water dispersibility, no organic solvents usage during fluorimetric and spectrophotometric studies, absence of intrinsic interference from the probe at an acidic pH, rapid ‘turn-on’ type of signal transduction and detection cum imaging of silica accumulation. This sensor proved to be biocompatible with effortless cellular penetrability that detected silica (specifically orthosilicic acid) accumulation as a function of dose and time with highest fluorescence output being noted on chronic exposure (8 days) to higher silica doses of ≥100 mg/L due to increased orthosilicic acid accumulation at such conditions. Therefore, this sensing material displayed promising potential for quick detection of silica accumulation in targeted renal proximal tubular cells and related nephrotoxicity associated with CKDu development in Canacona so that necessary preventive and therapeutic measures can be taken to reduce silica exposure preventing future rise in CKDu incidences.

Conclusion

This PhD research managed to provide significant and detailed amount of information on the CKDu scenario in the Canacona taluka of south Goa, India. Our research preliminarily defined the geographical prevalence of CKDu in Canacona wherein it was found to be restricted to two main villages namely Ponsulem and Chaudi. We also provided information on the demographic stratification of CKDu wherein the disease exhibited no existence of traditional causals like diabetes and hypertension, absence of gender and occupational predisposition and was found to target rural communities mainly younger adults in the age group of 30-50yrs.Moreover the risk factors for CKDu

development were identified to be presence of a non-operational mine in the vicinity, prolonged consumption of untreated well water, occurrence of skeletal disorders among the CKDu population and chronic intake of NSAID's for pain mitigation. Thus with the awareness of risk factors can help in the prediction of the risk of developing renal toxicity when exposed to the same and by appropriate intervention and modification of these CKDu risk factors in early stages could be beneficial in averting the advancement of this disease to renal failure. This thesis also established the type of renal pathology inflicted in CKDu of Canacona for the 1st time by analysis of the trend of presentation of various biochemical markers of tubular and glomerular pathology. Herein significantly elevated levels of urinary elimination of tubular injury biomarkers (viz. uBCR, uNCR) and normal levels of glomerular injury markers (viz. uPCR, uACR, uAlb/b2M ratio and uAPR) were noted in the CKDu affected subjects. Thus our results established the pathological manifestation of CKDu in Canacona to be of the tubular type specifically chronic tubulointerstitial nephritis (CTN) which was consistent with the disease presentation noted in other CKDu affected regions of Central America, Sri Lanka, Egypt and Balkan (Europe).Thus by illumination of the biochemical based pattern of CKDu presentation in Canacona could help in rapid and early screening and diagnosis of CKDu in susceptible populations allowing the adoption of suitable preventive or treatment measures to deter the progression towards end stage renal disease(renal failure), thus improving clinical outcomes, patient safety and morbidity reduction.

Since environmental nephrotoxin induced CTN was proved to be the pathological manifestation of CKDu, exposure to environmental nephrotoxins were presumed to be involved in CKDu causation in Canacona. From this PhD research we established a strong dose response link of silica and lead nephrotoxins at the significantly high and WHO limit surpassing internal exposure levels in the blood (that stemmed from chronic intake of groundwater polluted with high levels of these toxins) in the induction of tubular pathology typical of CKDu in Canacona, which was evident from the urinary tubular injury markers (i.e. uBCR and uNCR) rising in strong association with increasing levels of silica and lead in the blood of the CKDu subjects. Thus through our study we managed to prove that prolonged exposure to high levels of silica and lead routed through untreated nephrotoxin contaminated groundwater intake to be the major environmental nephrotoxins that are responsible for the etiological development of CKDu in Canacona This is the first ever report to establish the potential etiological agents for CKDu in Canacona since the past two decades and established groundwater to be the major route of exposure to these causal toxins. As the causal agents have been elucidated necessary preventive and remediative measures to reduce exposure to these toxins like groundwater treatment before consumption to free it from the toxins by techniques like reverse osmosis, chemical coagulation and ultrafiltration can be adopted to decrease the future rise in CKDu occurrences in Canacona.

Since silica and lead nephrotoxins were established to the major etiological agents of CKDu in Canacona we studied their nephrotoxicity mechanisms in detail. Since the renal toxicity mechanism of

lead has already been well-established no further analysis was done on the same. This PhD research focussed on deciphering the *in vitro* cellular and molecular toxicity mechanisms of the recently emerging nephrotoxin viz. silica using an ideal nephrotoxic assessment cell-model viz. normal human renal proximal tubular cells (HK cells) as it was not elucidated till date. Our findings established silica induced proximal tubular nephrotoxicity to be dose and time dependent with significant toxicity being noted only on chronic exposure to higher silica doses (i.e.≥100 mg/L). The toxicity mechanism was interceded by silica triggered mitochondrial mediated oxidative damage (i.e. unwarranted ROS generation and DNA damage) in proximal tubular cells being generated only on high dosing (i.e.≥100 mg/L) that ultimately resulted in the incessant induction of apoptosis and inflammation, which clinically exaggerated into the development of tubular atrophy and fibrosis, the major hallmarks of CTN that is also the pathological manifestation of CKDu. These nephrotoxicity-mechanisms were consistent with those inflicted by nephrotoxins (like heavy-metals) reported to be involved in CKDu development, thus validating the nephrotoxic potential of silica in CKDu causation. Our study also established 100 mg/L to be the nephrotoxic silica dose which was in consensus with the levels noted in the blood of Canacona's CKDu patients thus providing supporting evidence to the role of silica at internal exposure levels of 100 mg/L to be responsible for CKDu development in Canacona. Through this study we elucidated the sub-cellular targets of silica incited nephrotoxicity to be mitochondria and nucleus. Thus with this knowledge, suitable prophylactic and therapeutic modalities(like anti-oxidant therapy) can be adopted to guard these targets from toxin attack, possibly preventing stimulation of tubular nephrotoxicity and decrease the rise in CKDu incidences in Canacona.

Since silica was established to be the causal nephrotoxin for CKDu development in Canacona we decided to develop a biosensor for quick detection of accumulation of the same in targeted cells viz. human renal proximal tubular cells (i.e.HK cells) as such probes are not widely available. This sensor could help in the quick diagnosis of silica induced renal toxicity so that necessary measures can be taken to reduce toxin exposure and treat the manifested tubular damaging effects. Our developed sensor was focussed on detecting the accumulation of toxic silica species i.e. orthosilicic acid in the tubular cells as this species is the main form that has been reported to be involved in the nephrotoxicity induction. The sensor(Rh1@TiO_2) was prepared by simple physisorption of a fluorophore i.e. Rhodamine hydrazide (Rh1,which can detect H^+ ions), onto the biocompatible, non-toxic TiO_2 nanoparticles. This newly developed water-dispersible fluorescent nanoprobe demonstrated the ability to detect orthosilicic acid in aqueous media, and in biological systems(i.e. renal tubular cells) via interaction with the incessantly released H^+ ions from orthosilicic acid. The H^+ ions triggered the ring opening of the fluorophore which resulted in a strong orange fluorescence that was easily quantified. Our silica sensor offers several advantages like good water dispersibility, lack of organic solvents usage during fluorimetric studies, quick turn-on type signal transduction, biocompatibility and easy cellular penetrability. Due to the various merits conferred by this sensor it exhibited possible potential to be used as a medical tool by health professionals for early identification

of silica induced nephrotoxicity that can allow timely adoption of therapeutic measures, thereby reducing the global burden of CKD.

Overall, this thesis illuminated various aspects of the CKDu scenario in Canacona that have never been explored before such as the demographic and geographical distribution of the disease, significant risk factors for disease development, biochemical based pattern of disease presentation for assisting in quick diagnosis, major route of exposure to the causal factors, etiological agents(i.e. nephrotoxins) responsible for the causation of CKDu in the taluka, nephrotoxicity mechanism of the recently emerging toxin (viz. silica) involved in CKDu development in the taluka and a rapid method (i.e. biosensor) for the quick detection of silica induced nephrotoxicity.

We hope that through our research, the medical experts and the health regulatory bodies can take some cue and adopt necessary preventive and therapeutic measures to reduce exposure of the population to suspected causal nephrotoxins (viz. silica and lead) which could possibly help in averting the future rise in CKDu incidences in this taluka, thus providing some sort of relief to the residents suffering from this epidemic CKDu curse for the past two decades.