Role of Histone Ubiquitination and Angiotensin Converting Enzyme 2 in the Development of Renal Fibrosis under Type I Diabetic Condition

THESIS

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CERTIFICATE

This is to certify that the thesis entitled "**Role of Histone Ubiquitination and Angiotensin Converting Enzyme 2 in the Development of Renal Fibrosis under Type I Diabetic Condition**" and submitted by **Mr. Goru Santosh Kumar**, ID. No. **2013PHXF404P** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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Date: **Goru Santosh Kumar**

Abstract

Hyperglycaemia-induced expression of extracellular matrix (ECM) components plays a major role in the development of diabetic nephropathy (DN). Even though several novel therapeutic approaches have come up to prevent the progression of DN, the number of people with DN still increasing globally. This suggests us to find novel therapeutic strategies to prevent it completely. Recent reports have indicated the ubiquitin proteasome system (UPS) alterations in DN. Moreover, the proteasome inhibitor, MG132 was found to be protective in DN. In addition, renin angiotensin system (RAS) alterations were also implicated in the progression of DN. Existing reports highlighting the role of angiotensin converting enzyme 2 (ACE2) in the pathogenesis of diabetes. Moreover, the activity and expression of ACE2 was found to be reduced in kidneys of diabetic patients, as well as in animal models of diabetes. Several recent studies have also showed the potential role of recombinant ACE2 administration in preventing DN. However, the molecular and epigenetic mechanisms in the progression of renal fibrosis under diabetic condition were not understood completely.

The epigenetic mechanisms that modulate ECM gene expression in DN remain unclear. Therefore, we examined the role of histone H2A and H2B monoubiquitination on epigenetic chromatin marks, such as histone H3 lysine dimethylation (H3K4Me2, H3K9Me2 and H3K79Me2) in type 1 diabetic rat kidney. Hyperglycaemia increased collagen deposition and Col1a1 gene expression. In whole kidney of diabetic animals, both H2AK119 mono-ubiquitination (H2AK119Ub) and H2BK120 mono-ubiquitination (H2BK120Ub) were found to be increased, whereas, in glomeruli of diabetic animals, expression of both H2AK119Ub and H2BK120Ub was reduced. Changes in ubiquitin proteasome system components like increased Rnf2 (H2A-specific E3 ligase) and decreased H2A- and H2B-specific deubiquitinases (ubiquitin-specific proteases 7, 16, 21 and 22) were also observed. Globally increased levels of chromatin marks associated with active genes (H3K4Me2 and H3K79Me2) and decreased levels of repressive marks (H3K9Me2) were also observed. Hyperglycaemia also increased the protein expression of SET7/9 and decreased the expression of SUV39H1. We also showed the decreased occupancy of H2AK119Ub and H2BK120Ub on the promoters of SET7/9 and SUV39H1 in diabetic kidney. In addition, methylation marks regulated by H2AK119Ub (H3K27Me2 and H3K36Me2) and H2BK120Ub (H3K4Me2 and H3K79Me2) were also found to be altered on the promoters

of SET7/9 and SUV39H1. Taken together, these results show the functional role of H2AK119Ub and H2BK120Ub in regulating histone H3K4Me2 and H3K9Me2 through modulating the expression of SET7/9 and SUV39H1 in the development of diabetic renal fibrosis. Further, in our study, the known proteasomal inhibitor (Aspirin), was also found to prevent renal fibrosis in diabetic kidney, through H2AK119 and SET7/9 mediated pathway.

In addition, we observed the decreased levels of ACE2 in glomeruli isolated from diabetic animals and these levels were restored after treatment with ACE2 activator diminazene aceturate (DIZE). Moreover, in our study, DIZE administration prevented the renal fibrosis and apoptosis in diabetic kidney. Interestingly, chronic DIZE administration increased the protein and mRNA expression of AT2 receptor in diabetic kidney. DIZE treatment also decreased the levels of Ang II and increased the levels of Ang 1-7, indicating the increased ACE2 activation in diabetic kidney. Surprisingly, even after increased Ang 1-7 levels, we did not observed Mas1 receptor expression in renal tissues of DIZE treated animals. Further, all these protective actions of DIZE were prevented in presence of AT2 blocker (PD123319). These results clearly indicating that, DIZE mediated protective actions in diabetic kidney are through ACE2/Ang 1-7/AT2 axis.

In conclusion, these results clearly indicating the critical role of histone ubiquitination and ACE2 in the development of renal fibrosis under diabetic condition. Further, additional research is required to explore the role of histone ubiquitination and histone specific UPS components to find the novel hidden targets in the progression of DN. Moreover, our findings indicating the need of novel ACE2 and AT2 activators in future therapeutics to prevent DN.

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List of Abbreviations

AGEs	Advanced glycation end products
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
APC/c	Anaphase promoting complex / cyclosome
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
Ang II	Angiotensin II
Ang 1-7	Angiotensin 1-7
Ang 1-9	Angiotensin 1-9
c-Cbl	Casistas B-lineage lymphoma
CP	Core particle
CRLs	Cullin ring ligases
COX-2	Cyclooxygenase 2
DUBs	De-ubiquitinases
DN	Diabetic nephropathy
DKK1	Dickkopf related protein 1
DIZE	Diminazene aceturate
ECM	Extra cellular matrix
GMCs	Glomerular mesangial cells
H2AK119-Ub	H2AK119 mono-ubiquitination
H2BK120-Ub	H2BK120 mono-ubiquitination
HRV	Heart rate variation
HATs	Histone acetyl transferases
HDACs	Histone de-acetylases
HMTs	Histone methyl transferases
HMGB1	High mobility group box-1
Hrd1	Hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1
HUVECs	Human umbilical cord vein endothelial cells
IFN-γ	Interferon gamma
MCP-1	Monocyte chemo-attractant protein 1
NFκβ	Nuclear factor kappa-β
Nox	NADPH oxidase
PH	Pulmonary hypertension

PARP	Poly (ADP-ribose) polymerase
RAGEs	Receptors for AGEs
RAS	Renin angiotensin system
RUK/CIN85	Regulator of ubiquitous kinase / Cbl interacting protein of 85 kDa
Smurf-2	Smad ubiquitin regulatory factor 2
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
UCHL1	Ubiquitin carboxy terminal hydroxylase 1
UPS	Ubiquitin proteasome system
USP	Ubiquitin specific protease

Chapter 1
Introduction

1. Introduction:

Diabetic nephropathy (DN), one of the major microvascular complications is characterized by glomerular hypertrophy, increased basement membrane thickness and accumulation of extracellular matrix (ECM) in the glomerular mesangium and tubular interstitium[1,2]. The cumulative accumulation of collagen and fibronectin, important components of ECM, and elevated expression of transforming growth factor-β1 (TGF-β1) in the glomerular mesangium due to high glucose and elevated advanced glycation end products (AGEs) are critical for diabetic renal fibrosis, which eventually leads to DN [2-4]. However, the molecular mechanisms involved in the progression of diabetic renal fibrosis are not yet understood completely.

Dysregulation of ubiquitin proteasome system (UPS) has been implicated in pathogenesis of diabetes [5]. UPS, an essential degradation mechanism that regulates the quality and function of various proteins, and any abnormalities in UPS usually result in the pathogenesis of many diseases like cancer, neurodegenerative diseases, metabolic syndrome and inflammatory diseases [6]. Earlier it was thought that ubiquitination of substrate proteins by UPS leads to 26s proteasome mediated degradation. However, it is now clear that ubiquitination of proteins is also involved in regulation of several cell signalling pathways [6,7]. The process of ubiquitination is carried out by the sequential action of activating (E1), conjugating (E2) and ligating (E3) enzymes and it can be reversed by the action of deubiquitinases (DUBs) [6]. It is well documented that both ubiquitin E3 ligases and DUBs regulate the cellular events by acting on different protein substrates [8,9]. Increased UPS activity with increased NF-κB levels has been reported in diabetic atherosclerotic plaques and these changes could be reversed by Rosiglitazone treatment [10]. In another study, loss of histone H2A/H2B DUB, *Usp22* was found to increase fibrotic genes like fibronectin and *Tgfb1* in rat mesangial cells under hyperglycaemic condition [11]. In addition, inhibitor of UPS, MG132 has been proved to be protective in DN by preventing the degradation of Smad7 [12]. These reports demonstrate a critical role of UPS in diabetes. However, very little is known about UPS and its functional role in regulating the epigenetic mechanisms in the pathogenesis of DN.

Epigenetic modifications including DNA methylation and histone modifications alter the chromatin structure without changing the DNA sequence. The post-translational

modifications of nucleosomal histones such as histone H2A, H2B, H3 and H4, including ubiquitination, methylation, acetylation and phosphorylation at key lysine play a major role in regulating gene transcription process. Acetylation of histone H3 lysines (H3KAc) is associated with active gene transcription, whereas methylation (H3KMe) can be associated with, either active or repressed gene promoters depending on the position of lysine modified. H3KAc is mediated by histone acetyl transferases and H3KMe by histone methyltransferases (HMTs) [13]. Histone H3K4Me, H3K36Me and H3K79Me are usually associated with gene activation and transcriptional elongation. H3K9Me and H3K27Me on the other hand, are generally associated with gene repression. HMTs, such as SET7/9, SET1 and MLL1-4 promote active transcription through H3K4Me. Whereas, HMTs like NSD1, SMYD2 and SET2 act through H3K36Me. DOT1 is the only HMT that specifically acts on H3K79Me and participates in active gene transcription. HMTs, such as SUV39H1, G9A, and SET1/ESET suppress transcription through H3K9Me. HMTs, EZH2 and PRC2 promote H3K27Me and thereby repress transcription [13-15]. There should be a balance between active and repressive chromatin marks for normal gene transcription; any disruption in this may result in abnormal gene transcription and disease phenotypes. Recent evidence has also implicated the involvement of epigenetic mechanisms in diabetes and its complications [16]. Even though, various epigenetic mechanisms involving histone acetylation, methylation and other histone modifications have been well explored; more importantly, very little is known about the histone H2A and H2B mono-ubiquitination in DN pathogenesis.

Existing reports have demonstrated that histone H2AK119 mono-ubiquitination (H2AK119Ub) and H2BK120 mono-ubiquitination (H2BK120Ub) are also involved in transcriptional regulation [17]. Ubiquitination of H2AK119 is mediated by the BMI-1/RING-1A protein found in the human polycomb complex and is associated with transcriptional repression. In contrast, H2BK120 ubiquitination is mediated by human RNF20/RNF40 and UbcH6 and is required for active transcription [18,19]. BMI1, an E3 ligase, plays an important role in H2A ubiquitination and *Hox* gene silencing through H3K27Me by increasing methyltransferase EZH2 and H2AK119Ub [18]. Moreover, H2B ubiquitination is associated with the transcribed regions of highly expressed genes [20]. Overexpression of RNF20, an E3 ligase specific for H2B, subsequently increased the levels of H3K4Me and H3K79Me, and stimulation of *Hox* gene expression. In contrast, inhibition

of RNF20/40 complex reduced H2B mono-ubiquitination, lowers H3K4 and H3K79 methylation, and repressed *Hox* gene expression [19]. In addition, repressive chromatin mark histone H2AK119Ub and active mark H2BK120Ub were recently found to be involved in the expression of fibrotic genes like fibronectin and *Tgfb1* in rat glomerular mesangial cells under hyperglycaemic condition and MG-132, a potent UPS inhibitor was found to be protective through altering these changes [21]. Existing evidence, demonstrates that aspirin inhibits UPS, as potent as MG-132 [22,23]. Moreover, Aspirin was found to prevent experimentally induced DN [24]. However, the epigenetic mechanisms that involve H2AK119Ub and H2BK120Ub in DN and the effect of Aspirin on these mechanisms are least understood.

Moreover, accumulating evidence suggests that the activation of renal renin-angiotensin system (RAS) play an important role in the diabetic nephropathy progression through generating a pathological peptide angiotensin II (Ang II), and blockade of the RAS was proved to be protective in the development of diabetic kidney injury [25]. Ang II acts through two angiotensin receptors, Ang II type 1 receptor (AT1) and Ang II type 2 receptor (AT2). However, Ang II mediates its pathological actions in kidney through AT1 and includes cellular differentiation, proliferation, hypertrophy, fibrosis, renal vasoconstriction and increased tubular sodium reabsorption [26] whereas, the functional role of AT2 in the development of DN is not understood completely. Recent reports suggest that the actions like anti-inflammatory, antifibrosis, vasodilation, anti-hypertrophic and anti-apoptotic effects are due to activation of AT2 [27-29]. Although, chronic treatment with angiotensin receptor blockers (ARBs) and angiotensin converting enzyme (ACE) inhibitors are effective in retarding the progression of DN but it is not a cure [30,31], indicating requirement for finding additional pathways with in RAS system as potential drug targets.

A subsidiary part of RAS is angiotensin converting enzyme 2 (ACE2), shares 42% homology with angiotensin converting enzyme (ACE) but with different biochemical activities. ACE and ACE2 co-expressed in many tissues, ACE2 actions are counter regulatory to ACE and are required for reno-protection by degrading/cleaving Ang II, thereby generating a protective peptide angiotensin-(1-7) (Ang 1-7) [32]. In several animal models of diabetes, ACE2 expression and activity was found to be decreased in renal tissue [33,34]. In male

Akita mice, a model of type 1 diabetes, treatment with human recombinant ACE2 (hrACE2) reduced albuminuria, mild hypertension, plasma Ang II levels, nicotinamide adenine dinucleotide phosphate oxidase activation, glomerular hypertrophy, mesangial matrix expansion and there by prevented the progression of DN [35]. In another study, adenoviral (Ad)-ACE2 injection in STZ-induced diabetic rats for 4 weeks, improved the signs of DN [36]. In addition, patients with type 2 diabetes, glomerular and tubular ACE2 expressions were also found to be reduced [37]. Taken together, these studies suggest that ACE2 plays a protective role against the development of diabetic nephropathy.

Existing reports have also been demonstrated the protective role of ACE2 activator, Diminazene aceturate (DIZE) in various disease models. DIZE (15 mg/kg per day, s.c.) significantly attenuated the myocardial infarction induced decrease in fractional shortening, improved the maximal rate of rise of left ventricular pressure (LVP) and reversed ventricular hypertrophy. DIZE treatment was found to decrease the infarct area, LV remodeling post myocardial ischemia, and restored normal balance of the cardiac renin–angiotensin system. In addition, DIZE treatment increased circulating endothelial progenitor cells, increased engraftment of cardiac progenitor cells, and decreased inflammatory cells in peri-infarct cardiac regions. All of the beneficial effects associated with DIZE treatment were abolished by C-16, an ACE2 inhibitor [38]. DIZE treatment significantly prevented the development of pulmonary hypertension (PH) induced in male Sprague Dawley rats by monocrotaline, hypoxia, or bleomycin challenge due to an increase in the vasoprotective axis of the lung renin-angiotensin system, decreased inflammatory cytokines, improved pulmonary vasoreactivity, enhanced cardiac function which were abolished by C-16. The angiogenic progenitor cells derived from the bone marrow of monocrotaline-challenged rats were made dysfunctional under PH conditions and were repaired by DIZE treatment. The angiogenic progenitor cells isolated from patients with PH exhibited diminished migratory capacity toward the key chemoattractant stromal-derived factor 1a, which was corrected by in vitro DIZE treatment [39]. In another study performed by Rigatto and colleagues, it was demonstrated that DIZE (15 mg/kg/day) for a period of 21 days improved monocrotalin-induced PH is associated with a significant increase in sympathetic modulation and a decrease in heart rate variation (HRV) [40]. Recently, short term treatment with DIZE prevented the reduction in ACE2 activity in rats with subtotal nephrectomy [41]. In rats,

treatment with DIZE, found to improve the myocardial infarction through inhibiting cardiac inflammation and apoptosis [38]. In another study, DIZE prevented the hypoxia induced cardiomyocyte cell death through inhibiting high-mobility group box 1 (HMGB1) protein [42]. Accumulating evidence has also showed that DIZE is also beneficial in several diabetes induced pathologies. Chronic treatment with DIZE prevented the oxidative stress and endothelial damage in db/db mice by increasing ACE2 activity and Ang 1-7 levels [43]. ACE2 activator, DIZE, also found to prevent diabetes induced cardiac electrical changes in STZ induced diabetic rats [44]. However, it is not known till date, about the role of DIZE on molecular mechanisms in the progression of DN.

Therefore, the current study was designed mainly to explore the role of histone ubiquitination, proteasomal inhibition and ACE2 activation in the development of renal fibrosis in type 1 diabetic condition.

Chapter 2

Review of Literature

2. Review of literature:

2.1. Diabetic Nephropathy (DN): An over view

2.1.1. Global burden of DN:

Diabetes was first described in 1552 BC and it took more than three millennia to identify its association with DN, but it took only few decades for DN to become the leading cause of end stage renal disease (ESRD) [45,46]. This microvascular complication develops in approximately 30% of the patients with type 1 diabetes and 40% of the patients with type 2 diabetes [47]. The increasing prevalence of DN, correlates with the worldwide rise in diabetes. Worldwide, in the year 2015, 415 million people were estimated to have diabetes; by 2040, it is projected to increase to 642 million, with disproportionate growth in low to middle income countries [48]. DN attributed to diabetes is a major but under-recognized contributor to the global burden of disease. Between 1990 and 2012, the number of deaths attributed to DN rose by 94% [49]. This dramatic rise is one of the highest observed for all reported chronic diseases [50]. Notably, most of the excess risk of all cause and cardiovascular disease (CVD) mortality for patients with diabetes is related to the presence of DN [51].

2.1.2. Clinical features of DN:

The clinical diagnosis of DN usually depends on the detection of microalbuminuria (albumin excretion of more than 30 mg/g of creatinine in 2 out of 3 random urine samples collected in within a six month period) [52]. A subset of patients with microalbuminuria will develop advanced DN; referred as overt nephropathy, clinical nephropathy, proteinuria, or macroalbuminuria [53]. However, progression to microalbuminuria usually occurs after five years from the onset of diabetes. Pathogenesis of the disease is multifactorial, e.g., smoking, hyperglycemia, hypertension, male, genetic predisposition, advance age, retinopathy, macrovascular disease were the risk factors of diabetic nephropathy; and it involves genetic and environmental factors that affect multiple metabolic pathways not necessarily activated by hyperglycemia [54].

Progression of the diabetic nephropathy is divided in clinical stages depending on the duration of the disease [55,56]. The first stage starts prior to any renal damage. It is characterized by renal vasodilation and hyperfiltration that occur early in the onset of

diabetes. Several factors may lead to this hyperfiltration: including hyperglycemia, prostaglandins secretion and increased sodium/glucose reabsorption in the proximal tubule [57]. It has also been associated to increased urinary albumin excretion (UAE) related to physical activity [58]. During the second stage, morphologic lesions develop without signs of clinical disease. The earliest structural abnormality in diabetes is glomerular basement membrane (GBM) thickening. The kidney with early diabetes suffers significant hypertrophy; characterized by enlargement of the organ with a combination of hyperplasia and hypertrophy [59]. This occurs in nearly all patients 1.5 to 2.5 years after the onset of type 1 diabetes. Nonspecific vascular or interstitial changes are prevalent in these patients. Mesangial expansion and occlusion of glomerular capillaries lead to a loss of available surface area for filtration and to a decline in function [57]. Third stage is characterized by small amounts of albumin in the urine, not usually detected by conventional methods. This stage is also named incipient nephropathy [53]. A slow and gradual increase of albuminuria over the years is a prominent feature in this stage. According to the DCCT/EDIC Study, persistent microalbuminuria develops most frequently during the second decade after diagnosis of diabetes [60]. It reflects the existence of endothelial damage in the absence of specific renal lesions; and it is also associated with the beginning of advanced renal pathology [61]. Microalbuminuria could also represent podocytes loss; as podocyte number in patients with type 2 diabetes correlates with the change of albuminuria over time [62]. Although microalbuminuria has been considered a risk factor for macroalbuminuria, not all patients progress to this stage; some of them stay or even may regress to normoalbuminuria [63]. Microalbuminuria is considered to be predictive of progression to nephropathy in type 2 diabetes. However, that may not be the case in type 1 diabetes [57]. Normoalbuminuric patients with diabetes are extremely heterogeneous in renal function and structure [64]. Both, microalbuminuric and normoalbuminuric patients benefit from optimal glycemic control [65]; since it has been shown that about one third of the normoalbuminuric subjects develop diabetic nephropathy within few years after onset of diabetes [64,66-68]. The cause of albuminuria in patients without diabetic glomerulopathy is unclear. It might be related to early and very mild ultrastructural changes [69]. Overt nephropathy is characterized by persistent albuminuria (UAE > 300 mg/d or > 500 mg/d urinary protein excretion) that usually accompanies a decrease in GFR [70]. Macroalbuminuria has been associated to the

presence of proliferative retinopathy, coronary heart disease, and foot ulcers [57]. The prevalence of hypertension increases with higher levels of albuminuria [58]. Other risk factors to develop overt nephropathy include uncontrolled diabetes, smoking, advanced age and high lipids levels [71,72]. ESRD is defined by the presence of signs and symptoms of kidney failure requiring replacement therapy, regardless of the GFR level [73]. It has been described as an important independent predictor of hospitalization and death in adults with heart failure.

2.1.3. Histological changes in DN:

Once the presence of albumin in the urine is confirmed, patients should undergo complete evaluation; including work-up for other etiologies. Renal diseases other than DN have been reported in patients with diabetes. DN usually develops 10 years after onset of type 1 diabetes [63]; however, in type 2 diabetes this is variable [68]. An accurate estimate of damage in DN can only be achieved by the histological analysis of tissue samples [61]. Therefore, the kidney biopsy in patients with diabetes could represent a valuable procedure to establish the stage of the renal disease [74]. The relevance of this diagnostic tool is supported by the observation that when a renal biopsy is performed in patients with DM, results may vary from primary and secondary renal disease with changes unrelated to diabetes to changes of underlying DM [68]. Some of the earliest lesions are characterized by the thickening of the GMB visualized under electron microscopy, but with no findings under light microscopy. The morphologic lesions in type 1 diabetes predominantly affect the glomeruli, with thickening of the GBM and mesangial expansion; although the podocytes, renal tubules, interstitium, and arterioles also undergo substantial changes, especially at later stages of disease [75,76]. Nephropathy in patients with type 2 diabetes is associated with two distinctive patterns of glomerular pathology (nodular and non-nodular) [77]. Nodular type glomerulosclerosis (Kimmelstiel-Wilson nodules) was reported in 1936 by light microscopy. This lesion was initially identified as the only specific feature of DN [78]. It consists of nodular lesions containing areas of marked mesangial expansion forming large round fibrillar mesangial zones with palisading of mesangial nuclei around the periphery of the nodule and compression of the associated glomerular capillaries. Later on, diffuse type glomerulosclerosis was described as a different type of diabetic glomerular lesion [79]. All these diabetic glomerular changes are related to advanced or late DN associated to heavy

proteinuria and/or decreased renal function. Arteriosclerosis is also frequently associated to diabetic glomureolopathy [80].

It has been shown that there is not substantial difference in the injury caused in patients with type 1 diabetes in comparison to type 2 diabetes; and damages are considered basically similar in both types [81]. For this reason, there is a consensus classification combining type 1 and type 2 DN. It is divided into four classes of glomerular lesions. Class I: GMB thickening, composed of isolated GMB thickening and only mild, nonspecific changes by light microscopy that do not meet the criteria of classes II through IV. Class II: mesangial expansion; mild (II a) or severe (II b), without nodular sclerosis or global glomerulosclerosis in more than 50% of glomeruli. Class III: nodular sclerosis (Kimmelstiel-Wilson lesions); at least one glomerulus with nodular increase in mesangial matrix (Kimmelstiel-Wilson) without changes described in class IV. Class IV: advanced diabetic glomerulosclerosis, more than 50% global glomerulosclerosis with other clinical or pathologic evidence that sclerosis is caused by diabetic nephropathy [80,82]. Podocyte injury is also an important feature of DN [59,83-88]; and podocyte loss (podocytopenia) is considered an independent predictor of DN progression in patients with type 2 diabetes [62].

2.1.4. Molecular mechanisms involved in the progression of DN:

Multiple mechanisms contribute to the development and outcomes of diabetic nephropathy, such as an interaction between hyperglycemia induced metabolic and hemodynamic changes and genetic predisposition, which sets the stage for kidney injury [89]. Hemodynamic factors are the activation of various vasoactive systems, such as the renin–angiotensin–aldosterone and endothelin systems. In response, secretion of profibrotic cytokines, such as transforming growth factor β1 (TGF-β1), is increased and further hemodynamic changes occur, such as increased systemic and intraglomerular pressure. Metabolic pathway involvement, among other features, leads to nonenzymatic glycosylation, increased protein kinase C (PKC) activity, and abnormal polyol metabolism. Findings from various studies support an association between increased secretion of inflammatory molecules, such as cytokines, growth factors and metalloproteinases, and development of diabetic nephropathy [90,91]. Oxidative stress also seems to play a central part [92]. Studies that have used inhibitors of the pathways involved in genesis of diabetic nephropathy have shed light on the pathogenesis

of this condition but have not led to expansion of the therapeutic strategies to halt the disease process [91].

2.1.4.1. Hemodynamic pathways

The early signs of glomerular hyperperfusion and hyperfiltration result from decreased resistance in both the afferent and efferent arterioles of the glomerulus. The afferent arteriole seems to have a greater decrease in resistance than the efferent. Many factors have been reported to be involved in this defective autoregulation, including prostanoids, nitric oxide, vascular endothelial growth factor (VEGF), TGF-β1, and the renin–angiotensin system, specifically Ang-II. These early hemodynamic changes facilitate albumin leakage from the glomerular capillaries and overproduction of mesangial cell matrix, as well as thickening of the glomerular basement membrane and injury to podocytes [93]. In addition, increased mechanical strain resulting from these hemodynamic changes can induce localized release of certain cytokines and growth factors [94,95].

The renal hemodynamic changes are mediated partly by the actions of vasoactive hormones, such as Ang-II and endothelin. Glomerular hypertension and hyperfiltration contribute to the development of diabetic nephropathy because use of renin angiotensin blockers preserves kidney function and morphology. Blockade of the renin angiotensin aldosterone system antagonizes the profibrotic effects of Ang-II by reducing its stimulation of TGF-β1 [96]. Support that such profibrotic effects underlie diabetic nephropathy has also been provided by study of an animal model of diabetic nephropathy [97]. Transient blockade of the renin angiotensin system (for 7 weeks) in prediabetic rats reduced proteinuria and improved glomerular structure. Additionally, the administration of an angiotensin- converting-enzyme inhibitor to patients with type 1 diabetes and nephropathy lowered serum concentrations of TGF-β1 [98]. A correlation exists between decreased levels of TGF-β1 in serum and urine and renoprotection, as determined by changes in the glomerular filtration rate over time.

2.1.4.2. Hyperglycemia and advanced glycosylation end products

Hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an

increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. *In vitro* studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production [99,100] and mesangial cell apoptosis [101,102]. Mesangial cell expansion seems to be mediated in part by an increase in the mesangial cell glucose concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells [100]. Hyperglycemia might also upregulate VEGF expression in podocytes [95], which could markedly increases vascular permeability [103,104]. Hyperglycemia, however, does not account fully for the risk of diabetic nephropathy, as shown by studies in which kidneys from nondiabetic donors were transplanted into patients with diabetes and nephropathy developed irrespective of the glucose control [105]. Hyperglycemia might, therefore, be necessary for but not sufficient to cause renal damage. Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway [106,107]. Oxidative stress seems to be a theme common to all three pathways [108].

2.1.4.3. Glycosylation

Glycosylation of tissue proteins contributes to the development of diabetic nephropathy and other microvascular complications. In chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins. This non-enzymatic process affects the glomerular basement membrane and other matrix components in the glomerulus and initially leads to formation of reversible early glycosylation end products and, later, irreversible advanced glycosylation end products. These advanced products can be involved in the pathogenesis of diabetic nephropathy by altering signal transduction via alteration in the level of soluble signals, such as cytokines, hormones and free radicals. Circulating levels of advanced glycosylation end products are raised in people with diabetes, particularly those with renal insufficiency, since they are normally excreted in the urine [109]. The net effect is tissue accumulation of advanced glycosylation end products (in part by cross-linking with collagen) that contributes to the associated renal and microvascular complications [110].

Moreover, advanced glycosylation end products (AGE) interact with the AGE receptor, and nitric oxide concentrations are reduced in a dose-dependent manner [111].

2.1.4.4. Protein kinase C

Other proposed mechanisms by which hyperglycemia promotes the development of diabetic nephropathy include activation of PKC [112]. Specifically, activation of this enzyme leads to increased secretion of vasodilatory prostanoids, which contributes to glomerular hyperfiltration. By activation of TGF-β1, PKC might also increase production of extracellular matrix by mesangial cells [113]. The mechanism by which hyperglycemia leads to PKC activation involves *de novo* formation of diacylglycerol and oxidative stress [114]. PKC activation induces the activity of mitogen-activated protein kinases (MAPK) in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. The co-activation of PKC and MAPK in the presence of high glucose concentrations indicates that these two families of enzymes are linked [115].

2.1.4.5. Aldose reductase pathway

The polyol pathway is implicated in the pathogenesis of diabetic nephropathy. A number of studies have shown a decrease in urinary albumin excretion in animals administered aldose reductase inhibitors [116], but in humans these agents have not been studied widely and the results are inconclusive.

2.1.4.6. Prorenin

Initial clinical studies in children and adolescents suggest that increased plasma prorenin activity is a risk factor for the development of diabetic nephropathy [117,118]. The prorenin receptor in the kidney is located in the mesangium and podocytes, and its blockade has a beneficial effect on kidneys in animal models of diabetes. This effect is mediated by intracellular signals that are both dependent on and independent of the renin–angiotensin system. Prorenin binds to a specific tissue receptor that promotes activation of p44/p42 MAPK [119]. A possible pathogenic role for prorenin in the development of diabetic nephropathy was noted in an experimental model of diabetic mice with streptozotocin-induced diabetes. Sustained prorenin-receptor blockade abolished MAPK activation and prevented the development of nephropathy despite an unaltered increase in Ang-II activity [120]. If prorenin is a key player in the pathogenesis of this disease, use of renin inhibitors

for hypertension that increase prorenin concentrations should demonstrate a harmful effect. To date, no such adverse effects have been reported.

2.1.4.7. Cytokines

Activation of cytokines, profibrotic elements, inflammation, and vascular growth factors such as VEGF might be involved in the matrix accumulation that arises in diabetic nephropathy [121-123]. Although some evidence suggests that VEGF increases permeability of the glomerular filtration barrier to proteins [103], levels of this growth factor can be low in patients with diabetic nephropathy. Thus, the role of VEGF in the pathophysiology of nephropathy is unclear. Hyperglycemia is thought to stimulate VEGF expression and, therefore, act as a mediator of endothelial injury in human diabetes [121,122]. Studies showed initially that in patients with diabetic nephropathy the degree of neovascularization was increased and correlated with expression of VEGF and angiopoetin [124-126]. Later findings, however, showed that levels of VEGF messenger RNA were actually decreased in patients with diabetic nephropathy [127]. Evidence against the roles of VEGF and angiopoetin demonstrates promotion of vessel leakage and reduction in transendothelial electrical resistance; these two growth factors have key roles in development of retinopathy and contribute to nephropathy development in animal models. Further evidence to support a pathogenic role for VEGF in diabetic nephropathy is the observation that VEGF blockade improves albuminuria in an experimental model of the disorder [122,123]. Animal studies that used a neutralizing antibody to VEGF demonstrated the involvement of this growth factor in glomerular hypertrophy and mesangial matrix accumulation [122,128]. High glucose levels, TGF-β1, and Ang-II stimulate VEGF expression, which leads to the synthesis of endothelial nitric oxide. This action promotes vasodilatation and hyperfiltration, which are the early processes in diabetic nephropathy. VEGF also stimulates the production of the α3 chain of collagen IV, an important component of the glomerular basement membrane. Indirect evidence suggests that increased production of this collagen chain contributes to the thickening of the glomerular basement membrane observed in diabetic nephropathy. In animal studies, administration of an antibody to VEGF decreased urinary albumin excretion compared with that in untreated diabetic controls [103]. Findings from some studies refute a causative role for high VEGF levels in diabetic nephropathy. Instead, results imply that low levels are harmful. Eremina *et al*.[129] showed in a mouse model that VEGF is produced

by podocytes and is necessary for glomerular endothelial cell survival and differentiation as well as for mesangial cell development and differentiation. Gene expression of VEGF is decreased in humans with diabetic nephropathy [130], although whether this effect is due to podocytes loss, leading to reduced production of VEGF, has been questioned. Baelde *et al*. [127] showed that VEGF messenger RNA concentrations were decreased in the glomeruli of patients with diabetic nephropathy and correlated with reduction in the number of podocytes and progression of renal disease.

Hyperglycemia also increases the expression of TGF-β1 in the glomeruli and of matrix proteins specifically stimulated by this cytokine [123]. In the glomeruli of rats with streptozotocin induced diabetes, TGF-β1 levels are increased, and use of a neutralizing antibody to TGF-β1prevents renal changes of diabetic nephropathy in these animals. In addition, connective tissue growth factor and heat shock proteins, which are encoded by TGF-β1-inducible genes, have fibrogenic effects on the kidneys of patients with diabetes. However, diabetes is associated with decreased expression of renal bone morphogenetic protein 7, which in turn seems to counter the profibrogenic actions of TGF-β1 [98]. Evidence clearly shows that TGF-β1 contributes to the cellular hypertrophy and increased synthesis of collagen, both of which occur in diabetic nephropathy [98,123,131,132]. Further evidence for these actions is provided by studies in which the combination of an antibody to TGF β1 plus an angiotensin-converting-enzyme inhibitor normalized levels of protein in the urine of rats with diabetic nephropathy; proteinuria was only partly resolved with the use of an angiotensin- converting-enzyme inhibitor alone [133]. Glomerulosclerosis and tubulointerstitial injury were also improved by the combined therapy. The administration of hepatocyte growth factor, which specifically blocks the profibrotic actions of TGF-β1, ameliorates diabetic nephropathy in mice [134]. Inflammatory cytokines also contribute to the development and progression of diabetic nephropathy, specifically interleukin 1 (IL‑1), IL-6 and IL-18 and tumor necrosis factor. Concentrations of all these cytokines were increased in models of diabetic nephropathy and seemed to affect the disease via multiple mechanisms. In addition, raised levels of several of these cytokines in serum and urine correlate with progression of nephropathy, indicated by increased urinary albumin excretion [135]. Each cytokine has several different effects. IL-1 alters the expression of chemotactic factors and adhesion molecules, alters intraglomerular hemodynamics (by affecting

mesangial cell prostaglandin synthesis), might increase vascular endothelial cell permeability, and increases hyaluron production by renal tubular epithelial cells (which in turn could increase glomerular cellularity) [136]. IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relationships with increased endothelial permeability and mesangial cell proliferation. IL-18 induces the production of other inflammatory cytokines, such as IL-1, interferon γ and tumor necrosis factor, and might be associated with endothelial cell apoptosis. Tumor necrosis factor has the widest variety of biological activities and effects that contribute to development of diabetic nephropathy. Importantly, though, it causes direct renal injury as a cytotoxin, as well as affecting apoptosis, glomerular hemodynamics, endothelial permeability, and cell-cell adhesion. It also seems to play an important part in the early hypertrophy and hyperfunction of diabetic nephropathy [135,137,138].

2.1.4.8. Lipid mediators

Small lipids derived from arachidonic acid have been implicated in the pathogenesis of diabetic nephropathy. Cyclo-oxygenase 2 breaks down arachidonic acid into several different prostanoids. In a rat model of streptozotocin-induced diabetes, levels of inflammatory prostanoids, such as prostaglandins E2 and I2, were raised [139]. Furthermore, increased expression of cyclooxygenase 2 has been reported in animal studies of diabetes and in the macula densa of kidneys from humans with diabetes [140]. In diabetic rats, inhibition of cyclo-oxygenase 2 is associated with decreased glomerular hyperfiltration [141]. A more detailed characterization of how the production of prostanoids affects the pathogenesis of diabetic nephropathy is needed. Arachidonic acid can also be oxidized by lipoxygenases [142]. Evidence is accumulating that some of the products derived from the actions of lipoxygenases contribute to diabetic nephropathy. Specifically, levels of lipoxygenases 12 and 15 are increased in diabetic animals. In addition, high glucose levels increase expression of lipoxygenases 12 and 15 in cultured mesangial cells. To conclude, this pathway has a key mediatory role in the critical processes of mesangial cell hypertrophy and extracellular matrix accumulation mediated by TGF-β1 and Ang-II [142].

2.1.4.9. Oxidative stress

Generally, metabolic activity within the nephron produces a large amount of reactive oxygen species that are counterbalanced by a large number of antioxidant enzymes and free radical scavenging systems. Reactive oxygen species mediate many negative biological effects, including peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction and damage to DNA. Unfortunately, hyperglycemia tips the balance towards production of reactive oxygen species, most of which seem to be generated in the mitochondria [143]. The metabolism of glucose through harmful alternate pathways, such as via PKC activation and advanced glycation end product formation, in the setting of hyperglycemia also seems partly dependent on reactive oxygen species [143-145]. Hyperglycemia specifically induces oxidative stress, even before diabetes becomes clinically apparent. Concentrations of markers of DNA damage induced by reactive oxygen species are higher in patients with more-severe nephropathy (i.e. proteinuria versus microalbuminuria). Furthermore, histological analysis of human kidney biopsy specimens has detected products of glyco-oxidation (combined products of glycation and protein oxidation) and lipoxidation in the mesangial matrix and glomeruli, whereas these lesions are much less common in specimens from individuals without diabetes [145,146].

However, even after demonstration of several molecular mechanisms in the development of DN, still the pathology of DN remained enigmatic. Moreover, there is no specific therapy till date, to prevent DN. Further, the number of people with DN tend to increase globally, even after the success of existing therapy. This, indicating the urgent need to identify the novel mechanisms, therapeutic targets to understand and to prevent DN completely in future.

2.2. Ubiquitin proteasome system:

Ubiquitin is an evolutionarily conserved 76 amino acid protein and it targets the proteins for degradation through ubiquitin proteasome system (UPS) by the process of ubiquitination and 80% of intracellular eukaryotic proteins undergo this degradation process [147]. The ubiquitination of substrate proteins occurs mainly by the activity of three enzymes, including an ubiquitin activating E1 enzyme, ubiquitin conjugating E2 enzyme, and ubiquitin E3 ligase. First step of ubiquitination utilizes ATP and forms a thioester bond between a single

ubiquitin moiety and with the active-site Cysteine residue within the E1 ligase. Then, the activated ubiquitin is transferred to an ubiquitin conjugating E2 enzyme. With the help of E2 enzyme specific E3 ligase will transfer the ubiquitin chain to a particular substrate and finally presents the ubiquitinated substrate to 26S proteasome for degradation [7].

Besides, 26s proteasome, in mammalian cells various types of proteasomes exist, including standard, hybrid, immuno and thymoproteasomes with different proteolytic activity [148]. All these proteasomes contains central multi subunit, multi catalytic barrel shaped 20s core particle (CP) with a stack of four rings made up of two outer α-rings and two inner β-rings [148]. The α- and β-rings are each made up of seven structurally similar α1-α7 and β1-β7 subunits [149]. Three of the β-subunits, having active sites β1, β2 and β5 shows peptidyl-glutamyl-hydrolyzing or caspase-like, the trypsin-like, and the chymotrypsin-like proteolytic activity [150]. The binding of different regulators over α-subunits decides the function of the 20s CP and origin of different of proteasomes. For example binding of two PA700 (19s) regulator units to 20s CP (19s-20s-19s) in an ATP-dependent manner results in the formation of 26s proteasome, which mainly involves in the degradation of proteins tagged with poly-ubiquitin chain. Further, binding of PA28 (11s) regulator to 20s CP (11s-20s-11s) results in the formation of PA28 proteasome mainly involves in ATP-independent degradation of proteins. In addition binding of one 19s and one 11s regulatory units to 20s CP forms hybrid proteasome with both ATP dependent and independent proteasomal activity [151]. Exceptionally, under certain conditions, the catalytic subunits (β1, β2 and β5) of 20s CP can be replaced with immuno-subunits like β1i (LMP2), β2i (MECL-1) and β5i (LMP7) results in the formation of immunoproteasomes [152]. Immunoproteasomes mainly involves in the generation of substrate fragments with greater affinity to MHC class I molecules, which improves the antigen presentation. Immunoproteasomes largely found in various immune system specific tissues like spleen, small intestine, liver, thymus, lungs, kidney, colon and antigen presenting cells (APCs) [148]. In contrast, they can be induced in other cells upon exposure to inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) [148]. Moreover, substitution of active subunit β5 of 20s CP with β5t forms thymoproteasome and found only in cortical epithelial cells of thymus and plays a vital role in the selection of $CD8^+$ T-cells [148,153]. The major function of proteasomes is to maintain protein quality and function through proteolysis of unwanted

proteins; any alterations in it results in disease phenotypes. Existing studies have demonstrated the potential role of UPS in diabetes [154-156]. However, very little is known about UPS and its components including E2 conjugating enzymes, E3 ligases and deubiquitinases in the pathogenesis of DN.

2.3. UPS in DN:

Accumulating evidence demonstrates the UPS alterations in DN. Massimo Papale et al., identified free ubiquitin as a potential biomarker in urine samples of patients with DN compared to patients with diabetes who have other chronic kidney diseases [61]. Moreover, Dihazi et al. reported the presence of the ubiquitin fusion protein UbA52 in urine of patients with T2D with macro or micro albuminuria, suggesting its role as a tubular injury [157]. In the kidney, UbA52 protein was more prominently localised in renal tubules, and its expression in diabetic mice was found to increase proportionally with the increased glucose concentrations [158]. In addition increased 26S proteasomal activity, which mainly involves in degradation of ubiquitin tagged proteins, was also reported in DN. Further, proteasomal inhibitor MG132 also showed beneficial effect in preventing DN through inhibiting 26S activity and concentration [159]. These reports suggesting the potential role of UPS in DN (Figure 1).

Increasing evidence is implicating proteasomal alterations in DN. Zhi-Feng Luo et al., demonstrated proteasome inhibition protects DN, through reducing oxidative stress [160]. Oxidative stress plays a crucial role in the development of DN [161]. Several mechanisms, including auto oxidation of glucose, AGEs through acting on RAGEs, glycolysis, polyol pathway, glucose-6-phosphate dehydrogenase, hexosamine pathway and increased activity of NADPH oxidase (Nox) involves in the generation of ROS [162,163]. Nevertheless, of all these pathways, increased Nox activity is a major contributor in ROS generation in diabetic kidney [161,164]. Seven active subunits of Nox exists, includes Nox1-5, Duox1-2, of which Nox4 highly expressed in kidney and also found to be upregulated diabetic kidneys with increased oxidative stress [165]. Other subunits of Nox, like p22phox, p47phox were also found to be upregulated in DN [160,166]. Recently, Nox4 inhibitors were proven to be protective in DN [165]. Moreover, in human umbilical cord vein cells (HUVECs), nontoxic proteasome inhibition increased the levels of antioxidant enzymes and reduced the ROS

generating Nox4 levels and stabilized the antioxidant transcription factor Nrf-2 [167,168]. In addition, proteasomal inhibition using MG132 in STZ induced DN model, found to protect renal tissue through inducing Nrf-2 levels and thereby increasing the antioxidant enzymes like GSH, SOD and catalase. MG132 also reduced the levels of Nox subunit p47phox and reduced oxidative stress in DN [160]. These reports indicating the therapeutic potential of other proteasome inhibitors like bortezomib, carfilzomib, ixazomib, salinosporamide A (NPI-0052), MLN9708, CEP-18770 (Figure 1). Bortezomib, carfilzomib and ixazomib were FDA approved proteasomal inhibitors, whereas NPI-0052, MLN9708 and CEP-18770 are under clinical trials [169]. However, all these proteasome inhibitors inhibit one of the catalytic β-subunits of 20S CP [169], including MG132 [170] and hence, they may interfere with other proteasomes and important cellular functions of UPS. Hence, there is a need to develop specific proteasome inhibitors in preventing DN.

However, in DN, PA28 (11S) regulatory subunits of 20S CP, PA28-α/β expression was found to be increased in the glomeruli of 8 week old $Ins2^{Akita/+}$ diabetic mice [154]. Further, Knock out of PA28-α and PA28-β genes, found to protect the STZ induced diabetic mice from renal damage [171]. Stefanie GRIMM et al., demonstrated that AGEs can induce immunoproteasomal subunit β1i (LMP2) in RAW264.7 cell lines [172]. AGEs are the common end products of chronic hyperglycemia, which promotes DN through increasing oxidative stress. Recently, β5i (LMP7), another immunoproteasomal subunit was found to activate NF-κβ in the progression of DN [173]. These reports suggesting the increased activity of PA28 and immunoproteasomes in DN. Hence, specific inhibitors of PA28 and immunoproteasomes will be benificial in preventing DN (Figure 1). Specific PA28 inhibitors were not reported till now. However, immunoproteasome inhibitors, like UK-101, IPSI-001 and YU-102, specific for LMP2, whereas ONX 0914 (PR-957) and PR-924, specific for LMP7 were developed [174] and further research is required using these compounds to further understand the new underlying mechanisms in the progression of DN.

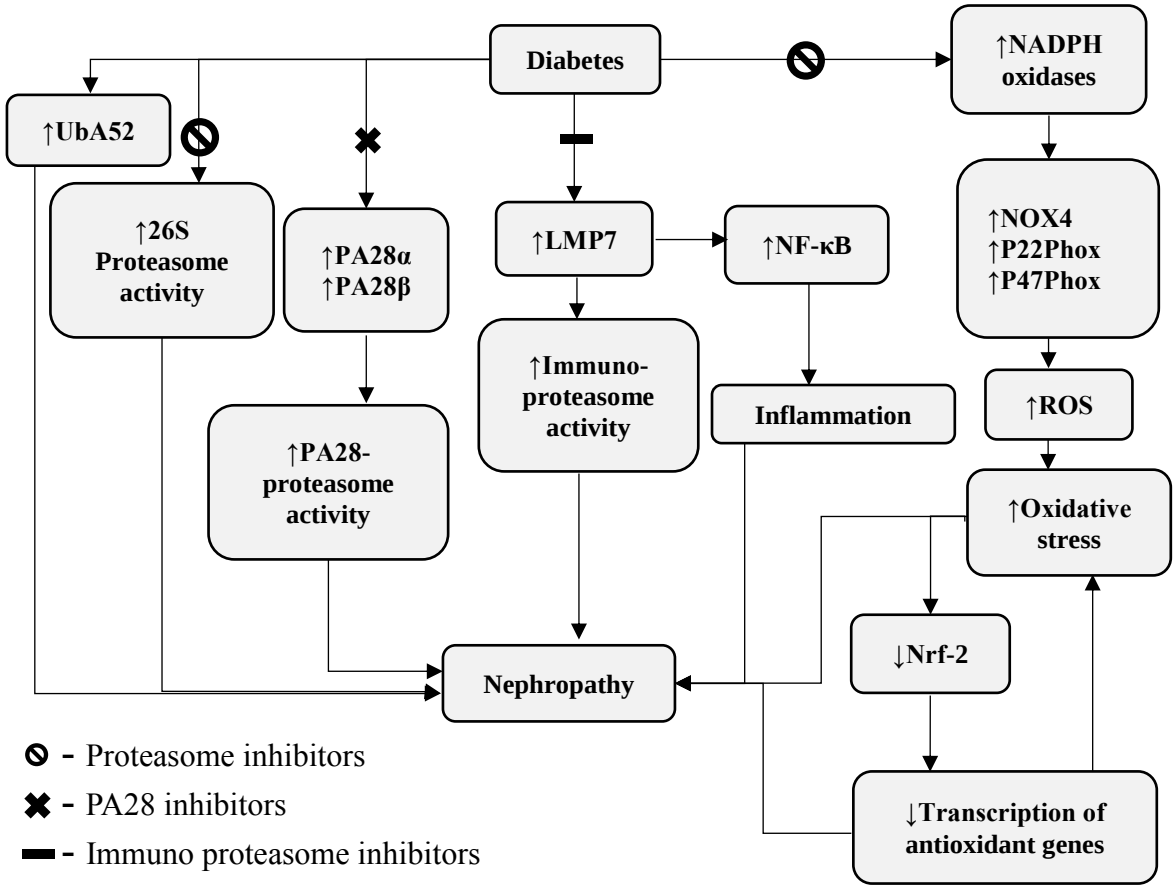


Figure 1: Ubiquitin proteasome system (UPS) and proteasomal inhibitors in diabetic nephropathy (DN): Increased ubiquitin fusion protein and 26S proteasome activity indicating the alterations of UPS in DN. In diabetic increased activity of different proteasomes like PA28 (PA28-α and PA28-β) and immunoproteasomes (LMP7) results in renal damage and nephropathy. Proteasomal inhibitors prevent DN through inhibiting diabetes induced oxidative stress

2.4. E2 conjugating enzymes in DN:

Previously, the E2 enzymes were thought to be involved in the ubiquitination reaction largely through its association with a given E3 ligase [7]. Present literature states that E2 enzymes are independently capable of ubiquitination on selected lysine residues [175]. In eukaryotic genome 16-35 E2 enzymes identified and whereas in humans, 35 active E2 enzymes were identified till now [176]. Functionally, E2 enzymes also participates in various cellular processes. For example, E2 enzyme Appolon (BIRC6) over expression prevented apoptosis through targeting SMAC and Caspase-9 for ubiquitination and

proteasomal degradation in mouse embryonic fibroblast cells [177], E2 enzyme UBE2O involved in reticulocyte maturation and hematopoiesis [178]. Recently, E2 enzyme variant UBE2v1 was found to play a crucial role in the development of DN [179] (Figure 2).

The ubiquitin-conjugating E2 enzyme variant 1, UBE2v1, also called UEV1 or MMS2, is a cofactor of UBC13. UBC13 is the only known E2 Ub-conjugating enzyme that polyubiquitinates the substrate directly at Lys63 [180]. Woroniecka et al. reported an increased mRNA expression of UBE2v1 in renal tubules of DN pateints [181]. Recently, Paola Pontrelli et al., demonstrated the increased UBE2v1 protein expression and increased Lys63-ubiquitinated proteins in kidneys of type 2 diabetic patients. Similarly, in HK2 tubular cells HG (high glucose) also induced UBE2v1 protein expression and increased 30 Lys63-ubiquitinated proteins, which are mainly involved in maintaining cytoskeletal structure including β-actin [179]. All these changes were reversed by the NSC697923, the inhibitor of Ubc13/UBE2v1 complex [179]. UBE2v1 silencing using UBE2v1 siRNA, normalized the Lys63- β-actin ubiquitination levels in HK2 cells under HG condition [179]. Increased expression of UBE2v1 and Lys63-ubiquitination in DN patients and in HK2 cells, correlated with increased α-SMA expression. However, UBE2v1 silencing reversed the HG induced α-SMA expression in HK2 cells [179]. This suggesting the potential role of E2 conjugating enzyme UBC13 and Lys63-ubiquitination in DN.

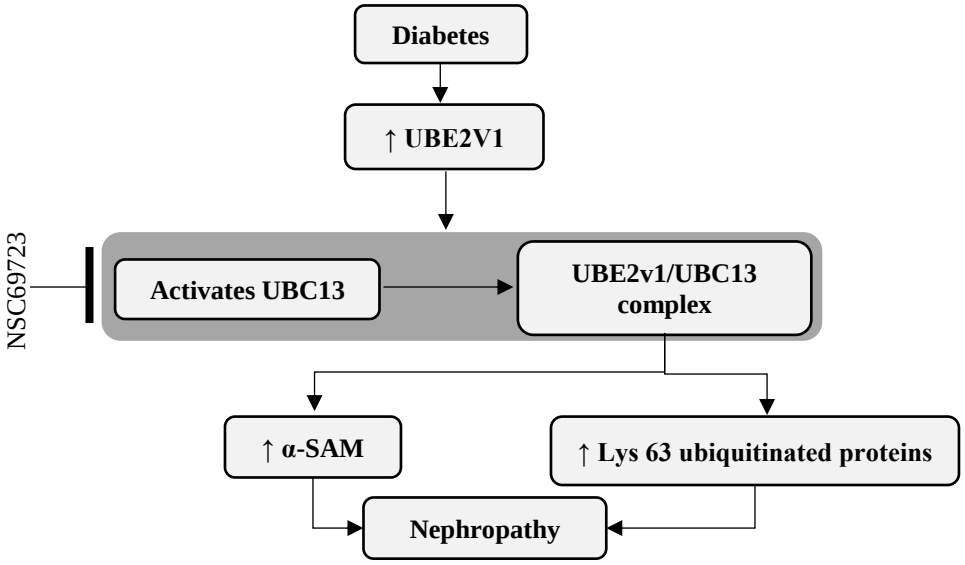


Figure 2: E2 conjugating enzyme UBE2v1 promotes lysine 63 polyubiquitination and accelerates DN: Increased UBE2v1, an activator of E2 enzyme UBC13, increased the expression of lysine 63 polyubiquitination of various substartes and promotes DN.

2.5. E3 ligases in DN:

E3 ligases are the enzymes mainly involved in the ubiquitination pathway, they interact with ubiquitin, E2 enzymes and finally with substrate proteins in the last step of ubiquitination process. E3 ubiquitin ligases are variety of proteins actively involved in substrate ubiquitination to decide the fate of a protein, for example, K11-linked polyubiquitination is essential for cell cycle regulation [182]. K27-linked polyubiquitin chains are required for mitophagy [183], K48-linked polyubiquitin chains mainly targets proteins for proteasomal degradation [184] and K63-linked polyubiquitination plays a role in regulation of NFκB signaling [185]. E3 ubiquitin ligases are classified into three types including Homologous to the E6AP carboxyl terminus (HECT) domain type, Really interesting new gene (RING) E3 ligases and RING-related E3 ligases [186]. The mammalian genome codes 30 HECT domain and more than 600 RING E3 ligases, which play crucial roles in various biological pathways like cell cycle progression, immunity and protein transport [187,188]. E3 ligases were found to play a critical role in various diseases like cancer, neurodegenerative disorders and inflammatory diseases [186,189,190]. Moreover, E3 ligases including Arkadia, Anaphase promoting complex/Cyclosome (APC/C), Casitas B-lineage lymphoma (c-Cbl), Cullin E3 ligases (Cullin 1 and 3), Hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (Hrd1), Smad ubiquitin regulatory factor 2 (Smurf2) were found to play a key role in the progression of DN (Figure 3).

2.5.1. Arkadia:

Arkadia is a member of the RING E3 ubiquitin ligase, which positively regulates the TGF-β signaling pathway by targeting Smad7, the negative regulator of TGF-β for ubiquitination and proteasomal degradation [191]. Konstantinos J. Mavrakis et al., reported that Akardia, directly ubiquitinates p-Smad2/3 and leads to its degradation, which also results in the activation of TGF-β [192]. In another study, Yoshiko Nagano et al., demonstrated that negative regulators of TGF-β, SnoN and c-Ski were found to be targeted by Akardia for proteasomal degradation [193]. TGF-β is a key mediator of fibrogenesis, which induces ECM accumulation and tubulointerstitial fibrosis by the TGF-β/Smad signaling pathway in DN [194]. Recently, Lirong Liu et al., reported increased protein expression of Akardia in normal rat renal tubular epithelial cells (NRK52Es), when cultured in HG medium [195].

Increased expression of Arkadia, increased the ubiquitination of SnoN and reduced its expression in NRK52Es and resulted in over expression of TGF-β1 [195]. NRK52Es treated with Oxymatrine, an alkaloid with anti-fibrotic properties found to reduce the expression of fibrotic markers α-SMA, Fibronectin and TGF-β1 under HG condition through decreasing Arkadia expression and ubiquitination of SnoN [195]. Hence, blocking Arkadia may have beneficial role in preventing DN.

2.5.2. Anaphase promoting complex/Cyclosome (APC/C):

The APC/C is a RING-type E3 ligase which plays a key role in cell cycle (G1 and mitosis phase) regulation through targeting cell cycle linked proteins for proteasomal degradation. The activity of the APC/C maintained by two activators Cdc20 and Cdh1, with the help of these two activators APC/C recognizes and targets various substrates for degradation in a cell cycle dependent manner [196]. Substrate specificity for APC/C-activator complex determined by two degradation motifs mainly D-box and Ken-box. Substrate with D-box motif can be recognized by both Cdc20 as well as Cdh1, but Ken-box motifs can only be recognized by Cdh1 [197-199]. APC/C-Cdc20 complex is essential in metaphase to anaphase transition and regulates the proteins involved in this phase. However, APC/C-Cdh1 complex activation is essential to enter the cells from late mitosis to G1 phase. Whereas, inactivation of APC/C-Cdh1 complex is required for the cells to enter into S phase from G1 phase, where DNA is replicated and chromosomes get doubled [200]. Hence, there is a possibility that, if unexpected inactivation of APC/C at late mitosis may results in mitotic arrest and cell death. Moreover, Cyclin B1 and Skp2 are the key regulators of mitosis and APC-C-Cdh1 complex targets them for proteasomal degradation [201]. In terminally differentiated neurons it was found that activated APC/C-Cdh1 prevented the neurons to enter into S phase through maintaining low levels of cyclin B1 expression [202], on the other hand loss of Cdh1 increased the Cyclin B1 mediated S phase entry and neuronal apoptosis [203]. Recently, Hua Su et al., demonstrated that loss of Cdh1, the activator of APC/C leads to podocyte injury, which eventually results in diabetic nephropathy [204]. In the same study, HG condition, found to inhibit APC/C-Cdh1 complex by upregulation MAD2B (inhibitor of Cdh1) in human podocyte culture, this results in the increased accumulation of APC/C-Cdh1 substrates, Cyclin B1 and Skp2 which are supposed to be degraded through proteasomal degradation [204]. The increased level of cyclin B1 and Skp2 leads to podocyte

injury through inducing caspase3 mediated apoptosis [204]. Silencing MAD2B found to reverse all the changes in podocytes cultured in HG, through increasing Cdh1 and thereby reducing the levels of Cyclin B1 and Skp2 [204]. This indicating the APC/C-Cdh1 activation is essential to prevent the DN progression.

2.5.3. Casitas B-lineage lymphoma (c-Cbl):

Casitas B-lineage lymphoma (c-Cbl) protein belongs to the Cbl family of proteins (e.g., c-Cbl, Cblb and Cbl-c) [205]. c-Cbl is a RING E3 ligase, mainly involved in the negative regulation of protein tyrosine kinases [206]. Evidence shows that c-Cbl plays a role in the development of insulin resistance by degrading IRS-1 and there is a well-established correlation between insulin resistance and diabetic nephropathy [207,208]. Deletion of c-Cbl or Cblb genes, protected the mice from developing adiposity and insulin resistance even after feeding with high fat diet [209]. Recently, it was shown that Regulator of ubiquitous kinase/Cbl-interacting protein of 85 kDa (Ruk/CIN85) was found to be upregulated in murine and human podocytes stimulated with HG and in kidneys of diabetic mice and diabetic patients [210]. Ruk/CIN85 is an interaction partner of c-Cbl and involves in substrate ubiquitination [205]. Increased expression of Ruk/CIN85 was found to induce nephrin ubiquitination and leads to its down regulation due to endocycytosis in murine and human podocytes stimulated with HG and in kidneys of diabetic mice and diabetic patients [210]. Nephrin is a 180-kDa transmembrane protein predominantly localized to the glomerular slit diaphragm. Its expression is essential for primary structural function of the slit diaphragm [211]. Nephrin may also act as a signaling adhesion molecule, triggering phosphorylation and activation of several kinase cascades [212]. Nephrin signaling is augmented by its interaction with podocin. During the early stage of human and experimental DN, podocytes shows decreased nephrin expression and lose their structural integrity and even detach from the GBM or undergo apoptosis, eventually results in albuminuria [161,213]. Absence of CIN85 Exon2 ($CIN85^{\Delta EX2}$) preserved the expression of nephrin under diabetic conditions [210]. C57BL/6J $CIN85^{\Delta EX2}$ diabetic mice showed reduced ubiquitin levels and increased nephrin in kidney sections when compared to diabetic wild type C57BL/6J mice. $CIN85^{\Delta EX2}$ diabetic mice also protected from induction of albuminaria and showed reduced Collagen IV deposition in glomeruli when compared to

wild type mice [210]. The above facts clearly indicating the role of c-Cbl in the development of DN.

2.5.4. Cullin-RING ubiquitin ligases (CRLs):

CRLs belong to RING-like E3 ligases, in addition contains a cullin scaffold protein, a RING subunit with catalytic activity and other sub-units like Rbx1 or Rbx2 [214]. Moreover, Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5 are the known cullin proteins were found to be coded by human genome till now. Cullin proteins when complexed with Rbx1 or Rbx2, originate in to various subfamilies of CRLs, CRL1 to CRL5 [215]. Cullin–RING ligases (CRLs) have been implicated in a multitude of cellular processes like cell cycle regulation, signal transduction, DNA replication and transcription [214]. Existing literature supports the involvement of Cullin 1 and 3 in the development of diabetic nephropathy [154]. Recently, protein expression of Cullins (Cullin 1 and 3) were found to be increased in glomeruli of $Ins2^{Akita/+}$ mice and this increase was found to match the severity of diabetic nephropathy [154]. However, the molecular mechanisms, how Cullins involved in progression of DN was not studied in detail. Additional research should be required to expose the role of Cullins in DN.

2.5.5. Hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (Hrd1):

Hrd1, is a member of RING E3 ligases and also called as synoviolin [216]. The name Hrd1 was named after its ability to ubiquitylate the endoplasmic reticulum transmembrane protein, hydroxymethyl glutaryl-coenzyme A reductase. It targets misfolded proteins and facilitates endoplasmic reticulum-associated degradation of proteins [217]. Moreover, Hrd1 expression was found to be beneficial in various neurodegenerative diseases, and pathological in other diseases, such as liver cirrhosis and rheumatoid arthritis [216,218,219]. Recently, Caifeng Yan et al., demonstrated the protective role of Hrd1 in DN model [220]. The kidney expression of Hrd1 was found to be decreased in db/db mice, which leads to the increased expression of IGF-1R. In the same study, using human proximal tubular epithelial (HCK8) cells they also found that Hrd1 promotes IGF-1R ubiquitination and degradation [220]. They have also demonstrated that the protective actions of resveratrol to prevent DN in db/db mice is due to its ability to increase Hrd1 expression and eventual polyubiquitination and proteasomal degradation of IGF-1R. Hrd1 silencing in HCK8 cells, prevented the

ubiquitinatin mediated proteasomal degradation of IGF-1R and also blocked the protective actions of resveratrol [220]. Thus, overexpression of E3 ligase Hrd1 may have a beneficial role in DN.

2.5.6. Smad ubiquitin regulatory factor 2 (Smurf 2):

Smurf2 belongs to the class of HECT E3 ligases, and mainly involved in the TGF-β signalling pathway. Smurf2 binds to Smad7 and leads to its ubiquitination and proteasomal degradation [221]. Smad7 is a negative regulator of TGF- β1, which causes degradation of TGF- β1R and other Smads involved in TGF- β1 [222]. Smurf2 not only degrades Smad7 but also SnoN, another negative regulator of TGF- β1 signaling pathway [221]. Similar to Smad7, SnoN is an another negative regulator of TGF-β1 by acting as a transcriptional repressor [223]. Moreover, Smad7 and SnoN expression was found to be decreased in the kidneys of diabetic rats, which results in the increased activity of TGF-β1 signalling [12,224]. Smurf2 expression was also found to be increased in the kidneys of diabetic animals. Increased Smurf2 leads to the decreased expression of Smad7 and SnoN, the negative regulators of TGF-β, in diabetic kidneys through increasing their ubiquitination and proteasomal degradation [12,224]. MG132, a proteasomal inhibitor was also found to be effective in preventing diabetic renal fibrosis through decreasing the expression of Smurf2, there by restoring the levels of Smad7, SnoN and thereby decreasing the levels of TGF-β1 [12,224]. Therefore, inhibiting Smurf2 may prevent the progression of DN.

Overall, these reports clearly indicating the role of E3 ligases in the development of DN. However, there is no specific therapy to treat DN till date. More importantly, very little is known about the role of E3 ligases in the development of DN. Hence, further research is required to identify the novel E3 ligases that are involved in the development of DN. In conclusion, these E3 ligases may serve as a specific and better therapeutic targets to prevent DN completely in future.

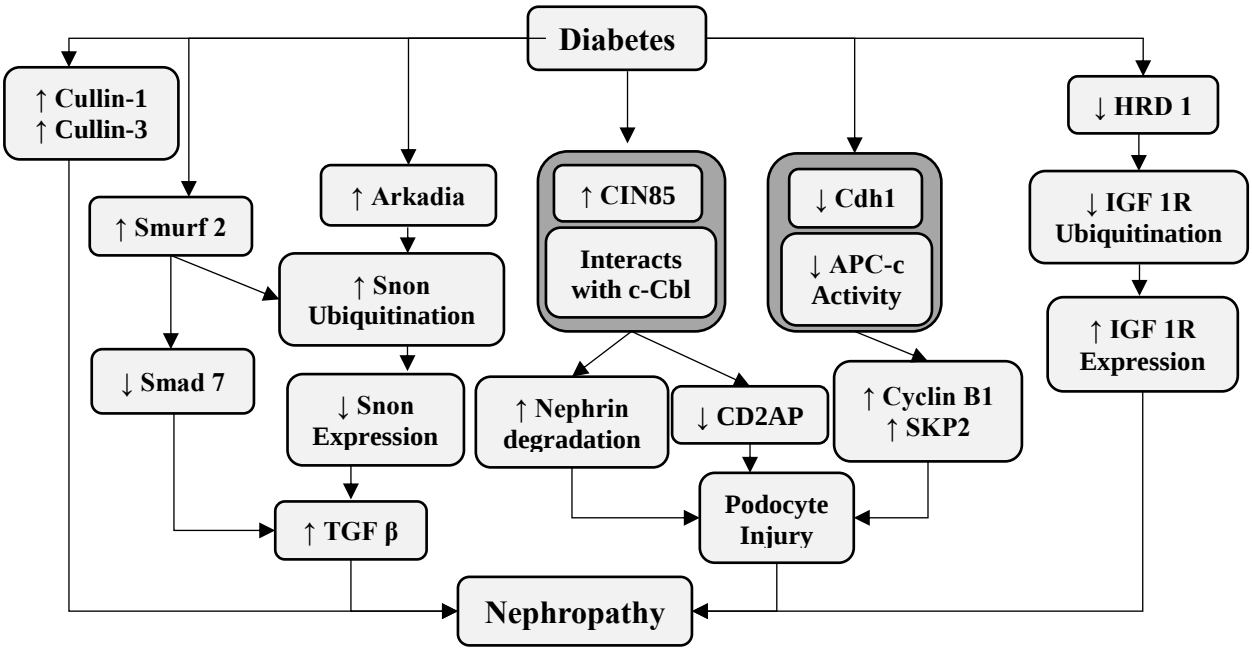


Figure 3: E3 ligases in the progression of DN: E3 ligases Cullin 1 and Cullin 3 expression was increased in DN. Increased expression of Smurf2 and Arkadia increases TGF-β1 activity in DN through proteasomal degradation of its negative regulators, Smad7 and SnoN. E3 ligase c-Cbl activity was found to be increased in diabetic kidney through the increased expression of its activator CIN85, leads to the podocyte injury through increased degradation of nephrin and decreased protein expression of CD2AP. In diabetic animals, reduced expression of Cdh1, the activating subunit of E3 ligase APC/C and decreased the activity of APC/C. This results in escape of its target cell cycle substrates Cyclin B1 and SKP2 from proteasomal degradation and leads to their accumulation, podocyte injury, eventually results in DN. Down regulation of another E3 ligase HRD1 in diabetic kidney helps in progression of DN through increasing IGF 1R expression.

2.6. Deubiquitinases (DUBs) in DN:

DUBs are the isopeptidase enzymes that can reverse the process of ubiquitination through removing ubiquitin moieties from the ubiquitinated proteins [225]. The human genome probably codes for 100 DUBs, of which 79 DUBs were found to be functionally active [226]. DUBs are mainly classified in to five subclasses: Ubiquitin Carboxy terminal Hydrolases (UCHs), Ubiquitin Specific Proteases (USPs), Josephin proteases, Ovarian Tumor (OTU) proteases, and JAB1/MPN/Mov34 (JAMM) metalloenzymes [227]. DUBs maintains the

free ubiquitin homeostasis by preforming three major functions including, removing ubiquitin moieties from precursor proteins, rescues the polyubiquitinated proteins from proteasomal degradation and by editing the ubiquitin signal through chopping or redefining the ubiquitin chains from various substrates [227]. Accumulating evidence also suggests the role of DUBs in the development of neurodegenerative diseases and cancer [228,229]. Recently, DUBs UCH-L1 and USP22 were found to be involved in the development of DN (Figure 4).

2.6.1. Ubiquitin carboxy-terminal hydrolase 1 (UCHL1):

UCHL1 belongs to the UCH class of DUBs, plays a prominent role in the regulation of various physiological events including cell cycle progression, proliferation and apoptosis [230]. Almost every UCH composes of N-terminal C12 peptidase domain, a C-terminal extension and an unstructured loop that regulates substrate recongnition for its catalytic site. UCH DUBs mainly cleave C-terminal and N-terminal conjugated ubiquitin from substrate proteins [231]. Increasing evidence revealed the role of UCH-L1 in the development of neurodegenerative disorders [232]. Moreover, UCH-L1 plays a role in podocyte differentiation and its upregulation in the podocytes causes various forms of nephritis [233-235]. Recently, UCH-L1 was found to be involved in the pathogenesis of DN. The protein expression of UCH-L1 was found to be increased in kidneys of DN patients as well as in the HG induced podocytes [236]. HG induced UCH-L1 altered the morphology and motility of podocytes. The HG induced changes in podocytes were reversed by treating with Wnt/β-catenin inhibitor dickkopf related protein 1 (DKK1) [236]. UCH-L1 overexpression in podocytes also altered the expression of structural proteins like snail, nephrin, synaptopodin and CD2AP [236]. These, facts suggests that inhibition of UCH-L1 may prevent the development of DN.

2.6.2. Ubiquitin specific protease 22 (USP22):

USP22 is a member of the USP deubiquitinase family. USP22 is required for transcriptional regulation and cell cycle progression [237]. USP22 is an essential DUB for histone H2A/H2B and also for a non-histone protein TBP [TATA box-binding protein]-related factor 1 (TRF1) and regulate the transcription of various genes necessary for apoptosis and cell ycle progression [238,239]. Other than histones, USP22 also targets several non-histone

proteins, of which, SIRT1 is one of them [240]. SIRT1 deubiquitination through USP22 is essential to inhibit p53 acetylation and p53-mediated apoptosis in mouse embryonic development [241]. Moreover, SIRT1 expression was found to be decreased in kidneys of diabetic animals and its protective effect in diabetic nephropathy has been reported [242,243]. Recently, Kai-Peng Huang et al., demonstrated that AGEs treatment increased the k48 linked polyubiquitination of SIRT1 and decreased its expression through proteasomal degradation in glomerular mesangial cells (GMCs), which in turn increased the expression of ECM markers fibronectin and TGF-β [11]. In the same study, AGEs treatment also decreased the expression of USP22 levels in GMCs and USP22 over expression in GMCs reversed all the changes induced by AGEs treatment [11]. This suggests the protective role of USP22 in preventing DN.

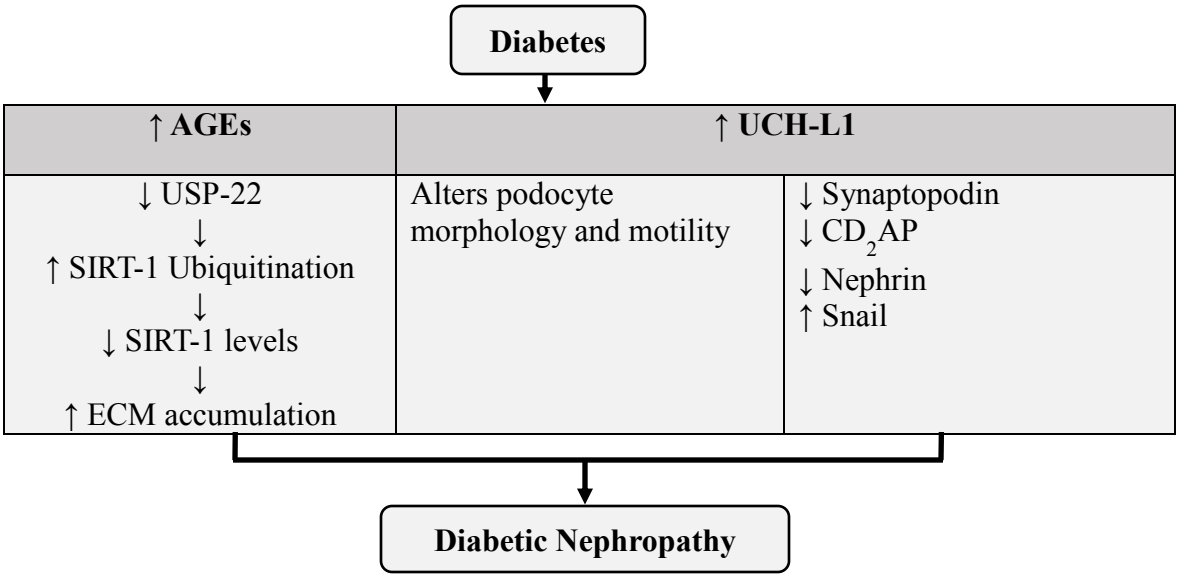


Figure 4: Deubiquitinases (DUBs) play a crucial role in the development of DN: Decreased expression of SIRT1 specific DUB USP22, resulted in the increased proteasomal degradation of SIRT1 and increased the expression of ECM genes. High glucose increased the expression of DUB UCH-L1, effected the podocyte morphology through altering the expression of various structural proteins (Synaptopodin, CD2AP, Nephrin and Snail).

2.7. Posttranslational modifications of histone H3 in diabetic condition

Regulation of gene expression relies on the accessibility of DNA to various transcription factors, coactivators/corepressors, and the transcriptional machinery. DNA is first wrapped around a histone octamer composed of a histone H3-H4 tetramer and two H2A-H2B dimers

followed by a histone H1 linker making up a nucleosome, the basic unit of chromatin [244]. Apart from the binding of transcription factors to their cognate promoter cis-acting elements, transcriptional activation or repression is also linked to the recruitment of protein complexes that alter chromatin structure via enzymatic modifications of histone tails and nucleosome remodeling. Therefore, gene transcription and activation depend on a chromatin structure that is very dynamic, depending on a multitude of posttranslational modifications of histones that allow for the conversion of inaccessible, compact, or repressive heterochromatin to the accessible or active euchromatin state of DNA. Posttranslational modifications that occur on the histone tails include acetylation, methylation, and phosphorylation to name a few [245].

Histone acetyl transferases (HATs) and Histone deacetylases (HDACs) have been found to play important roles in the regulation of several key genes linked to diabetes. HATs and HDACs can also modulate NFκβ transcriptional activity [246], resulting in changes in downstream inflammatory gene expression levels [247]. Interestingly, high glucose treatment of cultured monocytes increased recruitment of the HATs CPB and p/CAF, leading to increased histone lysine acetylation at the cyclooxygenase 2 and TNF-α inflammatory gene promoters, with a corresponding increase in gene expression. The in vivo relevance of histone acetylation in diabetes and inflammation was shown by demonstrating increased histone lysine acetylation at these inflammatory gene promoters in monocytes from both Type I and Type II diabetic patients [248]. p300 was also found to play a role in oxidative stress-induced poly (ADP-ribose) polymerase (PARP) and NFκβ signaling pathways in high glucose-treated endothelial cells and diabetic retina, kidney, and heart, leading to increases in extra cellular matrix (ECM) components related to diabetic complications. High glucose increased p300, leading to increased histone acetylation at promoters of key ECM genes [245]. Global histone acetylation is also found to be decreased in diabetic kidney [249,250]. Interestingly, the p300 inhibitor curcumin could prevent hyperglycemia induced changes by global hyper acetylation of histone H3 in STZ induced diabetic kidney [251]. These results further implicate a role for chromatin histone acetylation in promoting gene expression related to diabetic complications. Further in vitro and in vivo studies in diabetic kidneys have shown an important role for HDACs in TGF-β mediated ECM production and kidney fibrosis [252]. Histone methylation, on the other hand, can be generally more stable and there has been great interest in determining the role for key histone

methylation marks in diabetes and its complications. Knockdown of the H3K4 histone methyl transferase (HMT) SET7/9 in monocytes attenuated TNF-α induction of key inflammatory genes in an NFκβ dependent manner. Knockdown of SET7/9 also decreased NFκβ p65 subunit and p300 HAT occupancies at monocyte chemo attractant protein-1 (MCP-1) and TNF-α promoters in monocytes, with a corresponding decrease in promoter H3K4 methylation. These results suggest that SET7/9 might coactivate NFκβ transcriptional activity via promoter H3K4 methylation in response to high glucose induced inflammation in the diabetes [245]. Another important histone modification is histone H3 phosphorylation. Several reports showed that there is a decreased phosphorylation of histone H3 at serine 10 in diabetic kidney leads to mitotic arrest and cell death [243,251].

2.8. Histone ubiquitination in DN:

Accumulating evidence also states that histone H2AK119 mono-ubiquitination (H2AK119Ub) and H2BK120 mono-ubiquitination (H2BK120Ub) are also involved in transcriptional regulation [17]. H2AK119 ubiquitination is mediated by the BMI-1/RING-1A protein found in the human polycomb complex, which is associated with transcriptional repression. On the other hand, H2BK120 ubiquitination is mediated by human RNF20/RNF40 and UbcH6 and is required for active transcription [18,19]. E3 ligase, BMI1, promotes H2A ubiquitination and *Hox* gene silencing through H3K27Me by regulating methyltransferase EZH2 and H2AK119Ub [18]. Furthermore, H2B ubiquitination is associated with the transcribed regions of highly active genes [20]. H2B specific E3 ligase RNF20 overexpression, increased the levels of H3K4Me and H3K79Me, and induced *Hox* gene expression. In supporting with that, inhibition of RNF20/40 complex decreased H2B mono-ubiquitination, H3K4 and H3K79 methylation, and suppressed the *Hox* gene expression [19].The balance between active and repressive chromatin marks is essential for normal gene transcription, any alterations in this may result in abnormal gene transcription and disease phenotypes. Recent evidence has also implicated the involvement of epigenetic mechanisms in DN [253]. However, very little is known about the histone H2A and H2B mono-ubiquitination in DN pathogenesis. Recently, Chenlin Gao et al., reported the role of repressive chromatin mark histone H2AK119Ub and active mark H2BK120Ub in the expression of fibrotic genes like fibronectin and *Tgfb1* in rat glomerular mesangial cells

under hyperglycaemic condition [21]. In addition, loss of *Usp22*, a de-ubiquitinase specific for H2A/H2B ubiquitination, was found to increase fibrotic genes like fibronectin and *Tgfb1* in rat mesangial cells under hyperglycaemic condition [11]. These reports clearly indicating the role of histone ubiquitination in the progression of DN.

2.9. Renin angiotensin system in diabetic kidney:

Renin angiotensin system (RAS) in regulation of blood pressure and fluid and electrolyte homeostasis is well known. Vasodilator and vasoconstrictor effects are balanced by the actions of Ang-II, Ang 1-9 and Ang 1-7. Angiotensin I (Ang I) formed from angiotensinogen by the actions of renin and serine proteases (Kallikrein and Tonin), acts as a precursor for the formation of Ang II mediated by ACE and other serine proteases (Chymase and Cathespin G). Ang 1-9 is generated directly from the cleavage of Ang I with the help of the ACE2. Whereas, Ang 1-7 is obtained by the hydrolysis of Ang 1-9 and Ang I in presence of ACE and ACE2 [254]. Ang II acts through AT1 and AT2 receptors to mediate its actions. Detrimental actions of Ang II are due to its action through AT1 receptors. AT1 receptor activation has been implicated in the development and progression of DN, through release of several growth factors and cytokines, including transforming growth factor-β and tumor necrosis factor-α. Ang II has also been shown to activate and up regulate NFκβ signalling and related inflammatory genes through AT1 receptors. AT2 receptor activation has shown to be beneficial in DN. Protective actions mediated through AT2 receptors are completely opposite to AT1 receptors that include inhibition of cell growth, apoptosis, proliferation and inflammation [27,255]. Whole RAS cascade is explained briefly in the Figure 5 which is modified from Kobori *et al*., [254]. Clinically AT1 blockers and ACE inhibitors are proved to be effective in preventing DN. However, very little is known about ACE2 activators in preventing DN.

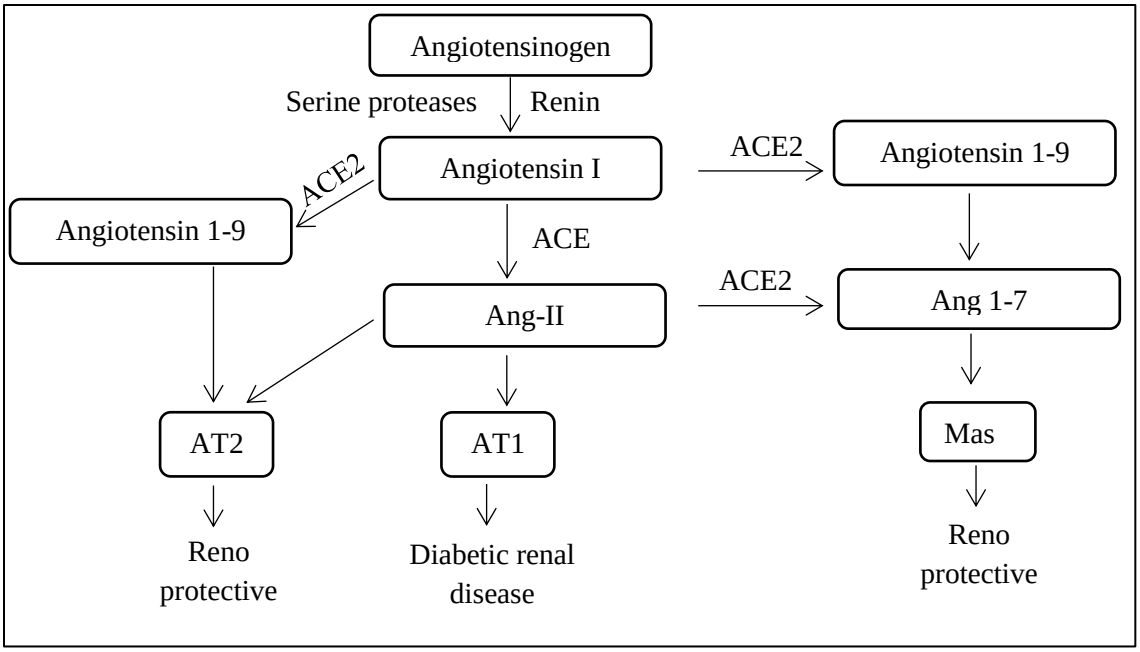


Figure 5: Schematic representation of renin angiotensin system in kidney

2.10. Importance of ACE2 in diabetic kidney:

ACE2 is a type 1 integral membrane protein that shares 42% homology with the metalloprotease catalytic domains of ACE. Unlike ACE, which consists of two catalytic domains, ACE2 contains only a single active domain and consists of 805 amino acids [256,257]. The catalytic mechanism of ACE2 closely resembles that of ACE. ACE2 functions as a monocarboxypeptidase by removing single amino acids from the C terminus of its substrates, whereas ACE functions predominantly as a peptidyl dipeptidase removing C-terminal dipeptides. ACE2 cleavage of angiotensin leads to the formation of Ang (1–7), whereas cleavage of Ang I leads to the formation of Ang(1–9). At least 11 peptides have been identified that are completely or partially hydrolyzed by ACE2 [258]. ACE2 has the highest catalytic efficiency for the degradation of Ang II. Other peptides that are hydrolyzed by ACE2 with high efficiency include the opioid peptide dynorphin A, des-Arg bradykinin, and ghrelin, a growth hormone secretagogue [259]. ACE2 has a more widespread distribution in mammalian tissues. Tissues with high levels of ACE2 include the kidneys, testes, intestines, and heart, whereas relatively low levels of ACE2 mRNA have been detected in various other tissues [257]. ACE2 is preferentially a tissue enzyme, and therefore is more likely to be involved in the degradation of Ang II in chronic conditions associated with over activity of this peptide, such as diabetic nephropathy. In this condition, ACE2

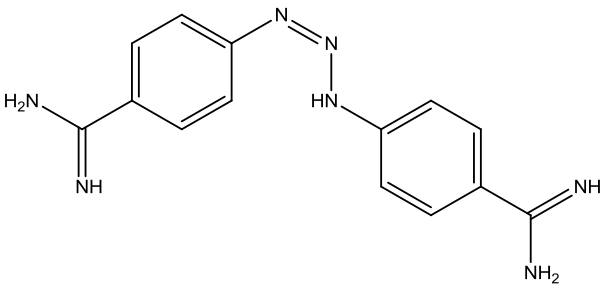
down regulation may cause excessive Ang II accumulation, particularly at the glomerular level, leading to increased albuminuria and glomerular damage [260]. A study in STZ induced diabetic rats found a decrease in glomerular ACE2 activity accompanied by a local increase in Ang II levels [34]. ACE2 deficiency has been associated with enhanced expression of inflammatory markers [261,262]. A study in male Akita mice, a model of Type I diabetes, showed that administration of human recombinant ACE2 could slow the progression of DN [35].

2.11. Diminazene Aceturate (DIZE): The ACE2 activator and its protective role in diabetes:

Diminazene aceturate (DIZE) is a US FDA approved small molecule which belongs to the group of aromatic diamidines used against babesiosis, piroplasmosis and trypanosomiasis since 1955 due to its DNA intercalating effect [263]. It has also been shown to exert an "off target" ACE2 activating effect [38]. DIZE (15 mg/kg per day, s.c.) significantly attenuated the myocardial infarction induced decrease in fractional shortening, improved the maximal rate of rise of left ventricular pressure (LVP) and reversed ventricular hypertrophy. DIZE treatment was found to decrease the infarct area, LV remodeling post myocardial ischemia, and restored normal balance of the cardiac renin–angiotensin system. In addition, DIZE treatment increased circulating endothelial progenitor cells, increased engraftment of cardiac progenitor cells, and decreased inflammatory cells in peri-infarct cardiac regions. All of the beneficial effects associated with DIZE treatment were abolished by C-16, an ACE2 inhibitor [38]. In addition, DIZE treatment significantly prevented the development of pulmonary hypertension (PH) induced in male Sprague Dawley rats by monocrotaline, hypoxia, or bleomycin challenge due to an increase in the vasoprotective axis of the lung renin-angiotensin system, decreased inflammatory cytokines, improved pulmonary vasoreactivity, enhanced cardiac function which were abolished by C-16. The angiogenic progenitor cells derived from the bone marrow of monocrotaline-challenged rats were made dysfunctional under PH conditions and were repaired by DIZE treatment. The angiogenic progenitor cells isolated from patients with PH exhibited diminished migratory capacity toward the key chemoattractant stromal-derived factor 1a, which was corrected by in vitro DIZE treatment [39].

Moreover, in another study performed by Rigatto and colleagues, it was demonstrated that DIZE (15 mg/kg/day) for a period of 21 days improved monocrotalin-induced PH is associated with a significant increase in sympathetic modulation and a decrease in heart rate variation (HRV) [40]. The treatment with DIZE was not able to reverse hyperglycaemia nor body weight loss but could successfully reverse hyperglycaemia-induced cardiac electrical changes in ventricular repolarization. DIZE treatment led to a shorter QT and QTc intervals. In addition, ACE2 activation was capable to shorten the cardiac action potential and also reverse the arrhythmic markers. Diminazene aceturate treatment did not induce arrhythmic events in normal, as well as in hyperglycaemic animals. This study showed that activation of ACE2 has a beneficial effect in hyperglycaemic rats, improving the cardiac electrical function. Therefore, DIZE represents a promising new therapeutic agent to treat hyperglycaemia-induced cardiac electrical changes in ventricular repolarization [264]. On the contrary, recent studies shows the controversy over direct ACE2 activation and expression by DIZE. Recently, Philipp K Haber et al., demonstrated that DIZE had no ability to activate ACE2 both in in-vitro and in ex-vivo experiments [265]. Similarly, Gábor Raffai et al., also showed, DIZE lacks ACE2 activation in an ex-vivo experiment using porcine coronary artery rings [266]. However, these studies were designed to study the acute effects of DIZE and failed to address the chronic administration of DIZE in in-vivo models on ACE2 activation. Recently, Yang Zhang et al., reported that chronic treatment with DIZE prevents oxidative stress and endothelial damage in db/db mice by increasing ACE2 activity and Ang 1-7 levels [43]. Hence, it is necessary to know the exact mechanism of DIZE in activating ACE2.

Table 1: Chemical details of Diminazene aceturate

Parameters	**Details**
Synonyms	Berenil; Azidin; Ganaseg; Diminazene aceturate; Azidine; Beronal; Ganasag; Diminazenaceturate
IUPAC Name	2-acetamidoacetic acid; 4-[2-(4-carbamimidoylphenyl)iminohydrazinyl] benzenecarboximidamide
Structure	
Molecular Weight	398.41908 g/mol
Molecular Formula	$C_{18}H_{22}N_8O_3$

Chapter 3
Background and Objectives

3. Background and Objectives

3.1. Background:

Diabetic nephropathy (DN) is one of the most common causes for the development of end stage renal disease [267]. Pathogenesis of DN is mainly due to uncontrolled or chronic hyperglycemia [268]. Moreover, there is no specific therapy till date to prevent DN suggesting the need of novel targets. Accumulating evidence demonstrating the ubiquitin proteasome system (UPS) alterations in diabetes. Increased UPS activity degrades Iκβ, a stabilizer of NFκβ and increases transcription of inflammatory and fibrotic genes through NFκβ/p300 mediated pathway [10,269]. Proteasomal inhibitors were also found to be protective in diabetic condition [270]. UPS components like E3 ligases and de-ubiquitinases were also found to be altered in diabetic condition. In addition, ubiquitinated H2A found to be over expressed under high glucose condition, which corresponds to the increased levels of fibronectin in mesangial cells. In contrast, ubiquitinated H2B expression was decreased [12,21,241]. More importantly, it is yet to be known, how histone ubiquitination involves in the development of renal fibrosis in diabetes. In addition, chronic treatment with angiotensin receptor blockers (ARBs) and Angiotensin Converting Enzyme (ACE) inhibitors are effective in retarding the progression of DN but their use remains limited because of the side effects, indicating requirement for finding additional pathways in RAS as potential drug targets. Recent studies highlighting the role of ACE-2 in diabetic nephropathy [35,37,271-273]. Based on the above facts, the study is conceived with the following major objectives.

3.2. Objectives

- To study the role of histone ubiquitination and its cross talk with other histone H3 modifications in the progression of renal fibrosis under type 1 diabetic condition.
- To study the effect of known proteasomal inhibitor (Aspirin) in preventing renal fibrosis in diabetic rats.
- To explore the effect of ACE2 activation on renal fibrosis under type1 diabetic condition.

Chapter 4
Materials and Methods

Chapter 4
Materials and Methods

4. Materials and Methods:

4.1. Materials

All the materials used throughout the study were enlisted in the below table (Table 2).

Table 2: List of instruments, biochemical kits and drugs

Name	Company	Country
Instruments/Minor accessories		
Vertical - electrophoresis unit	Bio-Rad	USA
Horizontal - electrophoresis unit	Tarsons	USA
Semi-Dry transfer apparatus	Bio-Rad	USA
Microscope	Olympus-BX51	Japan
Microtome	Leica	Germany
Light cycler-96	Roche	Germany
Thermocycler	BR-Biochem	India
High sensitive X-ray films	Thermofisher Scientific	USA
Cassettes	Thermofisher Scientific	USA
Cell strainers	Pluri select	Germany
DynaMag-2	Thermofisher Scientific	USA
Biochemical/ELISA Kits		
Glucose BUN Albumin Creatinine Total Protein — Biochemical KITS	Accurex	India
Angiotensin 1-7 Angiotensin II ACE-2 ACE — ELISA KITS	Fine Test	China
Drugs		
Streptozotocin Aspirin Diminazene aceturate	Sigma aldrich	USA
PD123319	Tocris	UK

4.2. Methods

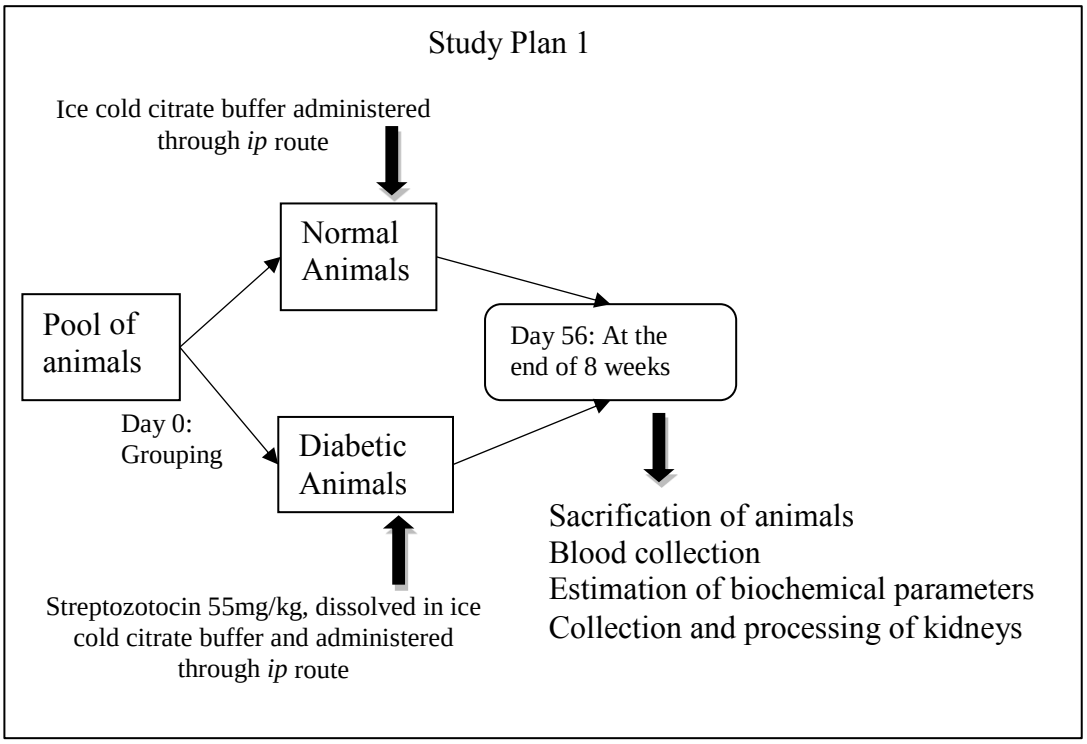
4.2.1. Experimental induction of diabetic nephropathy in male wistar rats and drug treatment

The male adult Wistar rats (180g – 220g) were procured from the central animal facility of Birla Institute of Technology and Science Pilani (BITS Pilani). Animals were maintained under standard environmental conditions and provided with feed and water *ad libitum*. Study was performed as per the protocol approved by the Institutional Animal Ethics Committee (IAEC), BITS, Pilani (Protocol No: IAEC/RES/18/05). Type 1 diabetes was induced as described by Tikoo et al. [251]. Briefly, diabetes was induced by injecting a single dose of STZ (55 $mg \cdot kg^{-1}$, i.p.), dissolved in ice-cold sodium citrate buffer (0.01M, pH 4.4) and the normal animals were injected only with ice-cold sodium citrate buffer. Animals with plasma glucose (PGL) levels >16 $mmol \cdot L^{-1}$ after induction of diabetes were included in the study as diabetic animals. All the diabetic animals received an i.p. injection of insulin (2–3 U) every 3 days to maintain blood glucose levels between 16 and 25 $mmol \cdot L^{-1}$ in order to prevent mortality induced by excessively high blood glucose levels. Detailed study plan for all the studies were given below schematically. **For study 1:** Only normal control (NC) and diabetic control (DC) were used till the end of 8 weeks. **For study 2:** At 4 weeks after the injection of STZ or saline, renal functional parameters were estimated in all the animals before separation in to experimental groups (8 rats per group). Normal animals (injected with saline) were divided in to normal control (NC), NC treated with high dose of Aspirin (NC + Asp50) and diabetic animals were divided in to diabetic control (DC), DC treated with low dose Aspirin (DC + Asp25) and DC treated with high dose of Aspirin (DC + Asp50). The low dose (25 $mg \cdot kg^{-1} \cdot day^{-1}$) and the high dose (50 $mg \cdot kg^{-1} \cdot day^{-1}$) of Aspirin were administered as single daily p.o. route for the last 4 weeks. **For study 3:** At 4 weeks after the injection of STZ or saline, renal functional parameters were estimated in all the animals before separation in to experimental groups (8 rats per group). Normal animals (injected with saline) were divided into normal control (NC), NC treated with high dose of DIZE (NC + HD) and diabetic animals (STZ-treated) grouped into diabetic control (DC), DC treated with low dose of DIZE (DC+ LD), DC treated with high dose of DIZE (DC + HD) and DC treated with high dose DIZE and PD123319 (DC + HD + PD). The low dose (5 $mg \cdot kg^{-1} \cdot day^{-1}$) and the high dose (15 $mg \cdot kg^{-1} \cdot day^{-1}$) of DIZE were administered as single

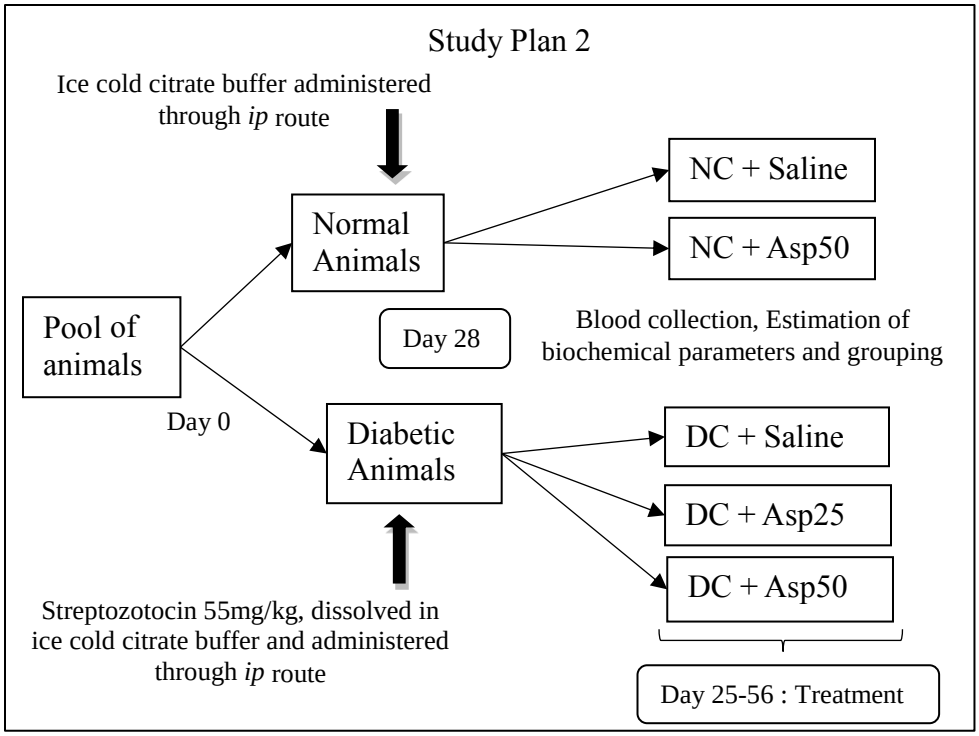
daily i.p. injections for 4 weeks; PD123319 (10mg·kg^{-1}·day^{-1}) was administered as single daily s.c. injections for the last 2 weeks as an addition to the treatment with DIZE. At the end of 8 weeks, kidneys from all the individual groups were processed separately for protein isolation, RNA extraction and for immunohistochemical studies.

4.2.2. Detailed study Plan:

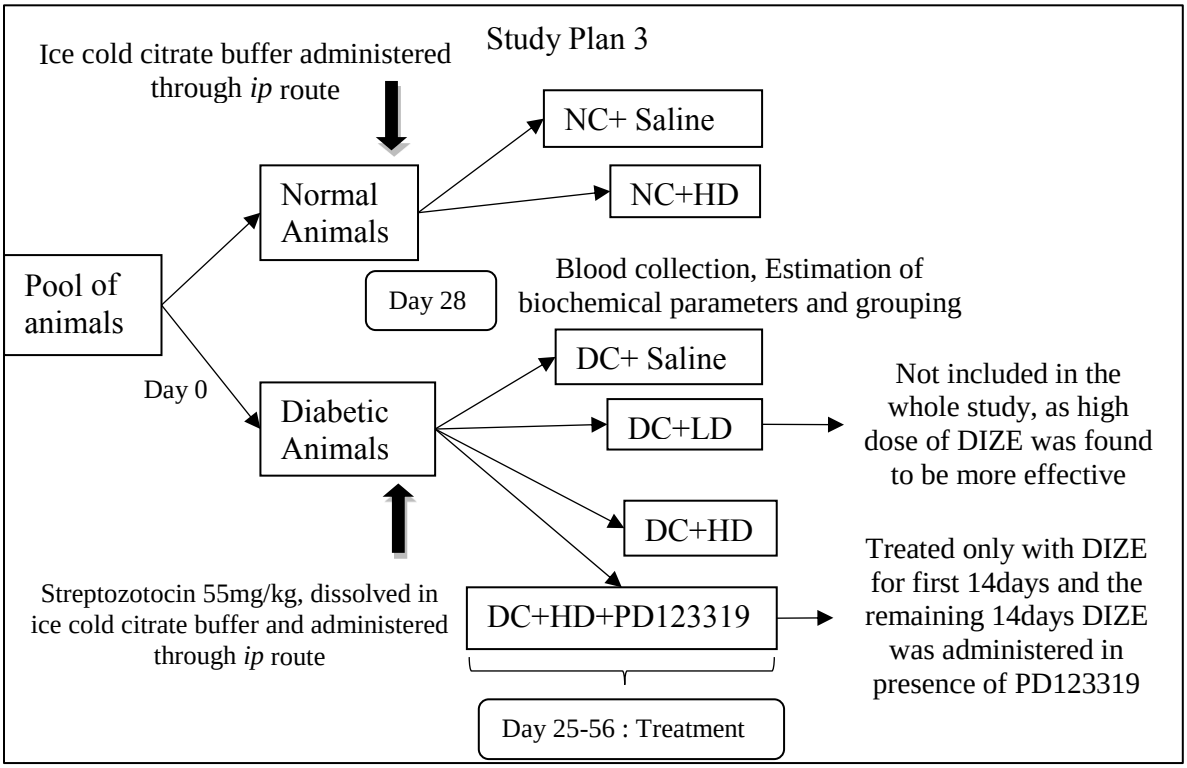
4.2.2.1. Study 1: To study the role of histone ubiquitination and its crosstalk with other histone H3 modifications in the progression of renal fibrosis under type1 diabetic condition.



4.2.2.2. Study 2: To study the effect of known proteasomal inhibitor (Aspirin) in preventing renal fibrosis in diabetic rats.



4.2.2.3. Study 3: To explore the role of ACE2 activation in the development of renal fibrosis under type1 diabetic condition.



4.3. Assessment of biochemical parameters

Venous blood (0.5mL) was taken at the time of group allocation (4weeks after STZ) and then again at the end of the whole experiment (8 weeks after STZ). All animals were fasted overnight before blood collection. Heparin (200 IU mL-1 of blood) was used as an anticoagulant. The plasma was separated from the blood by centrifuging the blood samples at 2000 x g for 15 min, at 4^{o}C. Plasma samples were analysed for glucose (PGL), BUN, albumin (PAL) and creatinine (PCr) by using commercially available kits (Accurex) [249].

4.4. Urinary total protein estimation

Urinary total protein estimation was performed as described by Sabbir khan et al. [274]. For urine collection, animals were kept in metabolic cages for 24 hours, urine was collected and the final volume was recorded for each animal, centrifuged at 2000rpm for 10 minutes. The supernatant was used for total protein estimation by using commercially available kit (Accurex, Mumbai, India).

4.5. Glomeruli isolation from whole kidney

Kidneys from both normal and diabetic animals were collected and placed in ice cold PBS, pH 7.4 and glomeruli were isolated using gradual sieving technique [275]. Briefly, kidneys were sliced in to thin portions, medullary portion was removed carefully and cortical area was minced with scalpel blade to a paste like consistency. This was passed through a stainless steel sieve with a pore size of 250µm. The material that passed through this sieve was suspended in ice cold PBS and passed through three consecutive cell strainers (200, 100 and 70µm), pluriStrainer set 3 (Pluriselect, Leipzig, Germany). The glomeruli retained on 100 and 70µm cell strainers were washed with ice cold PBS and resuspended in ice cold PBS. Thus obtained glomeruli were assessed under the light microscope and used for further analysis.

4.6. Histopathology

Histopathology was performed as per the protocol described by Gaikwad et al., [276]. Briefly, kidneys were fixed in 10% (*v/v*) formalin in phosphate buffered saline and embedded in paraffin. 5µm sections were deparaffinized with xylene (2 times, three minutes each) and rehydrated using gradient percentages of ethanol (100%, 95%, 70%, 50%; 3 minutes each). Then, the sections were washed under running tap water and stained with

Picrosirius Red (0.5% picrosirius red (PSR) in saturated picric acid solution in distilled water) for 1 hr. After staining with PSR, slides were treated with acidified water (5ml glacial acetic acid in 1000ml distilled water; 2 times, 5 minutes each), then the sections were washed under running tap water and dehydrated using ethanol (100%; 3 changes, 3 minutes each), placed in xylene and proceeded for mounting using Di-N-Butyle Phthalate in Xylene (DPX) media. Thus, stained sections with PSR were evaluated for collagen deposition. Further, Periodic acid and schiff reagent (PAS) stain was performed to evaluate extra cellular matrix (ECM) accumulation. Firstly, all the sections were deparaffinized, rehydrated, and treated with periodic acid (0.5% in distilled water) for 10 minutes and washed thoroughly under running tap water. Then, the sections were treated with Schiff's solution for 15 minutes, washed under hot running tap water, counter stained with hematoxylin (3 minutes), washed and proceeded for dehydration, mounting and analysis. At least 25 kidney sections from each group were observed and images were captured by using Olympus microscope (Model no. BX51, Tokyo, Japan). Collagen deposition was quantified by measuring the PSR positive area and ECM accumulation was measured by PAS positive area using ImageJ software.

4.7. Immunohistochemistry

Immunohistochemistry was performed as per the protocol described by Gaikwad et al., [276]. Briefly, kidney sections (5 µm) were taken from paraffin blocks and deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L for 30 minutes). After, antigen retrieval, the sections were cooled down to room temperature and treated with H_2O_2 (3%) for 15 minutes (to block endogenous peroxides), washed with tris-buffered saline (1X TBS) and blocked by using BSA (5%) solution. After blocking, the sections were incubated with the primary antibodies (12 hrs at 4^oC) listed in the following table (Table 3): anti-CollagenIV, anti-Fibronectin, anti-AT1, anti-AT2 (rabbit, 1:200 dilution; Santa Cruz Biotechnology), anti-TGF-β (rabbit, 1:200 dilution; Cell Signaling Technology, USA), anti-MAS1 (goat, 1:200 dilution; Santa Cruz Biotechnology) and rinsed thrice with TBS. Further, these sections were incubated (1 hr at room temperature) with respective anti-rabbit and anti-goat Horse Radish Peroxidase conjugated secondary antibodies, followed by detection with diaminobenzidine (DAB) as a chromogen. The sections were counterstained with hematoxylin, dehydrated with alcohol and xylene, and mounted in DPX (Sigma Aldrich). At least 25 kidney sections from each group were

observed and images were captured by using Olympus microscope (Model no. BX51, Olympus, Tokyo, Japan). All the images were analyzed using ImageJ software for calculating DAB positive area.

4.8. Estimation of systemic and tissue specific levels of Angiotensin converting enzyme (ACE), Angiotensin convertin enzyme 2 (ACE2), Angiotensin II (Ang II) and Angiotensin 1-7 (Ang 1-7)

Plasma, urine, whole kidney and isolated glomeruli samples were obtained from rats of all the groups. The levels of ACE, ACE2, Ang II and Ang 1-7 were assayed by use of ELISA kits following the manufacturer's recommendations (Wuhan Fine Biological Technology Co., Ltd. Wuhan, China) [277].

4.9. Protein isolation and western blotting

Western blotting was performed as per the protocol described by Gaikwad et al., [276]. Briefly, kidneys were dissected manually and processed for cytoplasmic and histone protein isolation. For, histone isolation kidneys were excised and washed with buffer containing 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl. Minced tissue was homogenized in a buffer containing 12% (w/v) sucrose, 10 mM Tris-HCl, pH 7.8, 2.5 mM EDTA and 1 mM PMSF. The homogenate was filtered through two layers of cheesecloth and centrifuged at 660 g for 5 min over a sucrose cushion [15% (w/v) sucrose in buffer A (10 mM Tris-HCl, pH 7.8, 10 mM NaCl, 1 mM PMSF)]. Thus obtained, crude nuclear pellets were washed with 12% (w/v) sucrose in buffer A, and then twice with Triton X-100 (0.2%) in buffer A, followed by pelleting over 15% (w/v) sucrose in buffer A. Pellets were further washed with 12% (w/v) sucrose in buffer A to remove traces of Triton X-100. Histones were isolated from thus obtained nuclear pellets using low salt buffer containing concentrated HCl through sonicating briefly and by centrifugation at maximum speed. Thus obtained supernatant contains basic histone proteins. Immunoblotting was performed by using antibodies (Table 3): H2AK119Ub, H2BK120Ub, H3K79Me2, H3K4Me2, H3K9Me2, SET7/9, SUV39H1, cleaved PARP, cleaved Caspase3, Smurf2, ACE2, AT2 and TGF-β; all antibodies were used in 1:1000 (*v/v*) dilutions. As secondary, anti-rabbit IgG, HRP-linked antibody was used in 1:20,000 (*v/v*) dilutions (Cell Signaling Technology, USA). Proteins were detected by using the electrochemiluminiscence (ECL) system and Hyperfilm. Immunoblots were quantified

by densitometric analysis using ImageJ software and the exposures were in linear dynamic range, H2AK119Ub, H3K4Me2 and H3K9Me2 were normalized using total H2A, H2BK120Ub and H3K79Me2 were normalized with total H2B and SET7/9, SUV39H1, cleaved PARP, cleaved Caspase3, Smurf2, ACE2, AT2 and TGF-β were normalized using β-actin. Then data analysis was performed by using Prism software (version 5.0; GraphPad, San Diego, CA, USA) and results were expressed as fold change over normal control (NC).

Table 3: List of antibodies used throughout the study

Primary Antibody (Used against)	**Dilution used**	**Company**	**Country**
For Immunohistochemistry			
TGF-β	1:200	Cell Signaling Technology	USA
AT1-receptor AT2-receptor MAS1-receptor Collagen IV Fibronectin	1:200	Santa Cruz Biotechnology	USA
For Western Blotting			
H2AK119Ub H2BK120Ub H3K79Me2 H3K4Me2 H3K9Me2 SET7/9 SUV39H1 Cleaved-PARP Cleaved-Caspase3 Smurf2 TGF-β	1:1000	Cell Signaling Technology	USA
ACE2 AT2-receptor	1:1000	Santa Cruz Biotechnology	USA

4.10. RNA isolation and Real Time-Polymerase chain reaction (RT-PCR)

RNA was isolated from kidneys by using commercially available kit (Ambion™ PureLink™ RNA Mini Kit, Life Technologies, USA). Aliquots of the RNA (5 µg) for each group was taken and incubated with 1 µl (2U) of recombinant DNase1 for 30 min at 37°C [Ambion™ Recombinant DNase I (RNase-free), Life Technologies, USA] to remove the single or/and double stranded DNA, chromatin and RNA:DNA hybrids present in the sample. Further, DNase1 was inactivated by heating the samples at 75°C along with 5mM of EDTA. EDTA is added to protect RNA from chemical scission when heated. On continuing, cDNA was synthesized by using cDNA kit (GeneSure™ First Strand cDNA Synthesis Kit, Puregene, Genetix brand, USA). The reactions were incubated at 25°C for 5 min, 42°C for 60 min followed by inactivation at 70°C for 5 min. Quantitative real-time polymerase chain reaction of the samples was performed as per the protocol described by Gaikwad et al, [249] on LightCycler® 96 Real-Time PCR System (Roche, Germany) using the FastStart Essential DNA Green Master (Roche, Germany) and results were analyzed by LightCycler® Software (Roche, Germany). Primers for *Rnf2*, *Usp7*, *Usp16*, *Usp21*, *Usp22*, *Col1a1*, *ACE, ACE2, AT1, AT2 and MAS1* were designed and obtained from Eurofins, India (Table 1). After amplification, a melting curve analysis was performed to verify the specificity of the reaction. Levels of mRNA of the samples were normalized to their respective *18s* contents. The 18S gene was used as an internal control, and the results were determined by 2^{-DDCt} expressed as the fold change over control group.

4.11. Chromatin-Immunoprecipitation (ChIP) assay

ChIP assay was performed as per the protocol described by Gaikwad et al, [249] by using MAGnify™ Chromatin Immunoprecipitation System (Thermo Fischer Scientific, CA, USA) according to manufacturer's guidelines. Briefly, kidneys were sliced in to small pieces, re-suspended in phosphate buffered saline (PBS) and cross linked with 1% (*v/v*) formaldehyde for 10 minutes. Cross linking reaction was stopped by adding 0.125M glycine, washed thrice with PBS containing protease inhibitors and lysed in SDS lysis buffer (supplied in the kit). Chromatin was sonicated for 10 s and the lysate was allowed to cool for 60 s over ice. This procedure was repeated for six cycles to obtain chromatin size of 0.5-1 kb. Lysates were incubated for 2 hours with H2AK119Ub, H2BK120Ub, H3K4Me2, H3K9Me2, H3K27Me2, H3K36Me2, H3K79Me2 antibodies, respectively. Before the addition of antibody, input

samples were removed from the lysate and stored at -20^{o}C until extraction. Following incubation with antibody, protein-DNA complexes were eluted, and the protein-DNA complexes were reversed using cross linking buffer. DNA was purified using magnetic beads supplied in the kit. ChIP enriched DNA samples and input DNA samples were analyzed by qPCR with SYBR reagent in Light cycler 96 Real-time PCR machine (Roche Diagnostics) using promoter specific forward and reverse primers for *Set7/9*, *Suv39h1* and *Col1a1* (Eurofins, Mumbai, India) (Table 4). Anti-IgG antibody was used as negative control for ChIP experiment. Experiments were carried out for each sample and the results were determined by 2^{-DDCt} method, expressed as ChIP/Input values for each group.

4.12. Statistical analysis

Data are presented as means ± SEM and 'n' refers to number of animals in a particular group. Statistical analysis was performed using GraphPad Prism, version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). To evaluate differences between two group mean values, we used unpaired Student's t-test. Statistical significance was determined with the oneway ANOVA followed by the Tukey's test for multiple comparisons when F achieved $p < 0.05$. Results were considered significant if $p < 0.05$.

Table 4: Primers list for qRT-PCR and ChIP-qRT-PCR analysis

Gene Name	Primer Sequence For qRT-PCR	Accession ID.
Rnf2	Forward 5'-ACAGCGCACAGACCAGATACA-3' Reverse 5'-AGACCCCACCACACCACTTG-3'	NM_001025667.1
Usp7	Forward 5'-ATGGAGGACGACACCAGT-3' Reverse 5'-CACAAAACACGGAGGGCTA-3'	NM_001024790.1
Usp16	Forward 5'-GCCGTCTCACCGGATTGTA-3' Reverse 5'-CCCCTTTGTTCGTTTCTTTCCC-3'	NM_001100501.1
Usp21	Forward 5'-TGGAGCGAGAAGACAGCAAG-3' Reverse 5'-CGGTCACATACTGGGGCATT-3'	NM_001127638.1
Usp22	Forward 5'-AACTGCACCATAGGTCTGCG-3' Reverse 5'-GTACGGAATGTGTGGGGAGC-3'	NM_001191644.1
Col1a1	Forward 5'-TGGCAACCTCAAGAAGTCCC-3' Reverse 5'-ACAAGCGTGCTGTAGGTGAA-3'	NM_053304.1
ACE	Forward 5'-CGCAGCTCTTCGCTGAC-3' Reverse 5'-TCTCCTCCGTGATGTTGGTG-3'	NM_012544.1
ACE2	Forward 5'-ATGAAGCGGGAGATCGTTGG-3' Reverse 5'-TGGAACAGAGATGCAGGGTC-3'	NM_001012006.1
AT1	Forward 5'-CTCTGCCACATTCCCTGAGTT-3' Reverse 5'-CTTGGGGCAGTCATCTTGGA-3'	NM_030985.4
AT2	Forward 5'- CAAGGGGAACTACATAAGAT-3' Reverse 5'- AAACACTGGCAACTAAAAGA-3'	NM_012494.3
MAS1	Forward 5'-AAGACCAGCCCACAGTTACCA-3' Reverse 5'-TCGATCACAGGAAGAGAGCC-3'	NM_012757.2
Primer Sequence For ChIP-qRT-PCR		
Set7/9	Forward 5'-GGGACCTGGGAATGAGAAAG-3' Reverse 5'-CACAAGCCGGTTTCCTAGAT -3'	NM_001109558.1
Suv39h1	Forward 5'-AGGACATGGGTGGACATTG-3' Reverse 5'-GCAGCCTACTATTCCCTCAAG-3'	NM_001106956.1
Col1a1	Forward 5'-GCTTAGCTGCCTGGTTCTT-3' Reverse 5'-CTCTTGGCCATGTCTCATAGTC-3'	NM_053304.1

Chapter 5

Results

5. Results:

5.1. Role of histone ubiquitination and its crosstalk with other histone H3 modifications in the progression of renal fibrosis under type1 diabetic condition

5.1.1. Increased collagen deposition and Col1a1 gene expression in type 1 diabetic kidney

After Streptozotocin (STZ) administration, all the rats had developed diabetes. Plasma glucose level of diabetic rats was significantly higher than the normal control rats (Table 5). Diabetic animals showed significant decrease in body weight and increase in kidney weight (Table 6). Increased kidney weight/body weight ratio, a marker for the development of diabetic nephropathy (DN), was also observed in diabetic rats (Table 6). Increased levels of plasma blood urea nitrogen (BUN), creatinine, decreased plasma albumin and increased urinary protein levels in diabetic animals, indicates the progressive renal damage [2,4]. In our study, we observed a significant increase in plasma BUN, creatinine, significant decrease in plasma albumin and increased urinary total protein levels in diabetic animals when compared to normal animals (Table 5). Renal fibrosis was further confirmed by light microscopy using Picrosirius Red (PSR) staining of kidney sections (Figure 6). Accumulation of ECM components like collagen and fibronectin in the mesangial and tubular interstitial spaces is a key indication for development of fibrosis in diabetic kidney [4]. Glomerular and interstitial fibrosis was found to be increased in diabetic animals (Figure 6 A, B, D, E). In diabetic kidney, we also observed an increased mRNA expression of Col1a1 gene (Figure 6 C). Based on these results, we confirmed the development of renal fibrosis in diabetic animals.

Table 5: Alterations of plasma biochemical parameters in diabetic animals

Group	PGL (mmol l^{-1})	PCr (µmol l^{-1})	BUN (mmol l^{-1})	PAL (g l^{-1})	UTP (mg 24 h^{-1})
NC	5.95 ± 0.42	82 ± 8.48	6.72 ± 0.5	32 ± 1	46.72 ± 7.14
DC	23 ± 0.78*	198 ±10.86*	18.92 ± 1*	20 ± 0.8*	298.46±22.10*

Table 6: Morphometric changes in diabetic animals

Group	Body weight (gm)	Kidney weight (gm)	(Kidney weight/Body weight) X 100
NC	199 ± 5.4	0.57 ± 0.044	0.29 ± 0.028
DC	141 ± 6.6*	0.72 ± 0.024*	0.52 ± 0.021*

Note (Table 5, 6): All the values represented as mean(s) ± S.E.M. n=8; *P < 0.05 vs NC. NC: Normal Control; DC: Diabetic Control; PGL: Plasma glucose; PCr: Plasma creatinine; BUN: Blood urea nitrogen; PAL: Plasma albumin; UTP: Urinary total protein.

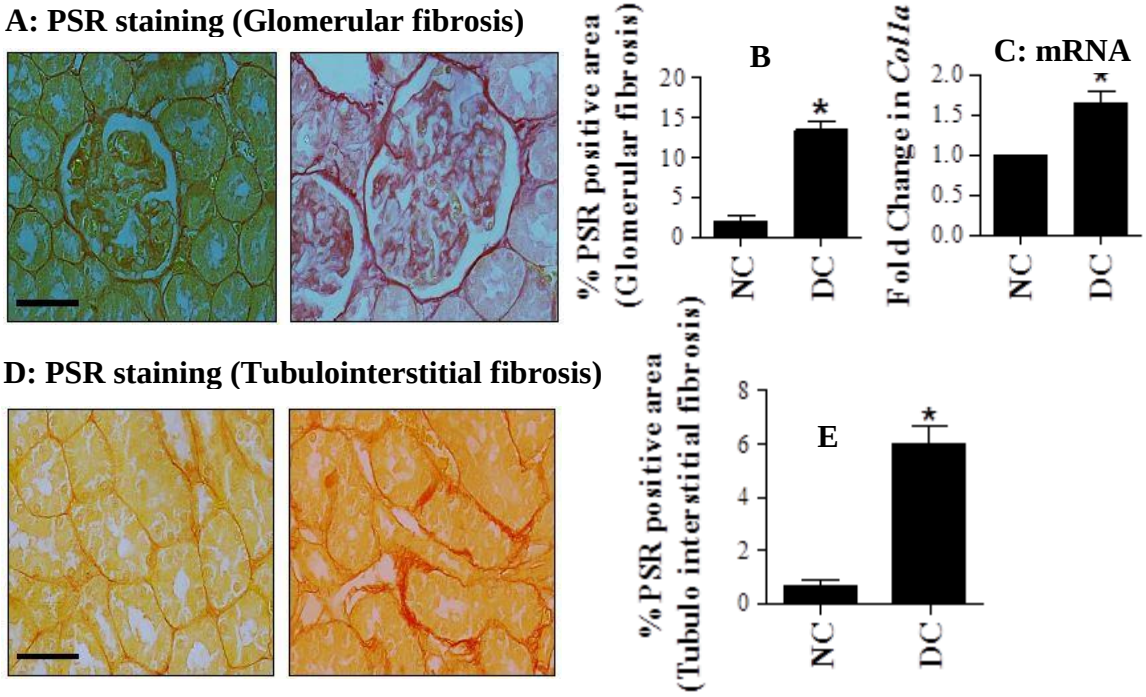


Figure 6: Hyperglycaemia increased collagen deposition and collagen1a gene expression in diabetic kidney. PSR staining of kidney sections (A, B) Glomerular collagen deposition and its semi quantitative analysis (C, D)tubulointerstitialcollagen deposition and its semi quantitative analysis was showed by measuring % PSR positive area using ImageJ software and (E) Increased mRNA expression of col1a gene in diabetic kidney. All the values represented as mean ± S.E.M. n=25 for B,D and n=3 for E; *P < 0.05 vs NC. NC (normal control) and DC (diabetic control).

5.1.2. Changes in histone H2AK119 mono-ubiquitination (H2AK119Ub), H2BK120 mono-ubiquitination (H2BK120Ub) and histone H3 dimethylation in isolated glomeruli and whole kidney of type 1 diabetic animals

It is well documented that the epigenetic mechanisms play a key role in diabetic nephropathy. We observed differential expression pattern of histone H2AK119Ub and H2BK120Ub in whole kidney and isolated glomeruli of type 1 diabetic animals (Figure 7, 10, 11). The expression of histone H2AK119Ub and H2BK120Ub was found to be increased in whole kidneys (Figure 7). On the contrary, the expression of histone H2AK119Ub and H2BK120Ub was reduced drastically in isolated glomeruli of diabetic animals (Figure 10, 11). Recently, H2AK119Ub and H2BK120Ub have been found to be involved in the increased expression of fibrotic genes in rat glomerular mesangial cells [21]. Histone H2B ubiquitination and histone H3 methylation cross talk is well documented. Therefore, we have checked the expression of histone H3K4Me2, H3K9Me2 and H3K79Me2 in diabetic kidney (Figure 7, 8, 9, 10, 11). Histone H2B ubiquitination is essential for H3K4Me and H3K79Me [19]. Our results also showed that increased expression of H3K4Me2 and H3K79Me2 in diabetic kidney was in parallel with the increased expression of H2B ubiquitination (Figure 7, 8). Decreased expression of repressive chromatin mark H3K9Me2 in diabetic kidney was also observed (Figure 8) whereas, in isolated glomeruli, only H3K4Me2 expression was found to be increased and no change was observed in the expression of H3K9Me2 in diabetic animals (Figure 10, 11). In addition, we also observed the increased protein expression of H3K4 specific histone methyl transferase (HMT) SET7/9 and decreased protein expression of H3K9 specific HMT SUV39H1 in diabetic kidney (Figure 9).

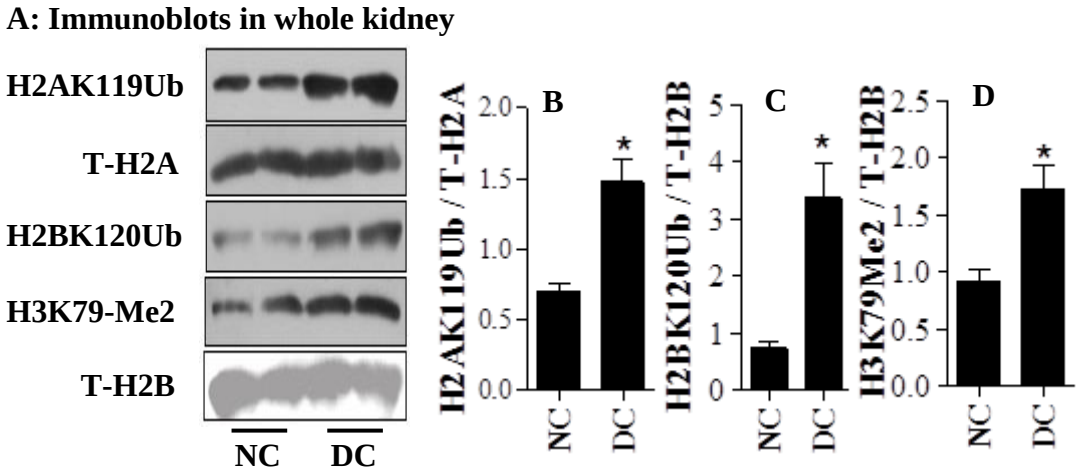


Figure 7: Increased expression of H2AK119Ub, H2BK120Ub and H3K79Me2 in whole diabetic kidney. (A) Western blot analysis of H2AK119Ub, H2BK120Ub and H3K79Me2; (B, C and D) Quantitative analysis of H2AK119Ub, H2BK120Ub and H3K79Me2 using ImageJ software. All the values are represented as mean ± S.E.M.; n=4; *P < 0.05 vs NC.

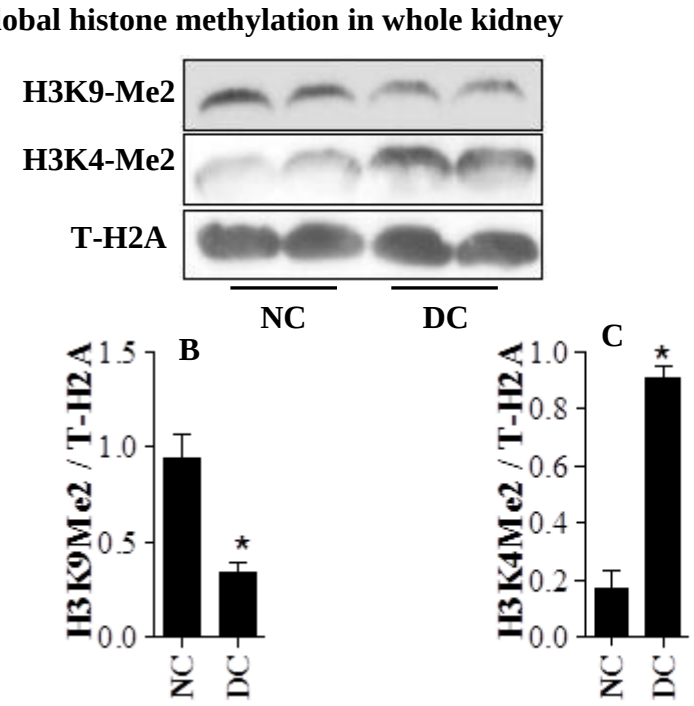


Figure 8: Decreased expression of H3K9Me2 and increased expression of H3K4Me2 in whole kidney. (A) Western blot analysis of H3K9Me2 and H3K4Me2; (B, C) Quantitative analysis of H3K9Me2 and H3K4Me2 normalized by T-H2A using ImageJ software. All the values are represented as mean ± S.E.M.; n=4; *P < 0.05 vs NC.

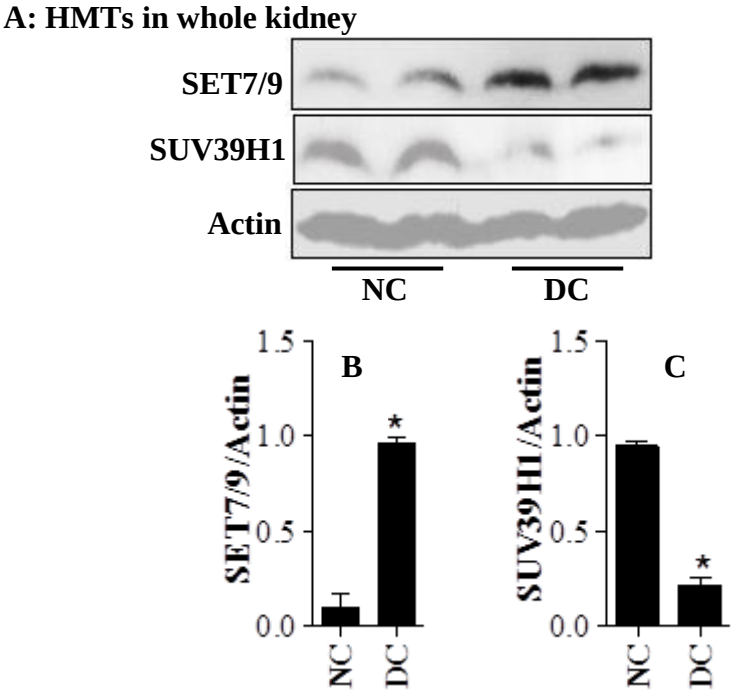


Figure 9: Increased expression of SET7/9 and decreased expression of SUV39H1 in diabetic kidney. (A) Western blot analysis of SET7/9 and SUV39H1; (B, C) Quantitative analysis of SET7/9 and SUV39H1 normalized by actin using ImageJ software. All the values are represented as mean ± S.E.M.; n=4; *P < 0.05 vs NC.

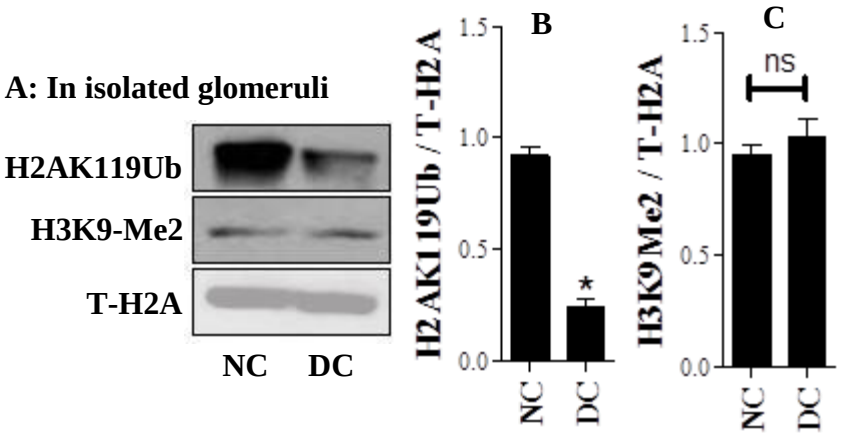


Figure 10: Expression of H2AK119Ub and H3K9Me2 in isolated glomeruli of diabetic animals. (A) Western blot analysis of H2AK119Ub and H3K9Me2; (B, C) Quantitative analysis of H2AK119Ub and H3K9Me2 normalized by T-H2A using ImageJ software. All the values are represented as mean ± S.E.M.; n=4; *P < 0.05 vs NC. ns (not significant)

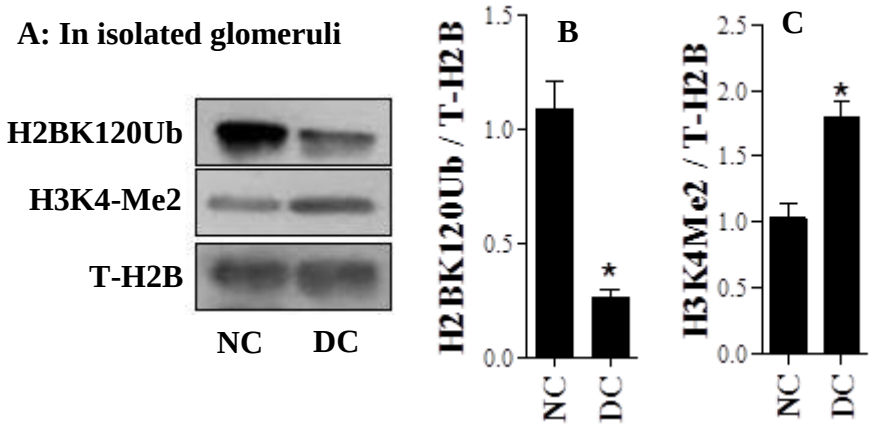


Figure 11: Expression of H2BK120Ub and H3K4Me2 in isolated glomeruli of diabetic animals. (A) Western blot analysis of H2BK120Ub and H3K4Me2; (B, C) Quantitative analysis of H2BK120Ub and H3K4Me2 normalized by T-H2B using ImageJ software. All the values are represented as mean ± S.E.M.; n=4; *P < 0.05 vs NC.

5.1.3. Increased expression of H2A specific E3 ligase Rnf2, decreased expression of H2A and H2B specific deubiquitinases (DUBs) in type 1 diabetic kidney

In diabetic kidney, we found increased mRNA expression of Rnf2 (Figure 12 A), an E3 ligase which specifically ubiquitinates histone H2A. Supporting to the above results, we observed the decreased mRNA expression of DUBs specific to histone H2A and H2B ubiquitination (Usp7, 16, 21 and 22) (Figure 12 B-E).

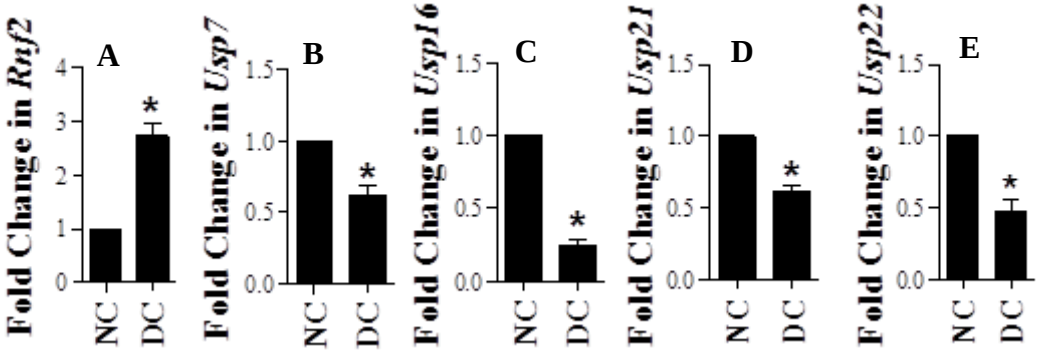


Figure 12: Changes in the mRNA expression of H2A specific E3 ligase Rnf2, H2A and H2B specific deubiquitinases in diabetic kidney. (A) Increased mRNA expression of H2A specific E3 ligase and (B-E) decreased mRNA expression of H2A and H2B specific deubiquitinases. All the values represented as mean ± S.E.M.; n=3; *P < 0.05 vs NC.

5.1.4. Epigenetic regulation of epigenetic enzymes: Changes in the occupancies of H2AK119Ub on the promoter of H3K4 HMT SET7/9 and H2BK120Ub on the promoter of H3K9 HMT SUV39H1 in type 1 diabetic kidney

Recent studies have indicated the role of histone H2AK119Ub in transcriptional repression [278]. Promoters of repressed genes like Serpina2, Lcn2 and Cyp2f2 were found to be enriched with H2AK119Ub in mouse regenerating liver [279]. Repressive actions of histone H2AK119Ub were, mainly by promoting repressive mark H3K27Me and by inhibiting active mark H3K36Me [280]. However, the signalling between H2B ubiquitination and histone H3 methylation is clearly linked to chromatin dynamics during transcription elongation. Histone H2BK120Ub is well known for promoting di- and tri-methylation of H3K4 and H3K79, modifications associated with transcriptionally active chromatin [19]. Interestingly, in our study, in diabetic kidney, repressive mark histone H2AK119Ub occupancy was found to be decreased over the promoter of SET7/9 (promotes H3K4Me) and also decreased occupancy of active mark H2BK120Ub on the promoter of SUV39H1 (promotes H3K9Me) (Figure 13 A, Figure 16 A), no change in the occupancies of H2AK119Ub and H2BK120Ub on the promoters of SUV39H1 and SET7/9 respectively (Figure 15 A, Figure 14 A).

In addition, we also observed the changes in H3 methylation marks that are regulated by H2AK119Ub (H3K27Me2 and H3K36Me2) and H2BK120Ub (H3K4Me2 and H3K79Me2) over the promoters of SET7/9 and SUV39H1 (Figure 13-16). Occupancies of H3K27Me2 was decreased and H3K36Me2 was increased over the promoter of SET7/9 (Figure 13 B, C) whereas occupancy of H3K27Me2 was decreased and no change in the occupancy of H3K36Me2 was seen over the SUV39H1 promoter (Figure 15 B, C). The occupancies of H3K4Me2 were remain unchanged and increased occupancies of H3K79Me2 were observed on the promoter of SET7/9 (Figure 14 B, C), whereas both H3K4Me2 and H3K79Me2 occupancies were decreased over the promoter of SUV39H1 (Figure 16 B, C).

In our study, we have observed the increased protein expression of SET7/9 and decreased expression of SUV39H1 in diabetic animals (Figure 9 A-C). Therefore the increased protein expression of SET7/9 and reduction in the expression of SUV39H1 in diabetic kidney may be due to the decreased occupancy of H2A and H2B ubiquitination over the promoters of

SET7/9 and SUV39H1. In renal mesangial cells, increased SET7/9 and its corresponding methylation mark H3K4 on the promoters of ECM genes like Col1a1, Ctgf and Pai1 were reported under hyperglycaemic condition [281]. In another study the decreased expression of SUV39H1 has been reported in VSMCs derived from db/db mice and this reduction resulted in the decreased occupancies of H3K9Me3 on the promoters of key inflammatory genes Il6 and Mcp1 [282]. Here, in this study, we also observed the increase in occupancies of H3K4Me2 and decrease in the occupancies of H3K9Me2 over the promoter of Col1a1 gene in diabetic kidney (Figure 17).

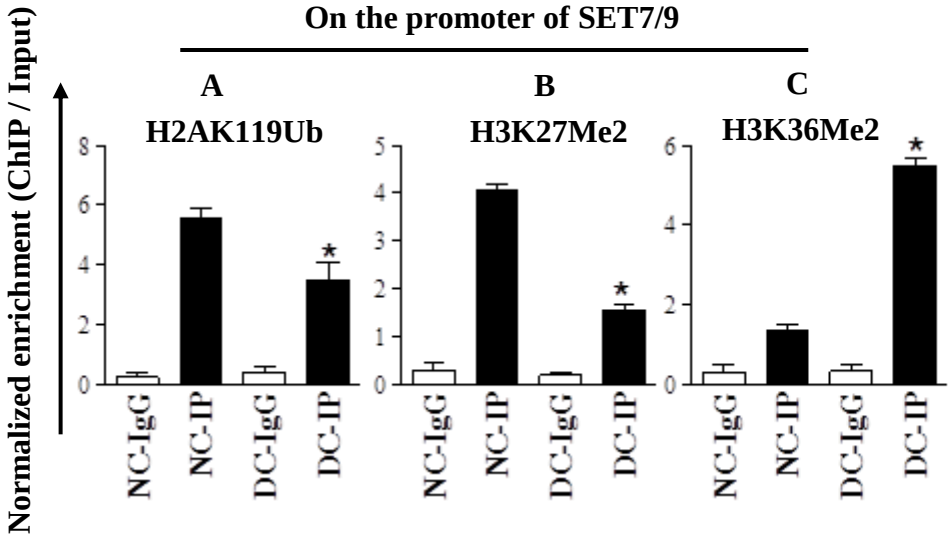


Figure 13: Effect of hyperglycaemia on the occupancies of H2AK119Ub and its regulated methylation marks (H3K27Me2, H3K36Me2) on the promoters of SET7/9. (A, B, C) Decreased occupancies of H2AK119Ub, H3K27Me2 and increased occupancies of H3K36Me2 on the promoter of SET7/9 gene. All the values represented as mean ± S.E.M.; n=3; *P < 0.05 vs NC.

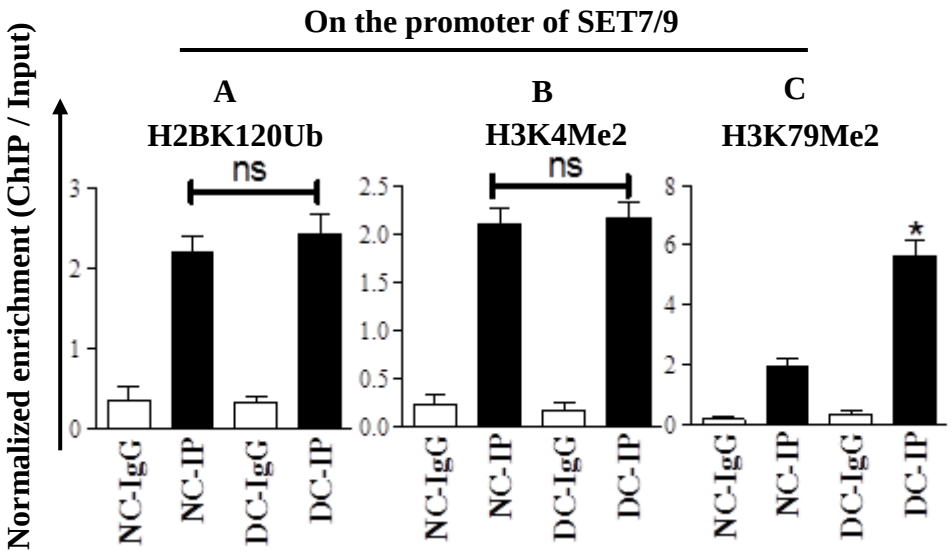


Figure 14: Effect of hyperglycaemia on the occupancies of H2BK120Ub and its regulated methylation marks (H3K4Me2, H3K79Me2) on the promoters of SET7/9. (A, B, C) No changes in the occupancies of H2BK119Ub, H3K4Me2 and increased occupancies of H3K79Me2 on the promoter of SET7/9. All the values represented as mean ± S.E.M.; n=3; *P < 0.05 vs NC. ns (not significant)

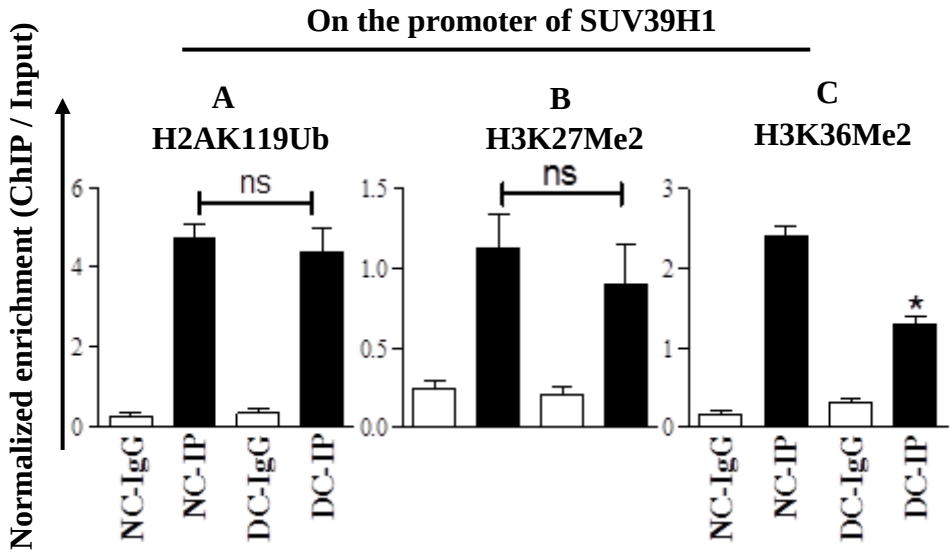


Figure 15: Effect of hyperglycaemia on the occupancies of H2AK119Ub and its regulated methylation marks (H3K27Me2, H3K36Me2) on the promoter of SUV39H1 gene. (A, B, C) No changes in the occupancies of H2AK119Ub, decreased occupancies of H3K27Me2 and no changes in the occupancies of H3K36Me2 on the promoter of SUV39H1. All the values represented as mean ± S.E.M.; n=3; *P < 0.05 vs NC.

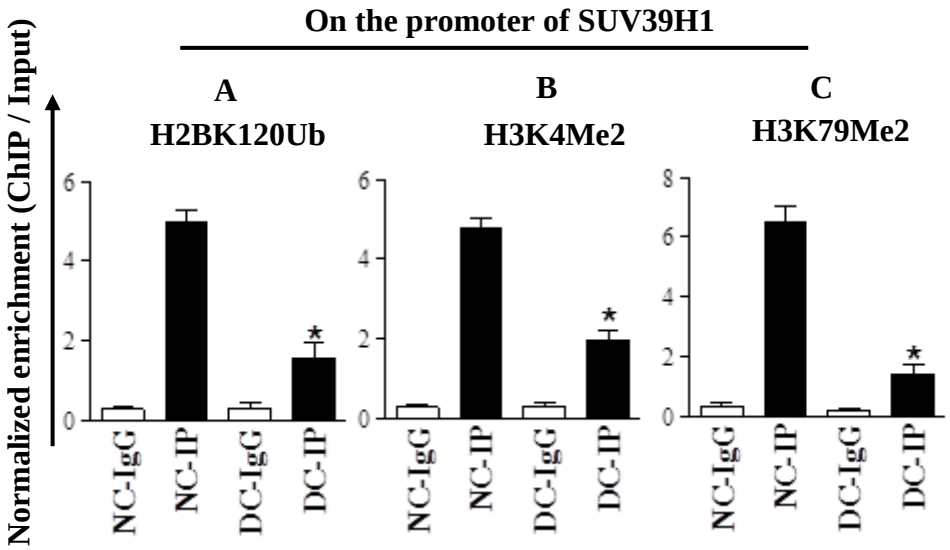


Figure 16: Effect of hyperglycaemia on the occupancies of H2BK120Ub and its regulated methylation marks (H3K4Me2, H3K79Me2) on the promoter of SUV39H1 genes. (A, B, C) Decreased occupancies of H2BK120Ub, H3K4Me2 and H3K79Me2 on the promoter of SUV39H1. All the values represented as mean ± S.E.M.; n=3; *P < 0.05 vs NC.

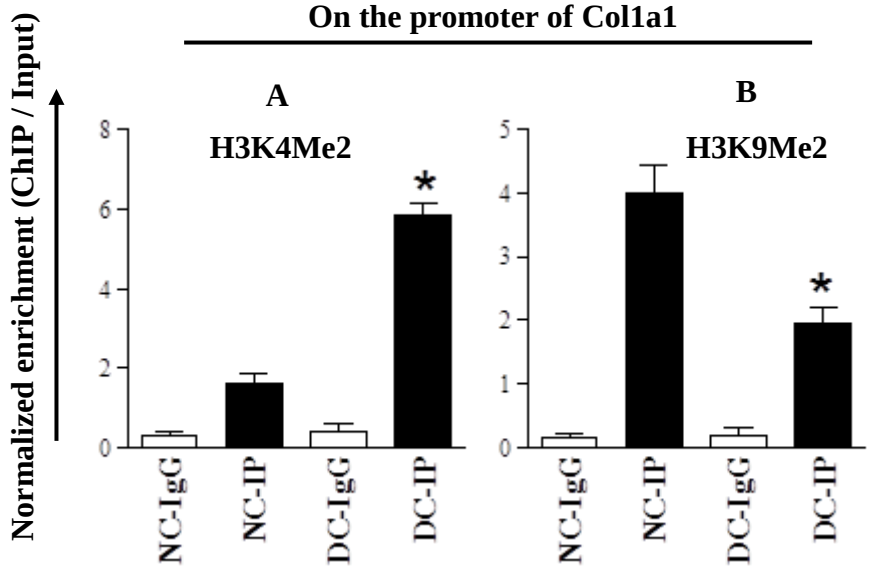


Figure 17: Increased occupancies of active chromatin mark H3K4Me2 and decreased occupancies of repressive chromatin mark H3K9Me2 on the promoter of Col1a1 gene. (A) Occupancies of active methylation mark, H3K4Me2 were increased and (B) occupancies of repressive methylation mark, H3K9Me2 were decreased over the promoter of Col1a1 gene. All the values represented as mean ± S.E.M.; n=3; *P < 0.05 vs NC.

5.2. Effect of known proteasomal inhibitor (Aspirin) on progression of renal fibrosis in diabetic rats

5.2.1. Aspirin improves morphometric and plasma biochemical parameters in diabetic animals

In this study, all the STZ induced diabetic animals showed marked reduction in body weight and this reduction was significantly reversed by the treatment with Aspirin at higher dose (Table 7). Further, diabetic animals also showed the key characteristic for the development of DN, which includes increased kidney weight and increased kidney weight to body weight ratio. Aspirin treatment decreased the kidney weight and kidney weight to body weight ratio in diabetic animals significantly at higher dose. In addition, all the diabetic animals also showed hyperglycaemia and Aspirin treatment, at high dose found to reduce the glucose levels to an extent. Further, we checked the plasma levels of blood urea nitrogen (BUN), creatinine (PCr) and albumn (PAL) in diabetic animals. Increased plasma levels of BUN, creatinine and reduced plasma albumin levels are the key markers for the development of renal dysfunction in diabetic animals. Moreover, we observed the increased plasma levels of BUN, creatinine and reduced plasma plasma albumin levels in diabetic animals and all these changes were normalized by Aspirin administration at higher dose (Table 8). Further, Aspirin treatment in normal animals did not affected the normal physiology (Table 7, 8).

Table 7: Effect of Aspirin on morphometric parameters

Group	Body Weight (gm)	Kidney weight (gm)	(Kidney weight/Body weight) X 100
NC	**232 ± 5.6**	**0.75 ± 0.04**	**0.32 ± 0.01**
NC+Asp	**234 ± 8.2**	**0.73 ± 0.04**	**0.30 ± 0.02**
DC	**155 ± 10***	**1.2 ± 0.1***	**0.80 ± 0.06***
DC + 25	**165 ± 7.6**	**1.2 ± 0.08**	**0.70 ± 0.03**
DC + 50	**205 ± 4$^{@}$**	**0.85 ± 0.1$^{@}$**	**0.42 ± 0.05$^{@}$**

Table 8: Effect of Aspirin on plasma biochemical parameters

Group	PGL (mmol l^{-1})	PCr (µmol l^{-1})	BUN (mmol l^{-1})	PAL (g l^{-1})
NC	**6.4 ± 0.6**	**47.0 ± 5**	**6 ± 0.6**	**47 ± 4**
NC+Asp	**6.2 ± 0.5**	**37.0 ± 2.3**	**5.5 ± 0.7**	**42.4 ± 4.3**
DC	**27 ± 0.7***	**151 ± 6.5***	**18.4 ± 1***	**21 ± 1.5***
DC + 25	**26 ± 1**	**140 ± 10.4**	**17 ± 1.2**	**23 ± 2**
DC + 50	**20 ± 1$^{@}$**	**62 ± 8.5$^{@}$**	**5.8 ± 1$^{@}$**	**38 ± 2$^{@}$**

Note (Table 7, 8): All the values are represented as mean ± S.E.M. n = 8. *P < 0.05 vs NC, NC+Asp and $^{@}$P<0.05 vs DC, DC+25. NC (normal control), NC+Asp [normal animals treated with high dose of Aspirin (50mg kg^{-1})], DC+25 [diabetic animals treated with low dose of Aspirin (25mg kg^{-1})] and DC+50 (diabetic animals treated with high dose of Aspirin (50mg kg^{-1})].

5.2.2. Aspirin treatment, reduced the expression of Mysm1, increased the expression of H2AK119Ub and thereby decreased the expression of SET7 in glomeruli isolated from diabetic animals

Further, at the molecular level, we observed the increased levels of histone H2A de-ubiquitinase, Mysm1 in glomeruli isolated from diabetic animals, which resulted in the decreased levels of histone H2AK119-Ub. Recently, we demonstrated that decreased levels of histone H2AK119-Ub involves in the increased expression of SET7, a histone H3 lysine 4 methyl transferase, mainly involves in the development of renal fibrosis. In addition, supporting to these results, in glomeruli isolated from diabetic animals, we observed increased protein expression of SET7. However, all these changes were significantly normalized by Aspirin treatment at higher dose in diabetic animals (Figure 18 A-D).

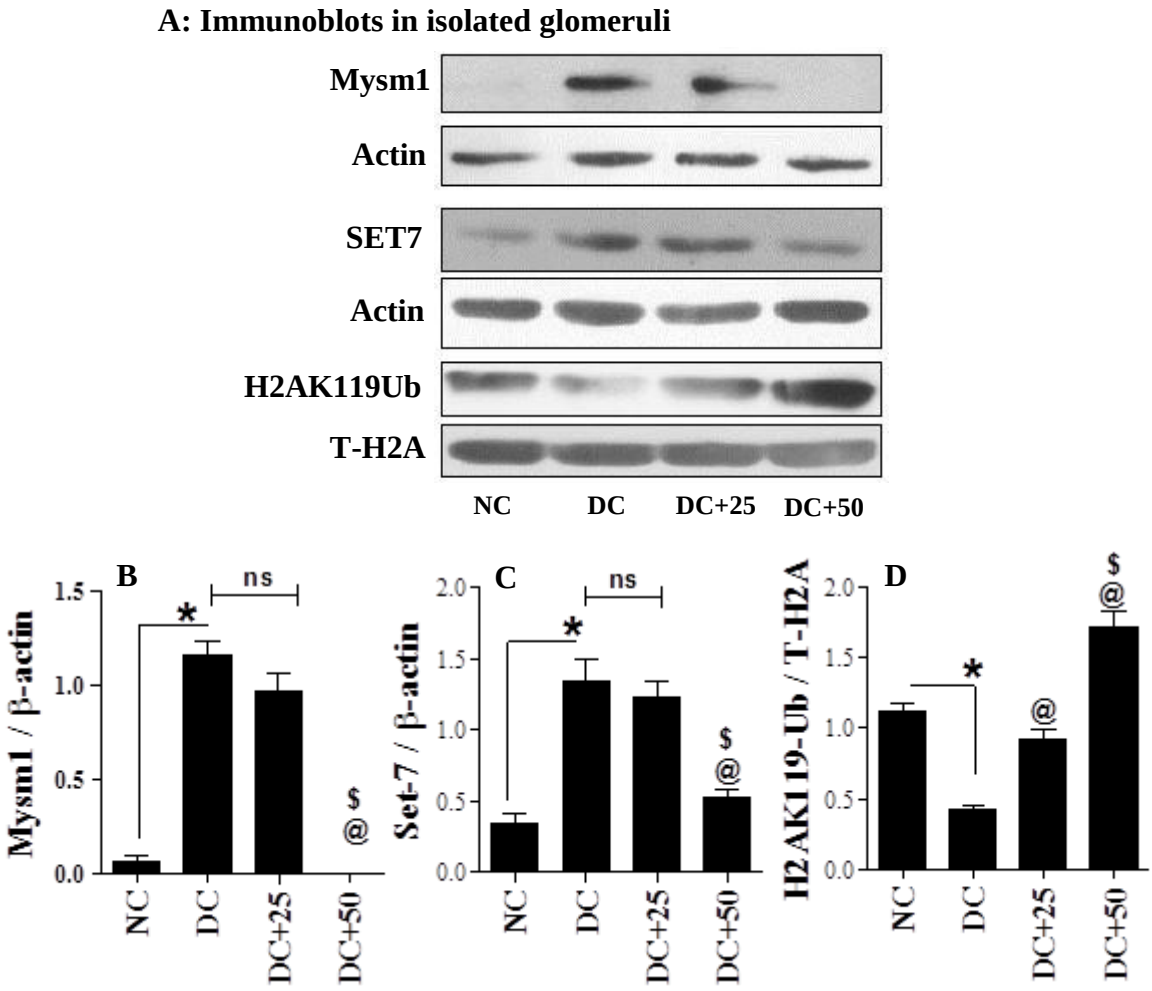


Figure 18: Aspirin treatment decreased the protein expression of SET7 in glomeruli isolated from diabetic animals through increasing the expression of histone H2AK119-Ub: In diabetic animals, increased expression of Mysm1 found to reduce the expression of H2AK119-Ub and further increased the levels of SET7. Further, aspirin treatment significantly reversed all these changes and reduced the expression of SET7 in glomeruli of diabetic animals at higher dose. Where (A) Western blot analysis of Mysm1, SET7, and H2AK119-Ub and (B-D) their respective quantitative analysis using ImageJ software. All the values are represented as mean ± S.E.M.; n = 6 rats per group; *P < 0.05 vs NC, $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+25. ns (not significant), NC (normal control), DC (diabetic control), DC+25 [diabetic animals treated with low dose of aspirin (25mg kg^{-1})] and DC+50 [diabetic animals treated with high dose of aspirin (50mg kg^{-1})].

5.2.3. Aspirin prevents extracellular matrix accumulation in diabetic kidney

Increased deposition of extracellular matrix (ECM) proteins like TGF-β, collagen and fibronectin in glomeruli and in interstitium is the key feature for the development of renal fibrosis in diabetes. In this study, we performed PAS staining of kidney sections, which mainly detects the ECM deposition. In diabetic animals, we observed increased PAS positive area and this PAS positive area was significantly reduced, when aspirin administered at higher dose (Figure 19 A, B). Further, we also checked the collagen deposition through PSR staining of kidney sections. Here, we also observed the increased PSR positive staining in diabetic kidney and further aspirin administration reduced the PSR positive area at higher dose (Figure 20 A, B). Moreover, aspirin treatment in normal animals did not show any effect on PAS or PSR positive area (Figure 19, 20).

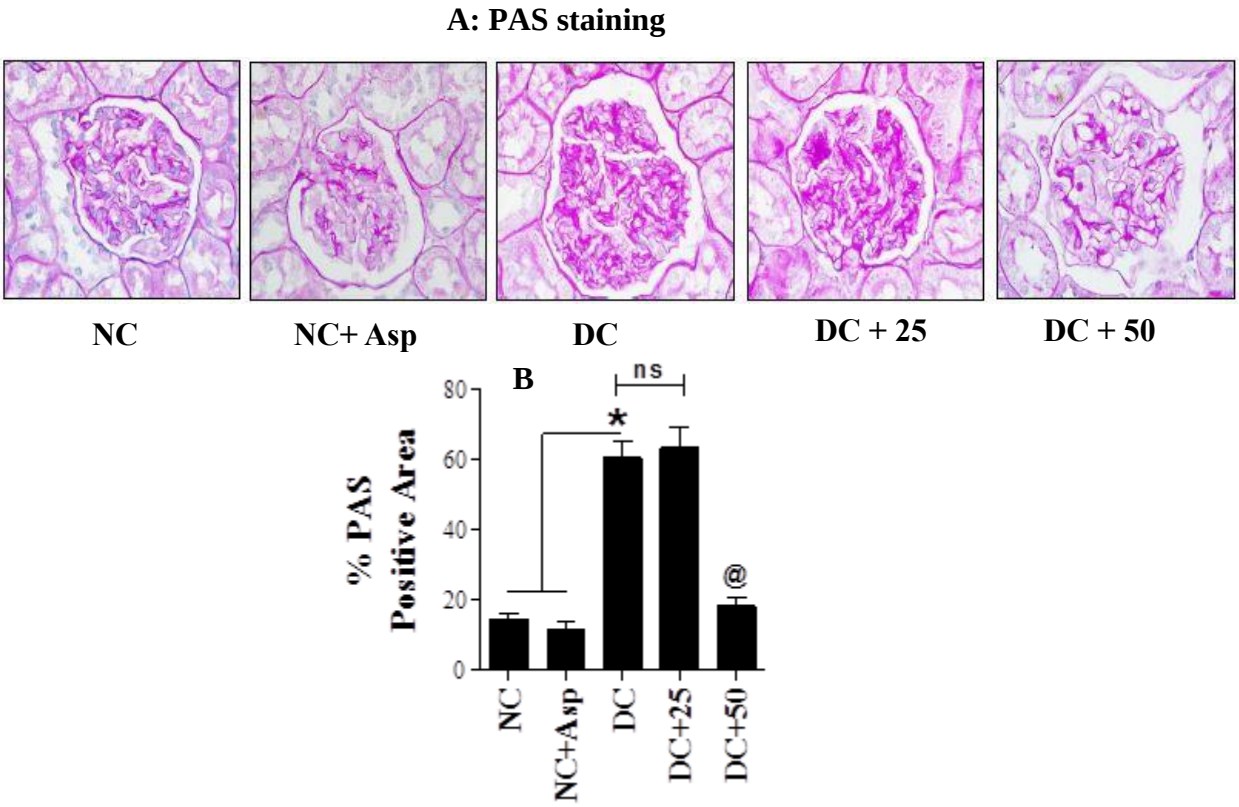


Figure 19: Aspirin treatment reduced the extracellular matrix depositon in diabetic rats: Where (A and B) PAS staining and its respective semi-quantitative analysis using ImageJ software. All the values are represented as mean ± S.E.M. n = 25. *P < 0.05 vs NC, NC+Asp and $^{@}$P<0.05 vs DC, DC+25.

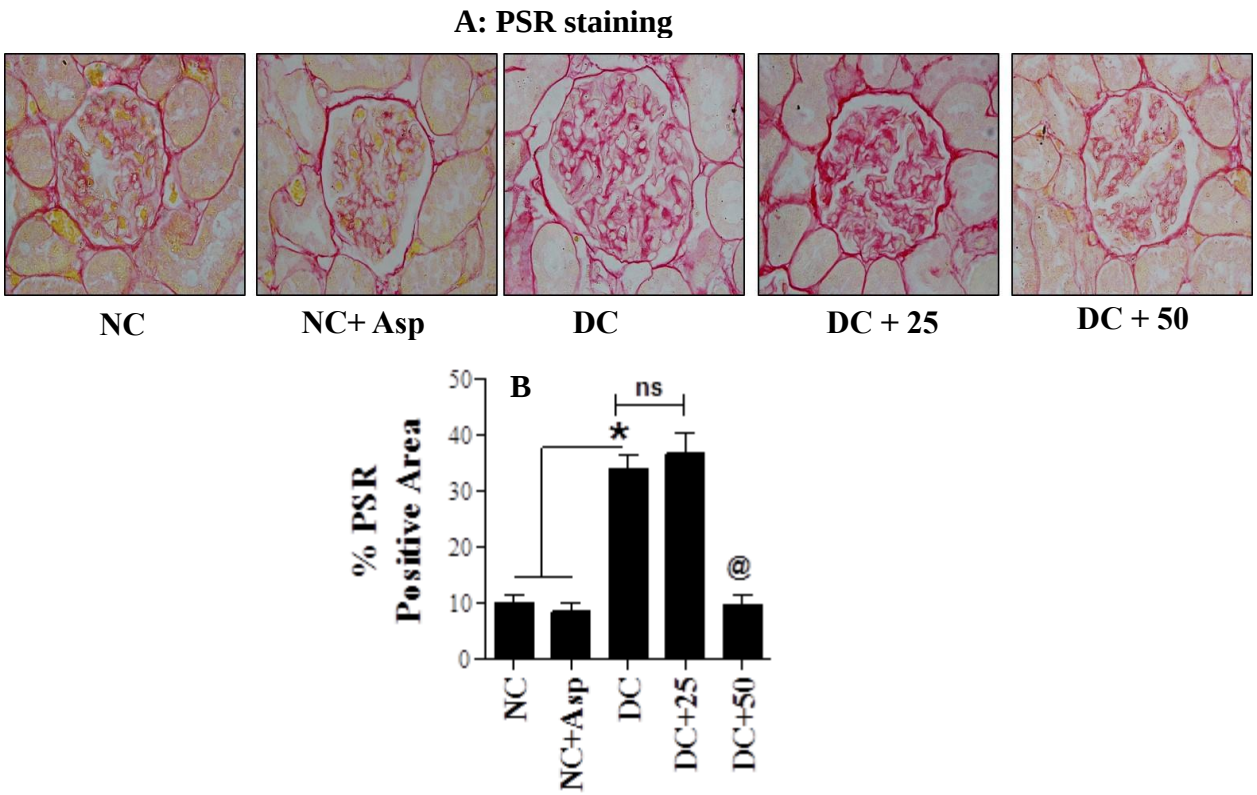


Figure 20: Aspirin treatment reduced the collagen depositon in diabetic rats: Where (A and B) PSR staining of kidney sections and its respective semi-quantitative analysis using ImageJ software. All the values are represented as mean ± S.E.M. n = 25. *P < 0.05 vs NC, NC+Asp and $^{@}$P<0.05 vs DC, DC+25.

5.3. Role of ACE2 activation in the development of renal fibrosis under type1 diabetic condition

5.3.1. Changes in body weight, kidney weight and kidney weight/body weight ratio by treatment with diminazene aceturate (DIZE) and DIZE in presence of AT2 blocker

Streptozotocin (STZ)-induced diabetic animals showed significantly decreased body weight and increased kidney weight, leading to an increase in the kidney/body weight ratio (Table 9). This ratio is a marker for the development of DN. Treatment of diabetic rats with the higher dose of DIZE (15 mg·kg^{-1}) reversed the increases in kidney weight and decreased the kidney /body weight ratio (Table 9). However, adding the AT2 receptor antagonist PD123319 for the last two weeks reversed these effects of DIZE (Table 9). Further, treatment of normal rats with the high dose of DIZE did not alter theses parameters (Table 9).

5.3.2. Effect of DIZE on plasma biochemical parameters in diabetic rats

After 8 weeks, plasma glucose levels of diabetic rats was significantly higher than in the normal control. Treatment with DIZE did not show any significant effect on plasma glucose levels in normal control and in diabetes induced rats (Table 10). Increased plasma creatinine (PCr) and blood urea nitrogen (BUN) levels are the indicators of the development of diabetic nephropathy in rats. DIZE at both the doses (5 and 15 mg kg^{-1}) significantly decreased the increased PCr and BUN levels in diabetic rats, and there is no difference observed between the two doses (Table 10). When compared to control animals, plasma albumin (PAL) levels were significantly decreased in diabetic control and this decrease is significantly increased by both the doses of DIZE without any difference (Table 10). Maintenance of these biochemical parameters closer to those in normal control by DIZE administration suggests that DIZE plays a role in protection against renal damage in diabetic condition. However, DIZE treatment in presence of AT2 blocker, failed to normalize the diabetes induced alterations in plasma (Table 10). In addition, DIZE did not altered any plasma biochemical parameters, when administered in normal rats (Table 10).

Table 9: Effect of Diminazene aceturate alone or in presence of AT2 blocker, on morphometric parameters

Group	Body Weight (gm)	Kidney weight (gm)	(Kidney weight/Body weight) X 100
NC	**208 ± 4.17**	**0.64 ± 0.02**	**0.31 ± 0.02**
NC + HD	**214 ± 6.8**	**0.59 ± 0.03**	**0.28 ± 0.01**
DC	**149 ± 5.6***	**0.8 ± 0.03***	**0.54 ± 0.03***
DC + LD	**162 ± 12**	**0.7 ± 0.04**	**0.44 ± 0.05**
DC + HD	**170 ± 7.2**	**0.56 ± 0.02$^{@}$**	**0.34 ± 0.03$^{@}$**
DC + HD + PD	**155 ± 7.5**	**0.82 ± 0.027*$^{\$}$**	**0.62 ± 0.04*$^{\$}$**

Note: All the values are represented as means ± S.E.M.; n = 8 rats per group; *P < 0.05 vs NC, NC+HD, $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD. LD: low dose of DIZE (5mg $kg^{-1}day^{-1}$), HD: high dose of DIZE (15mg $kg^{-1}day^{-1}$), PD: AT2 blocker PD123319.

Table 10: Effect of Diminazene aceturate alone or in presence of AT2 blocker, on plasma biochemical parameters

Group	PGL (mmol l^{-1})	PCr (µmol l^{-1})	BUN (mmol l^{-1})	PAL (g l^{-1})
NC	**6 ± 0.5**	**132 ± 7.6**	**7.7 ± 0.9**	**42 ± 1**
NC + HD	**7 ± 0.5**	**134 ± 5.8**	**5 ± 1.6**	**36.2 ± 1.2**
DC	**26 ± 1***	**214 ± 12***	**16.35 ± 0.7***	**21.76 ± 1.4***
DC + LD	**24.4 ± 1***	**171 ± 6.2$^{@}$**	**11 ± 0.8$^{@}$**	**31.7 ± 2.3$^{@}$**
DC + HD	**24 ± 1.2***	**145 ± 7.4$^{@}$**	**10.2 ± 0.5$^{@}$**	**34 ± 2.5$^{@}$**
DC + HD + PD	**27 ± 1.7***	**235 ± 5.2$^{\$}$**	**18 ± 0.9$^{\$}$**	**20.48 ± 2$^{\$}$**

Note: All the values are represented as means ± S.E.M.; n = 8 rats per group; *P < 0.05 vs NC, NC+HD, $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD.

5.3.3. ACE2 activation prevented renal fibrosis and apoptosis

Renal fibrosis and apoptosis are considered to be the underlying causes for the development of diabetic kidney disease. In this study we have observed the increased protein expression of profibrotic marker transforming growth factor- β (TGF- β) and increased markers of apoptosis like cleaved PARP and cleaved Caspase-3 in diabetic kidney. These changes were normalized significantly, by higher dose of DIZE administration (Figure 21 A-D).

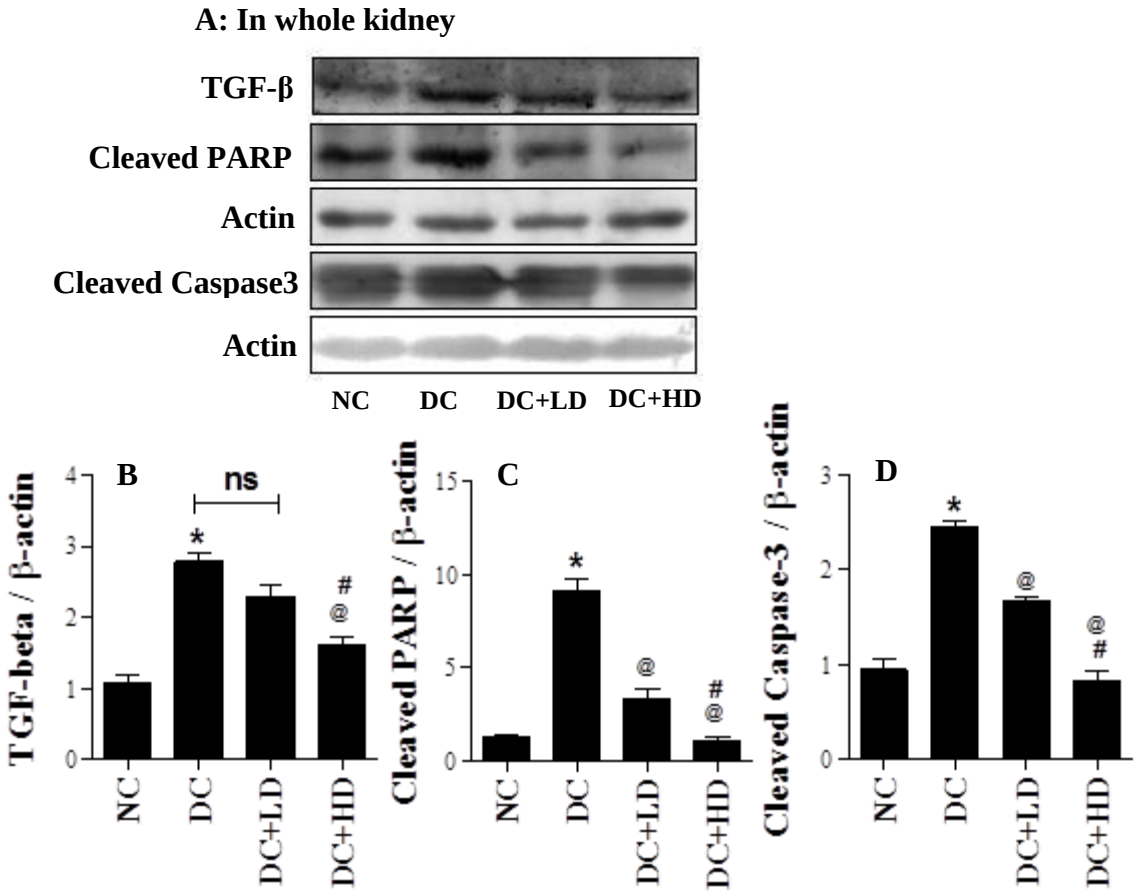


Figure 21: Diminazene aceturate inhibited diabetes induced renal fibrosis and apoptosis in diabetic rats. (A) Western blot analysis of TGF-β, cleaved PARP, cleaved Caspase-3 in whole kidney and (B-D) their respective quantitative analysis using ImageJ software. All the values are represented as mean ± S.E.M.; n = 6 rats per group; *P < 0.05 vs NC, $^{@}$P<0.05 vs DC and $^{\#}$P<0.05 vs DC+LD. NC (normal control), DC (diabetic control), LD (low dose of DIZE (5mg kg^{-1})) and HD (high dose of DIZE (15mg kg^{-1}).

5.3.4. DIZE treatment increased glomerular ACE2 and AT2 expression, decreased the cleaved PARP and Smurf2 expression

Further at molecular, in glomeruli from diabetic animals, expression of ACE2 protein was reduced and this was restored, dose-dependently, by DIZE treatment (Figure 22 A, B). The protein expression of cleaved PARP and Smurf2 was also increased in diabetic glomeruli, and this was reversed by the higher dose of DIZE (Figure 22 A, C, D). Smurf2 is an ubiquitin E3 ligase that degrades SMAD7, a negative regulator of TGF-β, and is the key molecule involved in the development of fibrosis. We also observed increased expression of AT2 receptor protein in diabetic glomeruli, which was further increased dose dependently by DIZE administration (Figure 22 A, E).

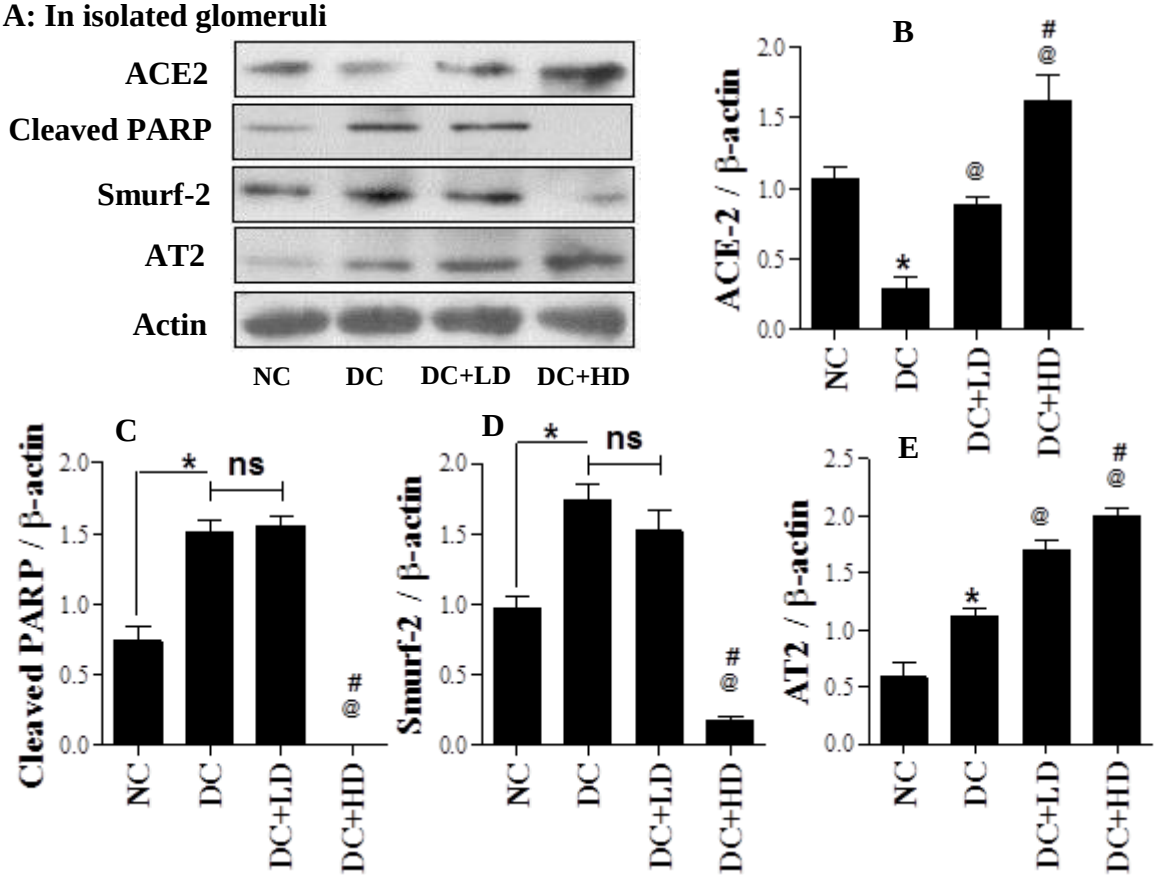


Figure 22: Diminazene aceturate treatment, increased the glomerular ACE2, AT2 and decreased the cleaved PARP, Smurf2 protein expression. (A) Western blot analysis of ACE2, cleaved PARP, Smurf2, AT2 in isolated glomeruli and (B-E) their respective quantitative analysis using ImageJ software. All the values are represented as mean ± S.E.M.; n = 6 rats per group; *P < 0.05 vs NC, $^{@}$P<0.05 vs DC and $^{\#}$P<0.05 vs DC+LD.

5.3.5. Effect of treatment with DIZE alone and in presence of AT2 blocker on systemic and tissue specific changes in RAS components (ACE, ACE2, Ang II, Ang 1-7)

RAS alterations are critical in the diabetes pathogenesis. In this study, we measured the protein levels of major RAS components including ACE and ACE2 in systemic fluids like plasma, urine and also in whole kidney, as well as in isolated glomeruli (Figure 23, 24, 25, 26). In diabetic animals, increased expression of ACE was observed in plasma, whole kidneys, and isolated glomeruli and DIZE administration reduced these levels in plasma (Figure 23 A-D). DIZE had no effect on increased ACE levels in whole kidney, isolated glomeruli and AT2 blocker further increased these levels in whole kidney and isolated glomeruli (Figure 23 C, D) whereas, urinary ACE expression did not alter in diabetic animals, as well as in diabetic animals treated with DIZE, but in diabetic animals where, DIZE administered in presence of AT2 blocker, increased urinary ACE protein expression was observed (Figure 23 B). In addition, we found no alterations in plasma ACE2 levels between any of the groups (Figure 24 A), whereas in urine, ACE2 levels were increased in diabetic animals and these levels were normalized in DIZE alone treatment group and again increased in DIZE in presence of AT2 blocker treatment group (Figure 24 B). In whole kidney, we found elevated levels of ACE2 in diabetic animals and DIZE alone, DIZE in presence of AT2 blocker treatment further increased these levels (Figure 24 C). Moreover, in isolated glomeruli, we observed the reduction of ACE2 protein expression in diabetic kidney and this was restored after DIZE treatment and AT2 blockade reversed the effect of DIZE and reduced the protein levels of ACE2 (Figure 24 D).

Further, we checked Ang II and Ang 1-7 levels in the plasma, whole kidney and isolated glomeruli. Ang II forms from Ang I through the enzymatic actions of ACE and ACE2 acts on Ang II to generate reno-protective peptide Ang 1-7. In our study, we observed increased levels of Ang II in plasma, whole kidney and in isolated glomeruli, this increase was significantly reversed after DIZE administration and AT2 blocker had no effect on this reversal in plasma and whole kidney (Figure 25 A, B) whereas, in isolated glomeruli AT2 blockade again increased the levels of Ang II (Figure 25 C). In addition, we found the decreased levels of Ang 1-7 in plasma, whole kidney and in isolated glomeruli, DIZE treatment improved these levels and plasma Ang 1-7 levels were not altered in presence of AT2 blocker whereas, AT2 blockade resulted in decreased levels of Ang 1-7 in whole kidney

and isolated glomeruli (Figure 26 A-C). In addition, DIZE did not show any effect, when administered in normal rats (Figure 23, 24, 25, 26).

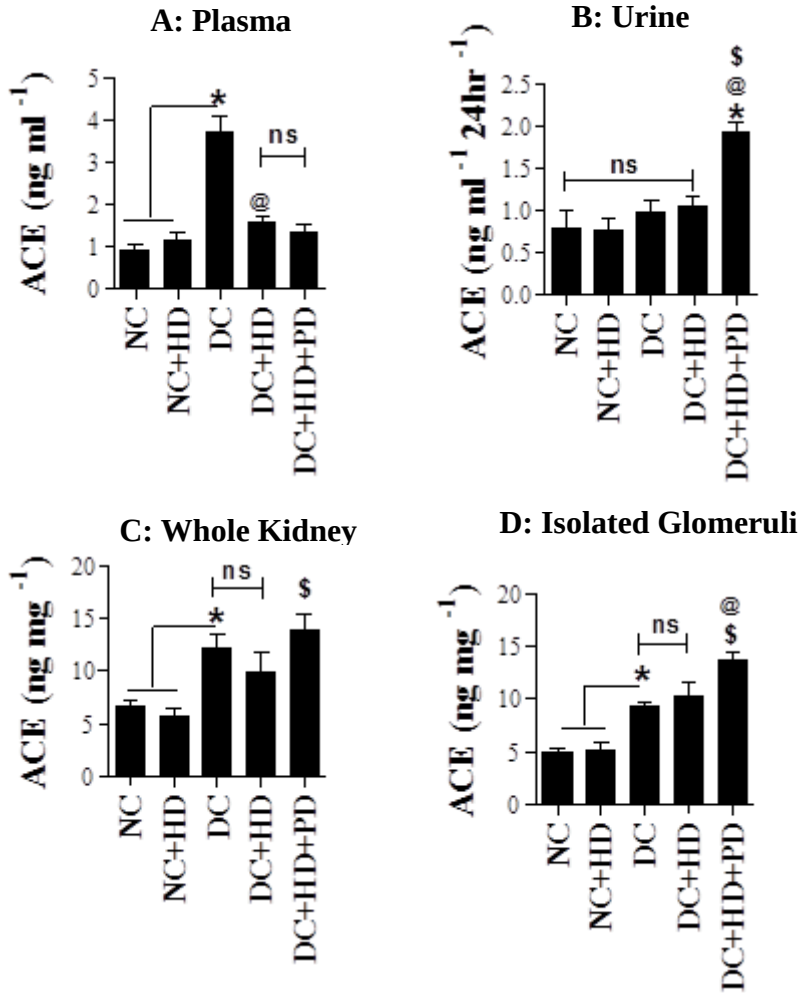


Figure 23: Effect of Diminazene aceturate alone and in combination with AT2 antagonist on systemic and tissue specific protein expression of ACE in diabetic animals. (A-D) protein expression levels of ACE in plasma, urine, whole kidney and glomeruli. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD. ns: not significant; NC (normal control), DC (diabetic control), and HD (high dose of DIZE (15mg kg^{-1}). PD: AT2 blocker PD123319.

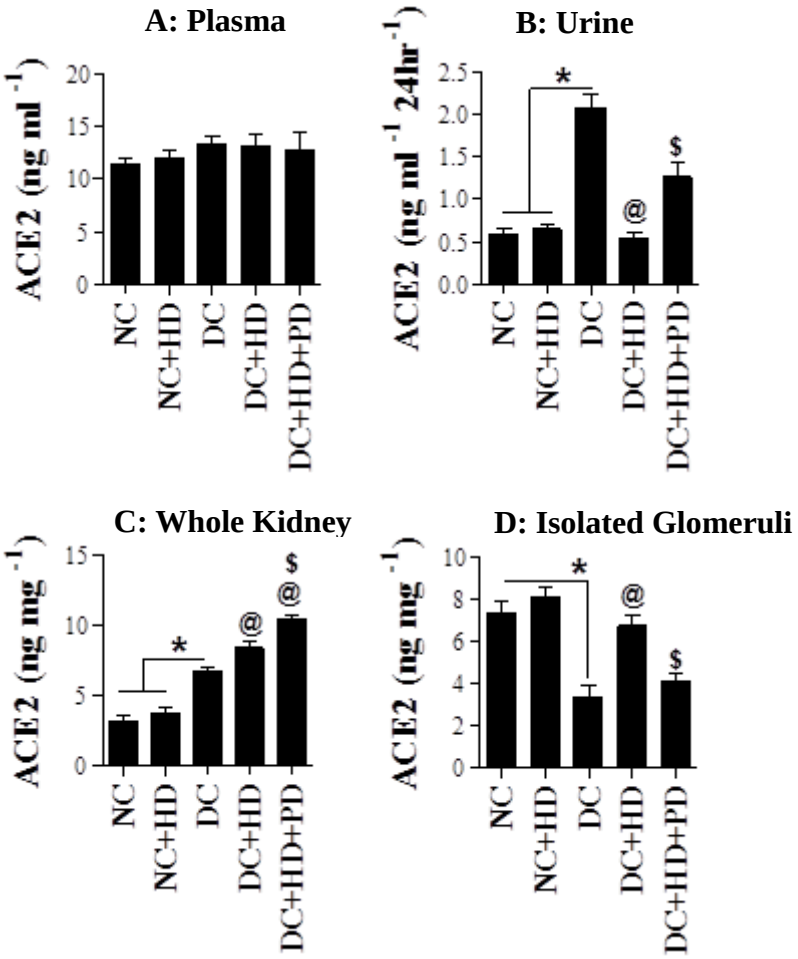


Figure 24: Effect of Diminazene aceturate alone and in combination with AT2 antagonist on systemic and tissue specific protein expression of ACE2 in diabetic animals. (A-D) protein expression levels of ACE2 in plasma, urine, whole kidney and glomeruli. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD; ns: not significant.

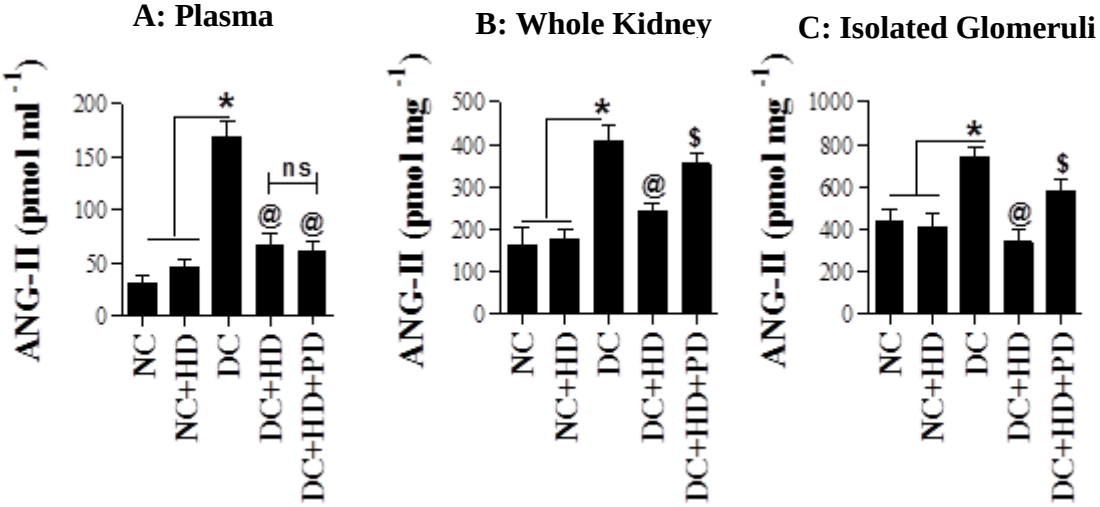


Figure 25: Effect of Diminazene aceturate alone and in combination with AT2 antagonist on systemic and tissue specific protein expression of Ang II in diabetic animals. (A-C) protein expression levels of Ang II in plasma, whole kidney and glomeruli. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD.

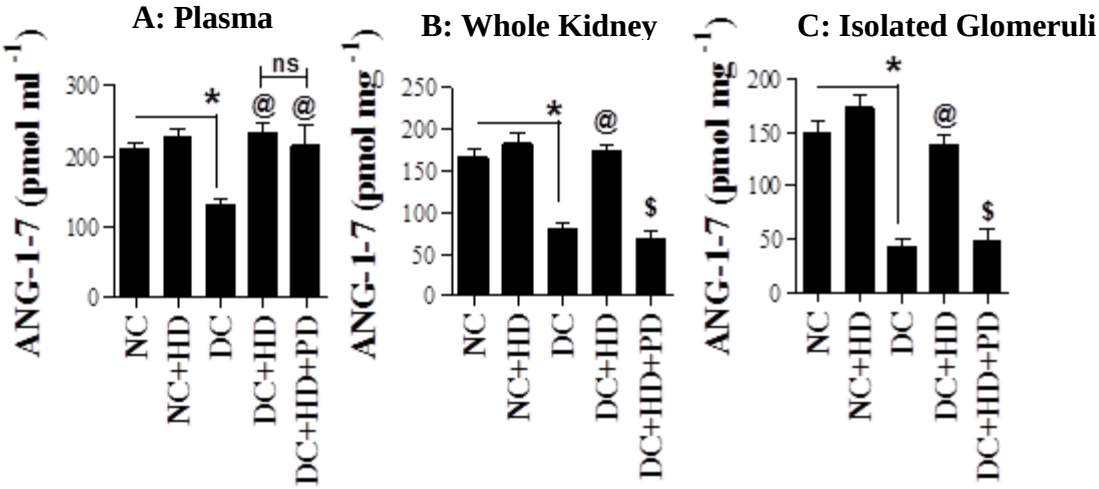


Figure 26: Effect of Diminazene aceturate alone and in combination with AT2 antagonist on systemic and tissue specific protein expression of Ang 1-7 in diabetic animals. (A-C) protein expression levels of Ang 1-7 in plasma, whole kidney and glomeruli. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD.

5.3.6. Changes in expression of mRNA for ACE, ACE2, AT1, AT2 and MAS1 receptors in whole kidney and glomeruli after treatment with DIZE alone and with AT2 blocker

To determine the effect of treatment with DIZE alone and in presence of AT2 blocker on ACE, ACE2, AT1, AT2 and MAS1 receptor genes, we have quantified their mRNA expression in whole kidney and isolated glomeruli. In whole kidney, increased mRNA expression of ACE was observed in diabetic animals and DIZE treatment normalized this increase, AT2 blocker reversed the effect of DIZE and increased these levels (Figure 27 A). However, we found the reduced gene expression levels of ACE2 in whole kidney of diabetic group and after DIZE treatment, these levels were increased significantly and even, AT2 blockade did not alter these increased levels of ACE2 (Figure 27 B). In whole kidney, we also observed the significant rise in AT1 gene expression in diabetic animals, DIZE had no effect on this rise of AT1 gene expression levels whereas, AT2 blockade, further elevated these levels (Figure 27 C).

Further, in whole kidney, we found increased mRNA expression of AT2, DIZE alone treatment and in presence of AT2 blockade further increased these levels (Figure 27 D). Surprisingly, we found significant reduction in mRNA expression of MAS1 receptors in whole kidney of diabetic group and treatment with DIZE alone or in presence of AT2 blocker, the mRNA expression remained same, as that of the diabetic group (Figure 27 E). In addition, mRNA expression of ACE, ACE2, AT1, AT2 and MAS1 showed similar pattern as that of whole kidney (Figure 28 A-E) and DIZE did not show any effect on gene expression, when administered in normal rats (Figure 27, 28).

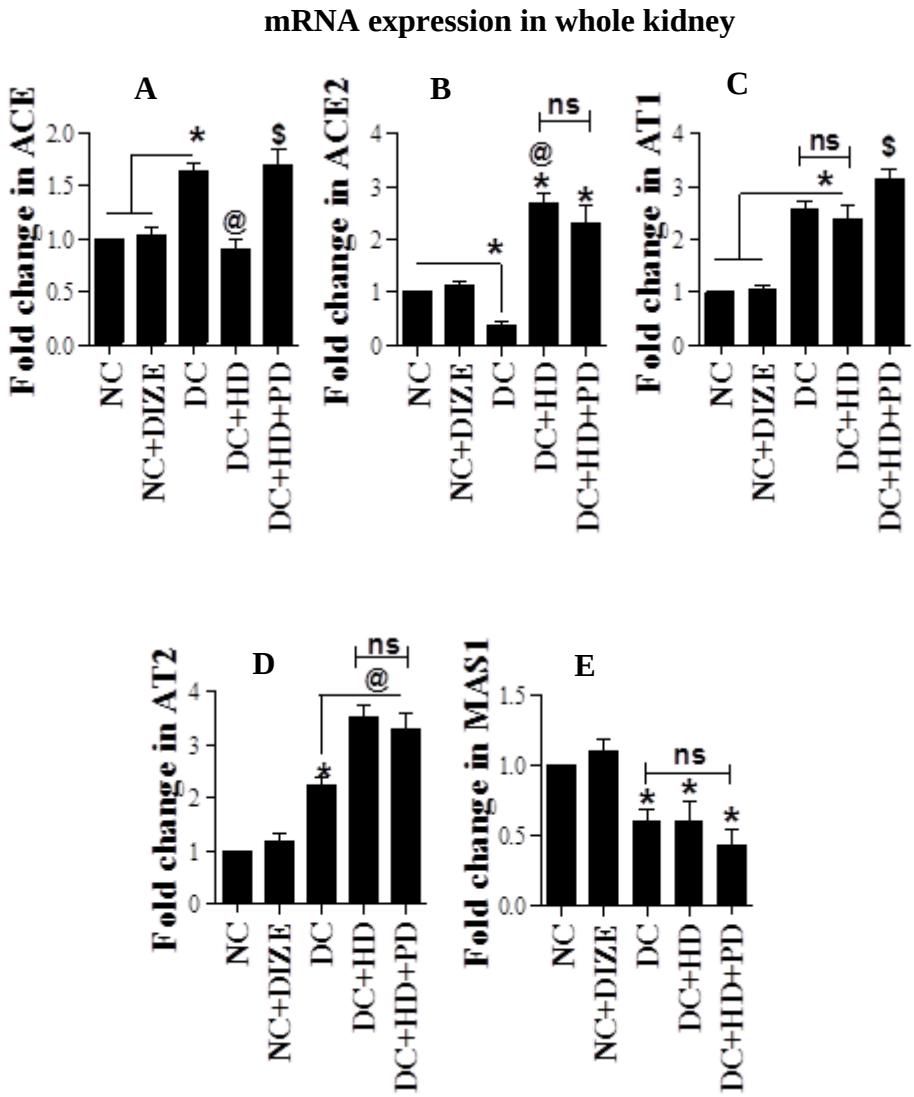


Figure 27: mRNA expression of ACE, ACE2, AT1, AT2 and MAS1 in whole kidney. (A) mRNA expression of ACE, (B) mRNA expression of ACE2, (C) mRNA expression of AT1, (D) mRNA expression of AT2 and (E) mRNA expression of MAS1. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD. ns: not significant. NC (normal control), DC (diabetic control), and HD (high dose of DIZE (15mg kg^{-1}). PD: AT2 blocker PD123319.

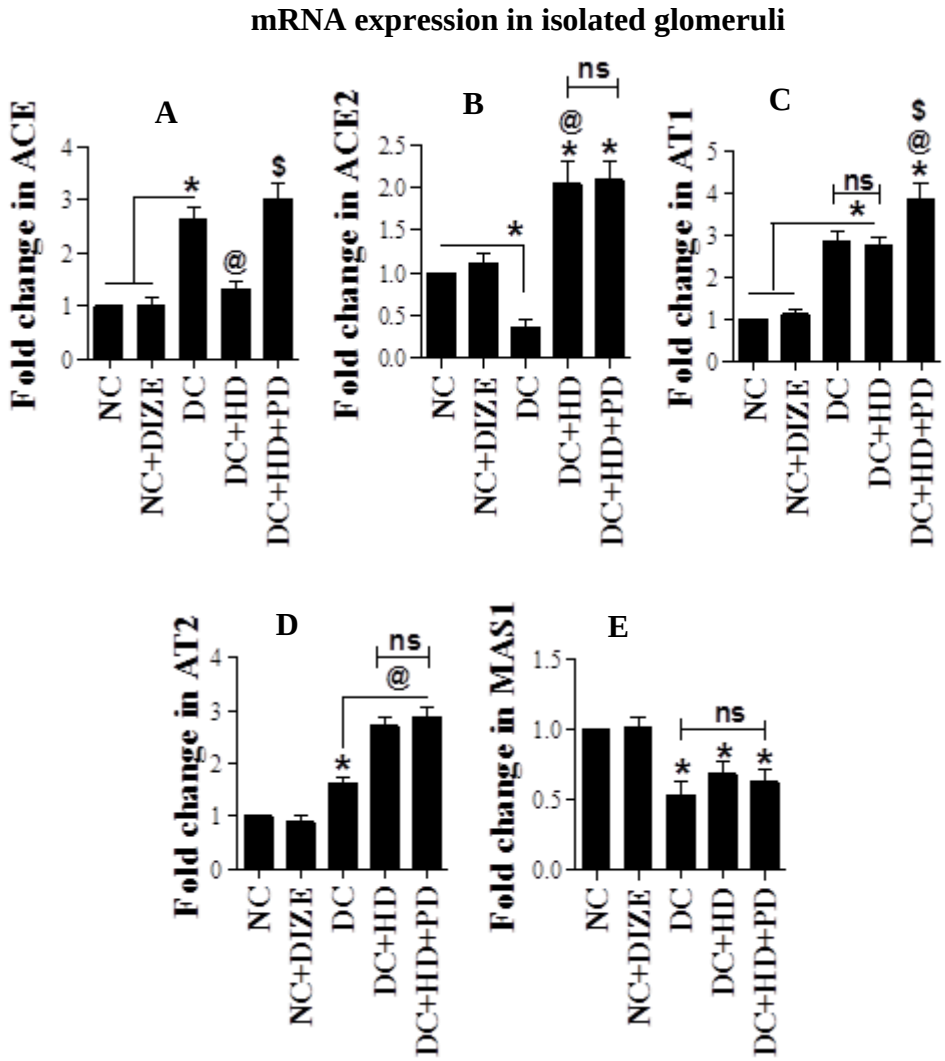


Figure 28: mRNA expression of ACE, ACE2, AT1, AT2 and MAS1 in isolated glomeruli. (A) mRNA expression of ACE, (B) mRNA expression of ACE2, (C) mRNA expression of AT1, (D) mRNA expression of AT2 and (E) mRNA expression of MAS1. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD. ns: not significant. NC (normal control), DC (diabetic control), and HD (high dose of DIZE (15mg kg^{-1}). PD: AT2 blocker PD123319.

5.3.7. Alterations in protein expression of AT1, AT2 and MAS1 receptors in renal tissue after treatment with DIZE alone and combined with AT2 blocker

Next, we used immunohistochemistry to monitor the expression of AT1, AT2 and MAS1 receptor protein, in our experimental groups. Expression of AT1 receptor protein was increased in diabetic kidney, DIZE alone significantly normalized this change and combining DIZE and PD123319 reversed the effects of DIZE (Figure 29 A, D). For AT2 receptors, expression in diabetic kidney was up-regulated and DIZE treatment further increased this expression. This effect of DIZE was blocked by adding PD123319, returning expression of AT2 receptor protein to the same level as that of diabetic control rats (Figure 29 B, E). Interestingly, we found down-regulation of MAS1 receptors in diabetic kidney. However, administration of DIZE alone or in presence of PD123319 had no effect on MAS1 receptor expression in diabetic kidneys (Figure 29 C, F).

5.3.8. AT2 receptor blockade re-induced renal fibrosis and apoptosis and prevented the protective actions of DIZE

Further, to assess the effects of AT2 receptor blockade with PD123319 on reno-protection mediated by DIZE, we quantified expression of three markers of fibrosis - TGF-β, fibronectin and collagen IV- along with two markers of apoptosis - cleaved PARP and cleaved caspase-3 - in our experimental groups. We observed increased expression of TGF β, fibronectin and collagen IV in diabetic kidneys, compared with kidneys from normal rats. Treatment of diabetic rats with DIZE significantly attenuated this increased expression of fibrotic markers and adding PD123319 to DIZE, reversed the DIZE-mediated attenuation (Figure 30 A–F). We measured the markers of apoptosis in isolated glomeruli and found cleaved PARP and cleaved caspase-3 were increased by diabetes and this increase was normalized by administering DIZE alone. However, DIZE combined with PD123319 was no longer able to reduce these apoptotic markers, and expression of cleaved PARP and cleaved caspase-3 was again markedly increased (Figure 31 A–C).

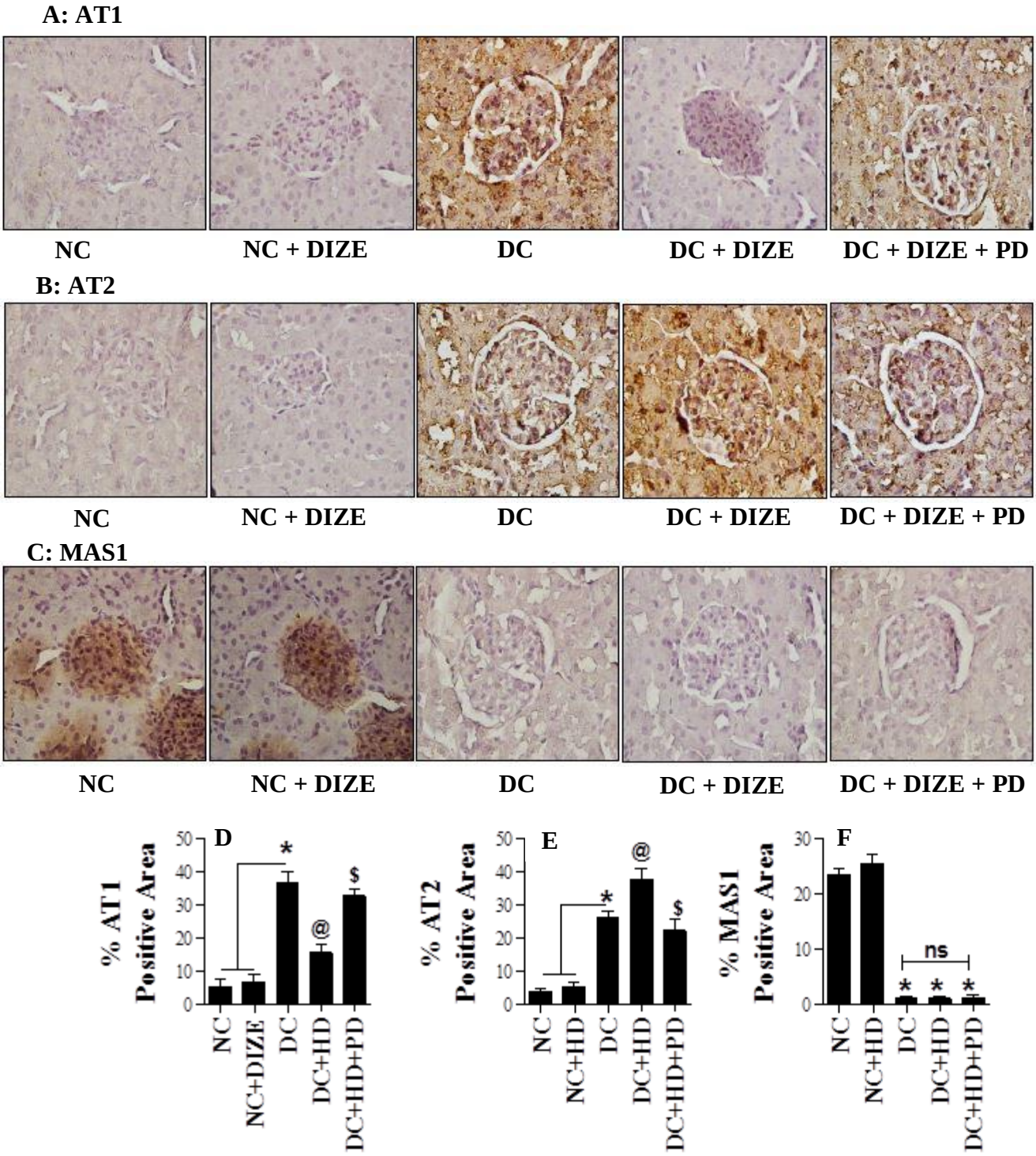


Figure 29: Renal protein expression of AT1, AT2 and MAS1. (A-C) Immunohistochemical staining of AT1, AT2 and MAS1 in kidney sections and (D-F) their respective semi-quantitative analysis using ImageJ software. All the values represented as mean ± S.E.M. n = 25. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD.

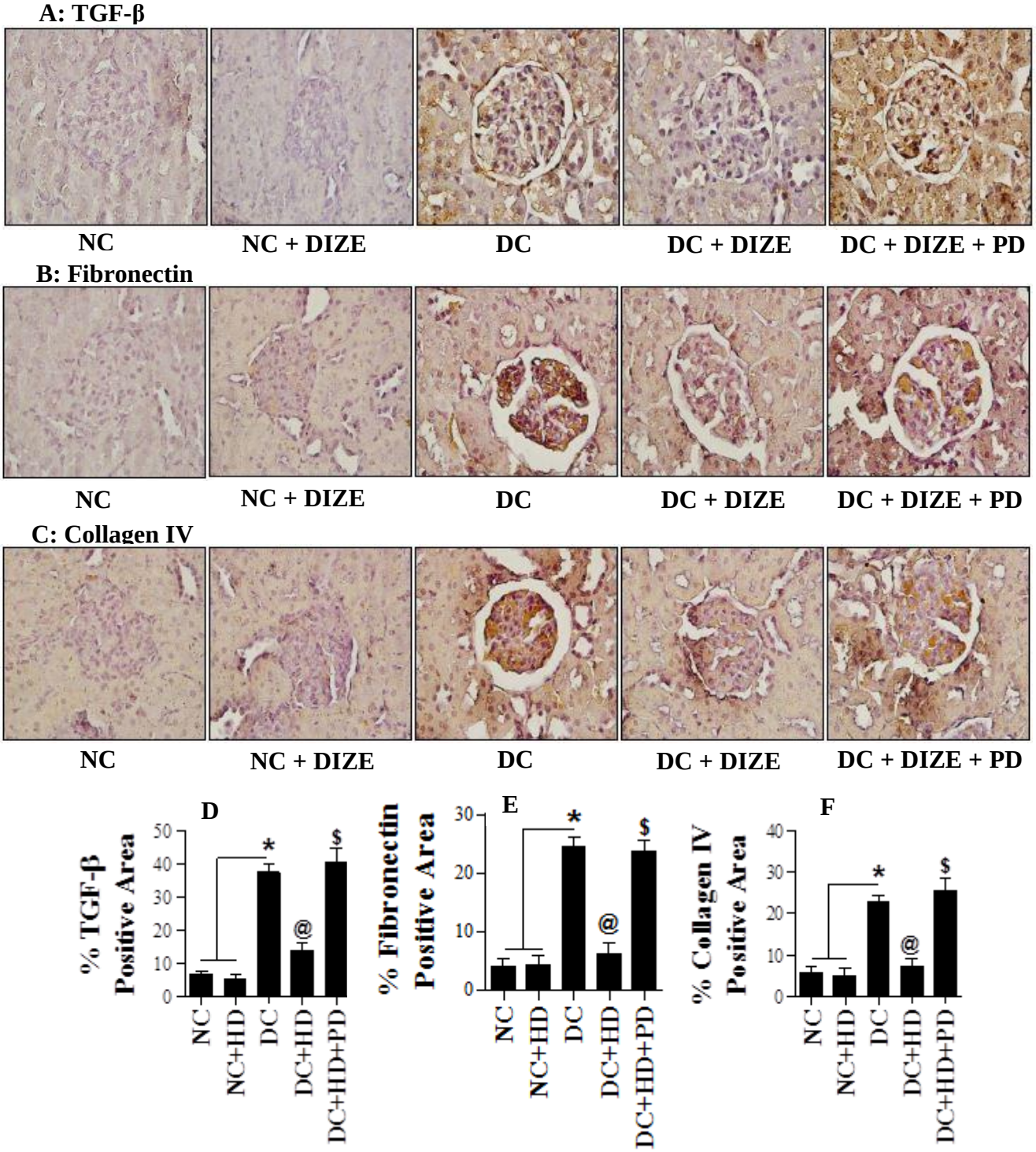


Figure 30: Blockade of protective actions of ACE2 activator in presence of PD123319 and re-expression of fibrotic markers. (A-C) Immunohistochemical staining of TGF-β, Fibronectin and Collagen IV in kidney sections and (D-F) their respective semi-quantitative analysis using ImageJ software. All the values represented as mean ± S.E.M. n = 25; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD.

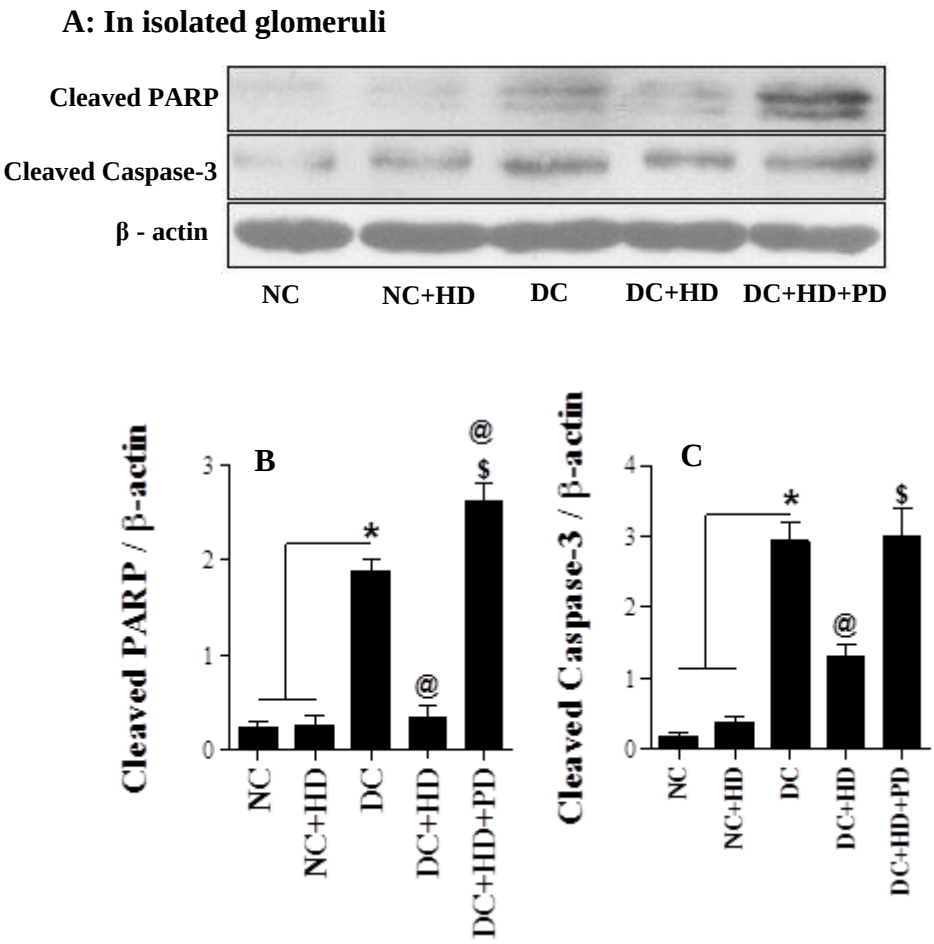


Figure 31: Prevention of Diminazene aceturate mediated protection and increased apoptosis in isolated glomeruli by AT2 blocker. (A) Western blot analysis of cleaved PARP, cleaved Caspase-3 in isolated glomeruli and (B, C) their respective quantitative analysis using ImageJ software. All the values represented as mean ± S.E.M. n = 6; *P < 0.05 vs NC, NC+HD $^{@}$P<0.05 vs DC and $^{\$}$P<0.05 vs DC+HD.

Chapter 6
Discussion

6. Discussion

Diabetes mellitus is the major cause for the development of DN, the major cause of ESRD. Even after the success of the current therapy to delay the progression of DN, the occurrence of it still continues to rise worldwide. Therefore, there is an urgent need to identify novel molecular mechanisms and novel therapeutic strategies to understand and prevent the progression of DN completely. Hence, we focused on novel epigenetic pathway histone ubiquitination in the progression of DN and on novel therapeutic strategies like ACE2 activation to prevent the progression of DN. Our results clearly demonstrating the role of histone H2AK119 and H2BK120 monoubiquitination in the development of renal fibrosis in type 1 diabetic rats. Further, ACE2 activator, DIZE was found to be protective in preventing DN through inhibiting renal fibrosis and apoptosis.

6.1. Histone H2AK119 and H2BK120 monoubiquitination modulate SET7/9 and SUV39H1 in type 1 diabetes induced renal fibrosis

Even though several molecular and epigenetic mechanisms involved in the progression of diabetic renal fibrosis are well explored, the current therapeutics is limited to prevent diabetic nephropathy. Hence, there is a need to focus on novel mechanisms that are involved in the development of diabetic renal fibrosis. Therefore, we focused on histone H2A and H2B ubiquitination, UPS components that are involved in histone H2A, H2B ubiquitination and histone H3 methylation. We found increased collagen deposition and increased mRNA expression of *Col1a1* in the diabetic kidney (Figure 6), which confirmed the development of renal fibrosis. In our study, we also observed the increased mRNA expression of histone H2A specific E3 ligase, *Rnf2* (Figure 12 A). *Rnf2* belong to the polycomb group protein (PcG) family and is involved in the transcriptional repression through histone H2A ubiquitination [283]. Recently, deletion of *Bmi1*, another member of PcG family, was found to improve insulin sensitivity and type 2 diabetes [284]. Here, in this study we can correlate that the increased *Rnf2* may be involved in the diabetic kidney damage by increasing repressive chromatin mark ubiquitinated histone H2A. We also examined the expression of DUBs, *Usp7*, *Usp16*, *Usp21* and *Usp22* in the diabetic kidney. All these USPs are capable to deubiquitinate histone H2A and H2B [285]. Other than histones, these USPs also deubiquitinate several ubiquitinated protein substrates and participates in different biological

functions. For example, upregulation of USP7 attenuates hepatic gluconeogenesis by deubiquitinating FoxO1 [286] and USP21 plays an important role in the down-regulation of TNF-α induced NF-κB activation through deubiquitinating receptor interacting protein1 [287]. Further, increased expression of USP22 reduced the Sirt1 ubiquitination and degradation, decreased fibronectin and TGF-β1 expression in glomerular mesangial cells under both basal and advanced glycation end products-treated conditions [11]. We observed the decreased mRNA expression of all these DUBs in diabetic kidney (Figure 12 B-E). Taken together, these results highlight the role of these UPS components (E3 ligases and DUBs) in the development of renal fibrosis.

Further, to understand the effect of changes in histone H2A and H2B specific UPS components, we examined the expression of H2AK119Ub and H2BK120Ub in diabetic kidney. Accumulating evidence shows that gene repression or activation by histone H2AK119Ub and H2BK119Ub, mainly due to their cross talk with histone H3 methylation. H2A ubiquitination represses transcription by promoting H3K27 di and tri-methylation [288]. Recently, it has been shown that H2A ubiquitination inhibits the methyl transferases ASH1L, HYPB, NSD1, and NSD2 specifically involved in H3K36 di and tri-methylation [280]. De-ubiquitination of histone H2A by USP21 is essential for transcriptional activation by promoting H3K4 di and tri-methylation [279]. Histone H2B shows its transcriptional activity mainly by increasing the levels of H3K4 and H3K79 di and tri-methylation through increasing their respective methyl transferase activity of SET1 and DOT1 [289,290]. Recently, histone H2A and H2B ubiquitination has been found to be involved in the increased expression of fibrotic genes in rat glomerular mesangial cells under hyperglycaemic condition [21]. Our results showed the increased expression of histone H2A and H2B ubiquitination in whole kidney of diabetic animals (Figure 7) and their expression is decreased in glomeruli isolated from diabetic kidney (Figure 10). These results clearly indicating the altered epigenetic mechanisms at glomeruli level in diabetic animals.

Further, to check the histone H2A/H2B ubiquitination and histone H3 methylation crosstalk, we examined H3K4Me2, H3K9Me2, and H3K79Me2 in diabetic kidney. Increasing evidence shows that histone H3 methylation involves in the development of renal fibrosis. H3K4Me2 and H3K79Me2 associated with gene activation, whereas H3K9Me2 can

correlate with gene silencing and transcriptional repression [13]. High glucose (HG) treatment caused the dynamic changes in H3K4Me2 and H3K9Me2 in human monocytes [291] and H3K4Me1 in endothelial cells [292], whereas H3K9Me3 levels were decreased in vascular smooth muscle cells (VSMCs) from diabetic *db/db* mice relative to control *db*/+ and in HG-treated VSMCs [293] and endothelial cells [294]. In VSMCs derived from diabetic *db/db* mice, H3K9Me3 levels were decreased at key inflammatory genes promoters and inversely correlated with the increased expression of these genes under basal and TNF-α treated conditions [293].Furthermore, human VSMCs and endothelial cells cultured under HG conditions also exhibited decreased levels of H3K9Me3 [293,294]. In addition to this, HG increased H3K4Me and decreased H3K9Me on the promoters of fibrotic genes like *Col1a11, Ctgf, Pai1* in rat mesangial cells [281]. These facts suggest that the increase in H3K4Me and loss of repressive H3K9Me mark can increase the expression of pathologic genes under diabetic condition. In addition to H3K4Me, H3K79Me is also an active chromatin mark. H3K79 hypermethylation was observed at the active rather than at the inactive loci in mammalian cells, and has been considered to be a conserved hallmark of active chromatin regions [295]. This is in line with our results showing global increase in H3K4Me2, H3K79Me2 and decrease in H3K9Me2 (Figure 7, 8, 10, 11) in diabetic animals. Further, in diabetic kidney H3K4Me2 expression was also found to be increased in isolated glomeruli. Whereas, H3K9Me2 expression was not altered in glomeruli of diabetic animals (Figure 10). In addition to these global changes, we also observed the increased occupancies of active chromatin mark H3K4Me2 and decreased occupancies of repressive chromatin mark H3K9Me2 on the promoter of *Col1a1* gene (Figure 17 A, B) and these changes may be the reason for the increased ECM deposition, increased *Col1a1* gene expression and renal fibrosis.

Next, we checked the protein expression of HMTs SET7/9 and SUV39H1 in diabetic kidney. SET7/9 is mainly involved in H3K4Me [296], whereas SUV39H1 is involved in H3K9Me3 [297]. In an *in-vitro* study, blocking SET7/9 markedly reduced the expression of ECM genes like Col1a1, CTGF and PAI-1 in cultured rat mesangial cells [281]. Lin SH et al., showed that reduced expression of SUV39H1 and increased the expression of fibrotic genes in response to high glucose in mice mesangial cells. Further, inhibiting SUV39H1, increased the expression of fibrotic genes under this condition. In addition, over expression of

SUV39H1, reversed high glucose induced expression of fibrotic genes in mice mesangial cells [298]. These studies highlighted the HMTs SET7/9 and SUV39H1 as potential targets in preventing DN. Enrichment of SET7/9 on fibrotic genes was reported in rat mesangial cells, when stimulated with TGF-β under hyperglycaemic condition [281]. Gene silencing of *SET7/9* with small interfering RNAs in monocytes significantly inhibited the TNF-α induced inflammatory genes and histone H3K4Me on these promoters [189]. On the other hand, SUV39H1 was found to be decreased in VSMCs of diabetic *db/db* mice, which leads to the enhancement of inflammatory genes like *Mcp1* and *Il6* by decreasing the H3K9Me on their promoters. The decreased expression of SUV39H1 in VSMCs of *db/db* mice was an epigenetic inhibition through micro RNA-125b [282]. This epigenetic inhibition created an interest in us to check the effect of histone ubiquitination on the regulation of HMTs SET7/9 and SUV39H1. Surprisingly, we observed a decreased occupancy of repressive chromatin mark H2AK119Ub on *SET7/9* promoter (Figure 13 A) and also reduced occupancy of active chromatin mark H2BK120Ub on the promoter of *SUV39H1* (Figure 14 A) in diabetic kidney. Not only H2AK119Ub but also the methylation marks that are regulated by it (H2AK119Ub promotes H3K27Me and inhibits H3K36Me) [280,288], were found to be altered over *SET7/9* promoter (Figure 13 A-C). Similarly, H2BK120Ub regulated methylation marks (H2BK120Ub promotes H3K4Me and H3K79Me) [289,290], were also decreased over the promoter of *SUV39H1* (Figure 14 A-C) with respect to H2BK120Ub. The methylation patterns (decreased H3K27Me2 and increased H3K36Me2 occupancies) on the *SET7/9* promoter supports the H2AK119Ub occupancy pattern on it (Figure 13 A-C). However, these patterns were not followed on the promoter of *SUV39H1* (Figure 15 A-C). The H2BK120Ub regulated methylation patterns (decreased H3K4Me2 and H3K79Me2 occupancies) were followed only on *SUV39H1* promoter (Figure 14 A-C), but not on the promoter of *SET7/9* (Figure 16 A-C). In support to this, the protein expression of SET7/9 was increased and SUV39H1 was decreased in diabetic kidney (Figure 9). These results suggesting that H2AK119Ub mainly involves with SET7/9, whereas H2BK120Ub with SUV39H1 regulation. Increased protein expression of SET7/9 and decreased expression of SUV39H1 HMTs has been reported in diabetic condition [282,299]. These results provided us lucid evidence that histone ubiquitination and methylation are involved in the development of diabetic renal disease. In this study, the increased expression of SET7/9 and

decreased expression of SUV39H1 could be correlated with the increased occupancies of H3K4Me2 and decreased occupancies of H3K9Me2 over *Col1a1* gene promoter (Figure 16 A,B) increased *Col1a1* gene expression and renal fibrosis in diabetic kidney .

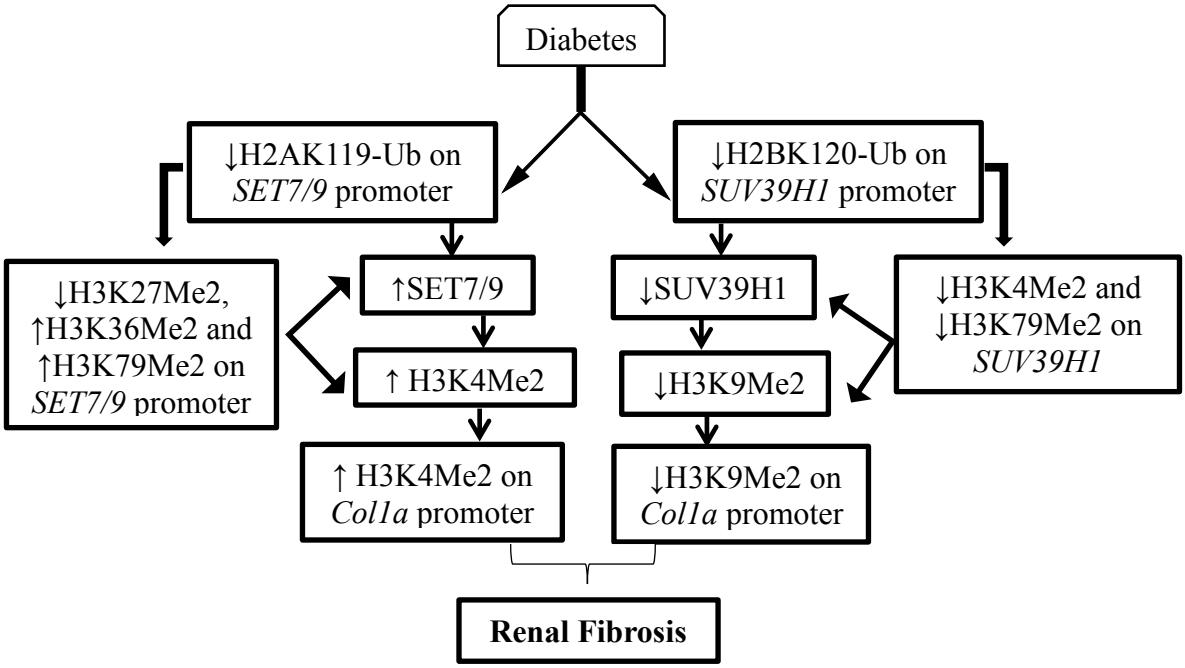


Figure 32: Epigenetic regulation of SET7/9 and SUV39H1 in the development of diabetic renal fibrosis. We propose that histone H2AK119 and H2BK120 ubiquitination regulates histone H3 methylation in the progression of diabetic renal fibrosis. In diabetic kidney decreased occupancies of repressive mark H2AK119Ub leads to decreased occupancies of H3K27Me2 and increased occupancies of H3K36Me2 methylation marks on the promoter of *SET7/9*, increased the protein expression of SET7/9. In addition, decreased occupancies of H2BK120Ub and its regulated methylation marks (H3K4Me2, H3K79Me2) on the promoter of *SUV39H1*, decreased the protein expression of SUV39H1. As a result, increased SET7/9, increased the global levels and promoter occupancies of H3K4Me2 on the promoter of *Col1a1*, whereas the decreased SUV39H1 also decreased the global levels and occupancies of H3K9Me2 on the promoter of *Col1a1* gene. These changes in methylation marks increased the *Col1a1* gene expression, ECM deposition and renal fibrosis in diabetic kidney. We suggest that epigenetic regulation of SET7/9 and SUV39H1 is a novel mechanism in the development of diabetic renal fibrosis.

6.2. Reno-protective mechanism of Aspirin involves H2AK119 monoubiquitination and SET7 in preventing type 1 diabetic nephropathy

Even though, several therapeutic approaches including renin angiotensin system (RAS) inhibitors, anti-inflammatory approach and reactive oxygen species (ROS) inhibitors have come up to curb the progression of DN, but not provided a complete cure. The number of people with DN still continues to increase globally, there is an urgent need to find novel targets and therapeutic strategies to prevent it completely. Recent studies highlighting the role of UPS in the development of DN and UPS inhibitor MG132 was proved to be protective in DN [12,21,159,224,300]. Previously, we have reported that reduced protein expression of histone H2AK119-Ub in glomeruli and reduced occupancies of H2AK119-Ub on SET7 gene results in decreased protein expression of SET7 in diabetic animals, which is the novel mechanism involves in the progression of DN [253]. Here, for the first time, we have demonstrated the protective actions of Aspirin in preventing renal fibrosis in type 1 DN rats involves histone H2AK119-Ub and SET7.

Hyperglycemia induced pathways like ROS, RAS and inflammation plays a major role in the development of DN [24,301,302]. Accumulating evidence also shows that the drugs which reverse hyperglycemia directly can be able to prevent the progression of DN [24,303]. Moreover, Aspirin was also found to reverse hyperglycemia and hyperglycemia induced changes in DN [24]. In line to these reports, in this study, Aspirin at a higher dose was found to reduce the glucose levels to an extent in diabetic animals and also improved the body weight. Morphologically, increased kidney weight and increased kidney weight/bodyweight ratio are the key markers for the development of diabetic nephropathy [24]. In addition, Aspirin administration significantly normalized the kidney weight and kidney weight/body weight ratio in diabetic animals at higher dose. Further, we also checked the kidney functional parameters like BUN, creatinine and albumin levels in plasma. Increased levels of plasma BUN, creatinine and reduced levels of plasma albumin indicates renal dysfunction in diabetes and all these changes were reversed significantly by treatment with higher dose of Aspirin. These results, indicating the protective actions of Aspirin in preventing DN.

Further, to check the protective actions of Aspirin at molecular level, we checked the expression of Mysm1, a H2A specific DUB and one of the component of UPS. Existing

reports also indicating a critical role of DUBs in the development of DN [300]. Recently, ubiquitin carboxy-terminal hydrolase 1 (UCH-L1), belongs to UCH class of DUBs was found to be increased in kidneys of DN patients as well as in the HG induced podocytes [236]. UCH-L1 overexpression in podocytes altered their structure and integrity through increasing the expression of snail, decreasing the expression of nephrin, synaptopodin and CD2AP [236]. Moreover, ubiquitin specific protease 22 (USP22), a member of the USP DUB family, found to be downregulated, when glomerular mesangial cells (GMCs) treated with AGEs and increased the expression of ECM proteins like fibronectin and TGF-β. Further, all the changes induced by AGEs treatment were reversed by over expressing USP22 in GMCs [11]. Previously, we also reported the alterations of histone H2A/H2B specific DUBs in diabetic kidney [253]. These reports, clearly indicating the role of DUBs in the development of DN. In addition, recent studies indicating that Mysm1 (H2A specific deubiquitinase) is the most susceptible gene and have a role in the development of diabetes [304]. Interestingly, in our study, we observed the increased protein expression of Mysm1 in glomeruli isolated from diabetic animals and Aspirin administration completely reversed this change (Figure 18). This indicating the protective role of Aspirin is through reducing the expression of Mysm1.

Accumulating evidence implicates the epigenetic mechanisms in the development of DN [29,253,305,306]. Recently, Chenlin Gao et al., reported the role of histone H2AK119-Ub in the expression of fibrotic genes like fibronectin and *Tgfb1* in rat glomerular mesangial cells under hyperglycaemic condition [21]. Histone H2AK119-Ub is a repressive chromatin mark and also involves in transcriptional regulation [17]. Previously, we also showed the reduced expression of H2AK119-Ub in glomeruli isolated from diabetic animals [253]. In addition, we also demonstrated the reduced methylation marks regulated by H2AK119-Ub (H3K27Me2, H3K36Me2) on *SET7* gene promoter, which resulted in increased protein expression of SET7 in diabetic kidney [253]. In supporting to our previous results, in this study, we also observed reduced expression of histone H2AK119-Ub in glomeruli isolated from diabetic animals and aspirin treatment significantly restored these levels.

Further, to check the effect of histone H2AK119-Ub on SET7, we checked the protein expression of SET7 in isolated glomeruli. SET7, is an epigenetic enzyme mainly involves

in lysine 4 methylation of histone H3 (H3K4Me). H3K4Me is an active chromatin mark, which mainly, involves in active gene transcription [299]. In diabetic nephropathy, increased protein expression of SET7 was reported [299]. In addition to this, high glucose increased SET7 and H3K4Me occupancies on the promoters of fibrotic genes like *Col1a1, Ctgf, Pai1* in rat mesangial cells and increased their expression [281]. Further, blocking SET7 markedly reduced the expression of these fibrotic genes in cultured rat mesangial cells under high glucose condition [281]. Previously, we also found the increased protein expression of SET7 in diabetic kidney resulted in the increased H3K4Me over *Col1a1* gene and increased collagen deposition [253]. These reports, clearly indicating the role of SET7 in the development of renal fibrosis in diabetic animals. Inline to these reports, we also observed the increased protein expression of SET7 in glomeruli isolated from diabetic animals and aspirin treatment, normalized this change significantly at higher dose. In addition, we also observed the increased ECM, collagen deposition in diabetic kidney and these changes were significantly reversed by Aspirin at higher dose (Figure 19, 20). Hence, the possible mechanism of aspirin involves Mysm1, H2AK119Ub and SET7 in preventing type 1 DN (Figure 33).

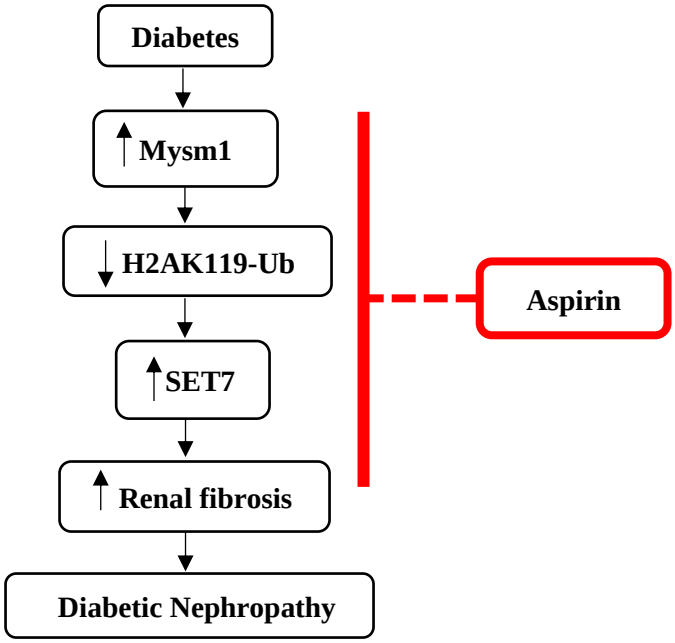


Figure 33: Reno-protective actions of Aspirin involves Mysm1, H2AK119-Ub and SET7: In diabetic kidney, increased expression of Mysm1, SET7 and reduced expression of H2AK119-Ub was observed and all these changes were reversed by aspirin administration. This is the novel mechanism of aspirin, in preventing renal fibrosis in diabetic animals.

6.3. Diminazene aceturate (DIZE) prevents renal fibrosis and apoptosis through activating ACE2/Ang 1-7/AT2 mediated pathway

To study the effect of ACE2 activation on the progression of DN, we have used a well-known off target ACE2 activator, DIZE. In our study, DIZE treatment significantly improved the renal morphometric parameters like kidney weight and kidney weight/body weight ratio at higher doses and it did not show any effect on reduced body weights in diabetic animals (Table 9). DIZE treatment also normalized the plasma biochemical parameters like blood urea nitrogen, plasma creatinine and plasma albumin levels in diabetic animals (Table 10). Further, at molecular level, DIZE treatment significantly reduced the expression of fibrotic markers like TGF-β in whole kidney and Smurf2 in isolated glomeruli at higher dose (15mg $kg^{-1}day^{-1}$) (Figure 21, 22). Similarly, DIZE administration also prevented diabetes induced renal apoptosis through reducing the expression of cleaved PARP in whole kidney, as well as in glomeruli and cleaved caspase3 in whole kidney (Figure 21, 22). Moreover, in isolated glomeruli from diabetic animals, we observed decreased expression of ACE2, increased expression of AT2 receptors and DIZE treatment restored the lost ACE2 levels and further increased the AT2 receptor expression dose dependently (Figure 22). These results led us to a conclusion that reno-protection of DIZE involves increased expression of ACE2 and AT2 receptors.

On the contrary, recent studies shows the controversy over direct ACE2 activation and expression by DIZE. Recently, Philipp K Haber et al., demonstrated that DIZE had no ability to activate ACE2 both in in-vitro and in ex-vivo experiments [265]. Similarly, Gábor Raffai et al., also showed, DIZE lacks ACE2 activation in an ex-vivo experiment using porcine coronary artery rings [266]. However, these studies were designed to study the acute effects of DIZE and failed to address the chronic administration of DIZE in in-vivo models on ACE2 activation. For instance, Yang Zhang et al., reported that chronic treatment with DIZE prevents oxidative stress and endothelial damage in db/db mice by increasing ACE2 activity and Ang 1-7 levels [43]. Moreover, in the recent past, Quaisar Ali et al., demonstrated that chronic AT2 activation leads to the increased ACE2 activity and reduced blood pressure in zucker obese rats. In the same study, they have also showed that AT2 activator significantly increased the ACE2 activity in HK-2 cells in-vitro and this effect was blocked in presence of AT2 blocker [271]. These reports raised the possibility that DIZE-mediated

renoprotection in our study might not be through increased ACE2 expression but through an AT2 receptor-mediated pathway. To test this, we treated diabetic animals with a high dose of DIZE, with and without the AT2 receptor antagonist PD123319.

Further, to check the chronic effect of DIZE on RAS components, we have quantified the tissue specific levels of ACE and ACE2. Although there are several reports on tissue-specific expression of ACE and ACE2 in diabetic kidney, these findings are still a matter of debate. Minghao Ye et al., demonstrated the reduced tubular ACE and increased glomerular ACE in 8 week old female db/db mice. In the same study, they observed the increased levels of ACE2 in renal tubules and decreased glomerular ACE2 in diabetic mice [260]. In another study, same group showed the reduced mRNA levels, protein levels and activity of ACE and increased ACE2 protein levels and activity but not its mRNA in renal tubules of female db/db mice [307]. Similarly, Jan Wysocki et al., also showed reduced mRNA levels, protein levels and activity of ACE and increased ACE2 protein levels and activity but not its mRNA levels in renal tubules of female db/db mice and STZ induced female diabetic mice [308]. In contradiction to this, Chris Tikellis et al., demonstrated the induction of diabetes in male c57bl6 mice led to a reduced cortical expression of ACE2 mRNA, ACE2 protein and this was associated with a significant reduction in cortical levels of Ang 1–7 [309]. In addition, Ju-Young Moon et al., showed the increased mRNA, protein expression of ACE in both tubules and glomeruli of STZ induced male diabetic rats. In addition, ACE2 expression was increased only in tubules and decreased in glomeruli, whereas mRNA expression was remain unaltered in these diabetic rats [273]. Previously, we have also showed the increased expression of ACE2 in kidney sections of type 2 diabetic rats [310]. These reports clearly indicating the site specific expression of ACE, ACE2, and the protein, mRNA expression and their activity are not well correlated in diabetic kidney and even species, strain, age, sex and the extent of glucose levels may also affects the expression of these two enzymes. However, drugs or agents that inhibit ACE activity and increase ACE2 activity or expression proved to be effective in diabetic nephropathy, irrespective of gender [35,311-314].

In our study, we observed the increased protein expression of ACE in plasma, whole kidney and also in isolated glomeruli, DIZE treatment only reduced the plasma levels of ACE but not in the whole kidney or glomeruli whereas, further increase was observed in both whole kidney and glomeruli when, DIZE administered in presence of AT2 blocker (Figure 23 A,

C, D). The urinary ACE levels were increased only in DIZE treated AT2 blocker group and remained unaltered in other groups (Figure 23 B). Further, we also observed the increased protein expression of ACE2 in whole kidney, this expression was increased in DIZE alone and DIZE in presence of AT2 blocker groups (Figure 24 C). On the other hand, ACE2 protein expression in isolated glomeruli was significantly reduced, DIZE treatment restored these levels and in presence of AT2 blocker, DIZE failed to restore these levels (Figure 24 D). However, no change was observed in the plasma levels of ACE2, in any of the groups (Figure 24 A). In addition, we observed increased mRNA expression of ACE and reduced mRNA expression of ACE2 in whole kidney, glomeruli of diabetic animals, DIZE significantly attenuated these changes and AT2 blockade had no effect on these effects of DIZE (Figure 27 A, B and Figure 28 A, B). Recent studies, demonstrated that the increased urinary ACE2 expression and activity in animal models of DN due to increased activity of ADAM17 and inhibition of ADAM17 reduced urinary ACE2 expression and activity [314,315]. In this study, DIZE significantly reduced the urinary ACE2 levels whereas, DIZE in presence of AT2 blocker lost this effect to some extent and resulted in increased urinary ACE2 levels (Figure 24 A). These results, suggesting that protective actions of DIZE were mainly through increasing renal ACE2 levels and the termination of protective actions of DIZE by AT2 blocker may relate to its increased expression of ACE in whole kidney, glomeruli and reduction of ACE2 expression in glomeruli.

Next, we checked the activity of ACE and ACE2 indirectly through quantifying the levels of Ang II and Ang 1-7 in plasma, whole kidney and isolated glomeruli. RAS activation is critical for the development of DN [25]. Increased ANG II, is a major outcome of RAS activation through the actions of ACE [254]. ANG II was found to be upregulated in plasma and renal tissue of DN [316]. On the other hand, Ang 1-7 was found to be downregulated in plasma and renal tissue of diabetic animals [277]. In line to these reports, we observed the increased levels of Ang II and reduced levels of Ang 1-7 in plasma, whole kidney and isolated gromeruli from diabetic animals (Figure 25, 26). This can be attributed to the increased and decreased ACE and ACE2 activity in plasma, whole kidney and isolated gromeruli of diabetic animals. However, high dose of DIZE administration decreased the levels of Ang II and increased Ang 1-7 in plasma, whole kidney and isolated glomeruli and further, AT2 blockade reversed the effect of DIZE in whole kidney and isolated glomeruli,

but not in plasma (Figure 25, 26). These results indicating the increased ACE2 activity in renal tissue after DIZE administration and this activity was lost in presence of AT2 blocker and suggesting the role of AT2 receptors in ACE2 activation.

Existing literature, shows the protective actions of Ang 1-7, which acts through MAS1 receptors and antagonizes the actions of Ang II and AT1 receptors. Recently, Kai Zhang et al., demonstrated that large dose of Ang 1-7 (800 ng/kg min) administered through subcutaneous injection, by an embedded mini-osmotic pump in STZ induced diabetic rats, for four weeks, significantly improved renal function, attenuated glomeruli sclerosis, reduced oxidative stress markers like NOX4, p47phox, decreased the expression of ECM markers like collagen IV, TGF-β1 through increasing the expression of renal MAS1 receptors and all these effects were blocked by MAS1 receptor antagonist (A779) [277]. In another recent study, Yixuan Shi et al., demonstrated the similar effects of Ang 1-7 in DN, when administered at a dose of 500 μg $kg^{-1}day^{-1}$ for six weeks in Akita mice and these effects were blocked by MAS1 receptor antagonist [317]. However, both the studies showed the downregulation of MAS1 receptors in diabetic animals and large amount of Ang 1-7 was administered to restore the MAS1 receptor expression. [277,317]. Additionally, downregulation of Ang 1-7 and MAS1 receptors, were also reported in human diabetic kidney [272]. Moreover, in our study, we also observed the loss of MAS1 receptors in diabetic kidney whereas, DIZE administration did not restore these levels even after increasing the Ang 1-7 levels, at both mRNA and protein levels in diabetic kidney (Figure 27 E, 28 E, 29 C, F). This suggesting that large doses of Ang 1-7 used by Kai Zhang et al., and Yixuan Shi et al., [277,317] in their study, may be required for the transcriptional and translational activation of MAS1 receptors. Hence, in our study, DIZE mediated reno-protective actions in DN are not through Ang 1-7/MAS1 axis. Further, we also observed the increased mRNA and protein levels of AT2 in diabetic kidney, after treatment with DIZE (Figure 27 D, 28 D and Figure 29 B, E). Previously, Sourris KC et al., showed that the protective effect of RAGE blockade in a mouse model of diabetic nephropathy is through increased expression of AT2 [318]. Ang 1-7 also known to show its protective actions through activating AT2 receptors and these actions were blocked in presence of AT2 blocker [319]. Therefore, our results, strongly suggesting that the DIZE mediated reno-protection may be through Ang 1-7/AT2 mediated pathway (Figure 34). Ang 1-7 and AT2, were also

known to regulate and antagonize the actions of AT1 receptors [319-321]. Further, in our study, DIZE administration, significantly decreased the diabetes induced AT1 receptors at protein level, but not in mRNA level and AT2 blockade again increased the AT1 protein and mRNA levels in diabetic kidney (Figure 29 A, D and Figure 27 C, 28 C). Moreover, recently, Pandey et al., demonstrated the chronic AT2 activation prevented caspase 3 mediated apoptosis and renal fibrosis in type 2 DN [29]. Interestingly, supporting to the above results, DIZE mediated reno-protection was lost in presence of AT2 blockade and revoked renal fibrosis through increasing fibrotic markers like TGF-β, Fibronectin and Collagen IV (Figure 30) and increasing glomerular apoptosis through elevating the levels of cleved PARP and cleaved caspase-3 (Figure 31) in glomeruli. In conclusion, the DIZE mediated reno-protection in type 1 diabetic animals was through ACE2/Ang 1-7/AT2 axis (Figure 34) and hence, DIZE administration will be beneficial in human DN as well as in other abnormalities, where ACE2/Ang 1-7/AT2 axis is dysregulated.

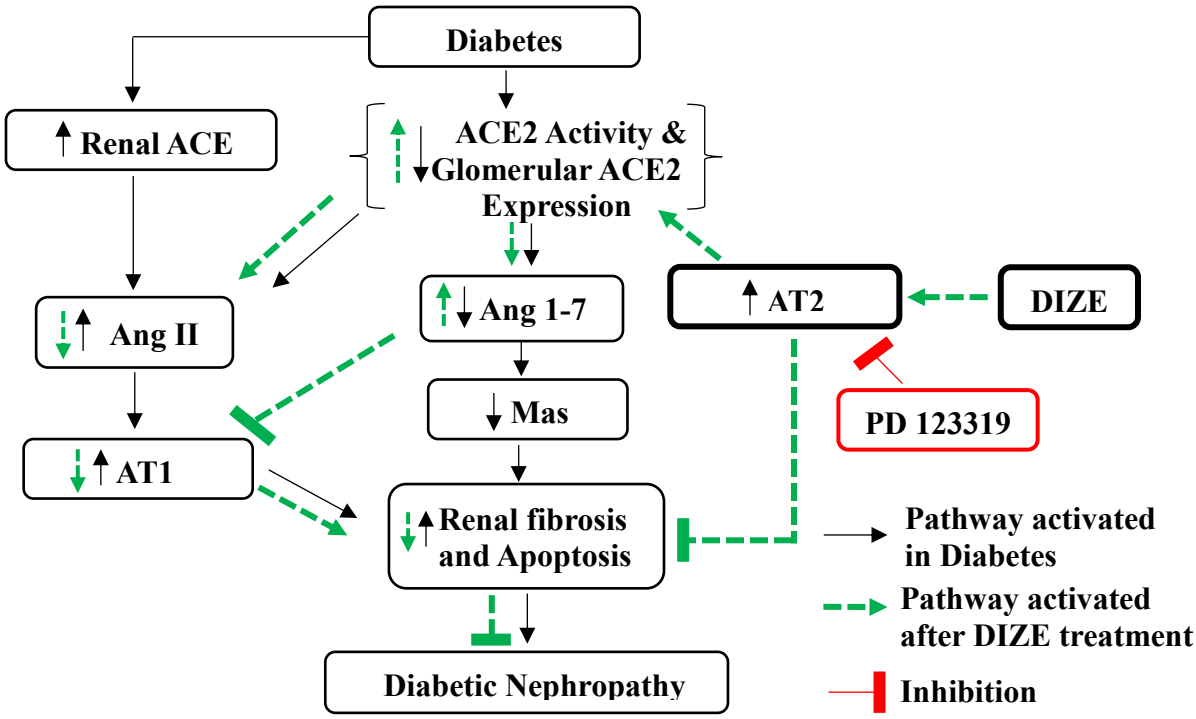


Figure 34: Proposed mechanism of action of DIZE. DIZE prevented diabetes induced renal abnormalities like decreased ACE2 activity, ACE2 expression in glomeruli, Ang 1-7 levels and increased AT1 receptors, fibrotic and apoptotic markers by acting through ACE2/ Ang 1-7/ AT2 axis.

Chapter 7
Conclusions

7. Conclusions:

- Even though there is a long standing relation between histone methylation and ECM gene expression in renal tissue under hyperglycaemic condition, the underlying mechanisms are still not clear. Our results showing the epigenetic regulation of HMTs SET7/9 and SUV39H1 through histone H2AK119Ub and H2BK120Ub. Further, our study leads us to the conclusion that histone H2AK119Ub and H2BK120Ub orchestrate diabetic renal fibrosis by regulating active (H3K4Me2) and repressive (H3K9Me2) chromatin marks through modulating the expression of their respective HMTs, SET7/9 and SUV39H1 (Figure 32). These results suggest that the epigenetic regulation of SET7/9 and SUV39H1 by histone H2AK119Ub and H2BK120Ub, may be a novel mechanism involved in the development of renal fibrosis in diabetes.
- Aspirin treatment significantly inhibited the renal fibrosis in diabetic animals. In our study, we observed increased expression of Mysm1, a histone H2A specific deubiquitinase in glomeruli isolated from diabetic animals. Further, we also observed the decreased expression of H2AK119Ub in glomeruli of diabetic animals. In addition, these changes resulted in the increased expression of HMT Set7 in glomeruli of diabetic animals. Set7, mainly involves in the active gene transcription and also involves in the development of renal fibrosis. Moreover, all these changes were significantly reversed by higher dose of aspirin treatment. In conclusion, these results clearly indicating that, aspirin prevents renal fibrosis in diabetic animals through a novel mechanism, which involves Mysm1, H2AK119Ub and Set7 (Figure 33). However, additional research is required to know the complete molecular and epigenetic mechanisms of aspirin involved in the progression of DN.
- ACE2, the enzyme which degrades pathological peptide, Ang II and genereates protective Ang 1-7 was found to be inactivated and downregulated in diabetic renal diseases. However, several studies indicating the protective role of DIZE, the ACE2 activator in various disease pathologies, the exact mechanism is still remained enigmatic. In our study, DIZE, significantly restored the reduced ACE2 levels in glomeruli isolated from diabetic animals. On the contrary, recent studies questions the mechanism of DIZE on direct activation of ACE2 whereas, they lack the

explanation over ACE2 activation on chronic DIZE administration. In addition, recent studies indicates that AT2 receptors and AT2 receptor activation involves in the activation and expression of ACE2 enzyme. Moreover, in our study, chronic DIZE administration increased AT2 receptor and mRNA expression in diabetic kidney. Further, DIZE treatment decreased the levels of Ang II and increased the levels of Ang 1-7, indicating the increased ACE2 activation in diabetic kidney. Interestingly, we observed the downregulation of Mas1 receptor expression in diabetic kidney and did not restore even after the DIZE treatment. Surprisingly, all the protective actions of DIZE were blocked by the AT2 blocker. Based on these results, we concluded that, DIZE mediates its reno-protective actions through ACE2/Ang 1-7/AT2 axis (Figure 34).

Chapter 8

Future prospective

8. Future prospective:

- Our study was mainly focused on histone ubiquitination and our results clearly indicating the role of histone ubiquitination in the development of renal fibrosis in DN. Aspirin was also found to prevent renal fibrosis in DN through histone H2AK119Ub and Set7 mediated pathway. Hence, development of novel compounds, which target this pathway will be beneficial in preventing diabetic renal fibrosis.
- Moreover, very little is known about E3 ubiquitin ligases and deubiquitinases in the progression of DN. Hence, further research is required to know their role in the progression of DN, to find novel therapeutic targets in future in preventing DN completely.
- Our results showed the dysregulation of ACE2/Ang 1-7/AT2 axis in diabetic kidney. In addition, Diminazene aceturate (DIZE), a wellknown ACE2 activator was found to improve the DN condition, acting through ACE2/Ang 1-7/AT2 axis. However, further toxico-kinetic studies and novel formulations of Diminazene aceturate are required to evaluate its safe usage in humans. Moreover, there are no approved ACE2 activators till date. Hence, development of novel ACE2 activators may be a benefit in future therapeutics to prevent DN.

Chapter 9

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Appendix

List of publications

From thesis

1. **Goru SK**, Kadakol A, Malek V, Pandey A, Sharma N, Gaikwad AB (2017) " Diminazene aceturate prevents type 1 diabetic nephropathy through increasing glomerular ACE2 and AT2 receptor expression". *British Journal of Pharmacology.* 174: 3118-3130 [Impact Factor: 5.491]
2. **Goru SK**, Kadakol A, Gaikwad AB. (2017) "Hidden targets of ubiquitin proteasome system: To prevent diabetic nephropathy". *Pharmacological Research.* 120: 170-179 [Impact Factor: 4.480]
3. **Goru SK**, Kadakol A, Pandey A, Malek V, Sharma N, Gaikwad AB (2016) "Histone H2AK119 and H2BK120 Monoubiquitination Modulate SET7/9 and SUV39H1 in Type 1 Diabetes Induced Renal Fibrosis". *Biochemical Journal.* 473: 3937-3949 [Impact Factor: 3.797]
4. Goru SK, Gaikwad AB. (2017) "Novel reno-protective mechanism of Aspirin involves H2AK119 monoubiquitination and Set7 in preventing type 1 diabetic nephropathy". *Pharmacological Reports* (In Press).

Other Publications

5. Kadakol A, **Goru SK**, Malek V and Gaikwad AB (2017) " Esculetin ameliorates vascular perturbation by intervening in the occupancy of H2BK120Ub at *At1*, *At2*, *Tgfβ1* and *Mcp1* promoter gene in thoracic aorta of IR and T2D rats". *Biomedicine & Pharmacotherapy.* 95:1461–1468. [Impact Factor: 2.759]
6. Kadakol A, Malek V, **Goru SK**, Pandey A, Sharma N and Gaikwad AB (2017) "Esculetin ameliorates insulin resistance and type 2 diabetic nephropathy through reversal of histone H3 acetylation and H2A lysine 119 monoubiquitination". *Journal of Functional Foods.* 35:256–266. [Impact Factor: 3.144]
7. Anuradha Pandey, Priyank Raj, **Goru SK**, Almesh Kadakol, Vajir Malek, Nisha Sharma, Gaikwad AB (2017) "Esculetin ameliorates hepatic fibrosis in high fat diet induced non-alcoholic fatty liver disease by regulation of FoxO1 mediated pathway". *Pharmacological reports.* 69: 666-672. [Impact Factor: 2.587]

8. Pandey A, **Goru SK**, Kadakol A, Malek V, Sharma N, Gaikwad AB (2016) "H2AK119 monoubiquitination regulates angiotensin II receptor mediated macrophage infiltration and renal fibrosis in type 2 diabetic rats". *Biochimie.* 131: 68-76. [Impact Factor: 3.112]
9. **Goru SK**, Pandey A, Gaikwad AB. (2016) E3 ubquitin ligases as novel therapuetic targets for inflammatory diseases. *Pharmacological Research*. 106: 1–9 [Impact Factor: 4.480]
10. Pandey A, **Kumar GS**, Kadakol A, Malek V, Gaikwad AB. (2016) FoxO1 inhibitors: The future medicine for metabolic disorders? *Current Diabetes Reviews.* 12:1-8.
11. Kadakol A, Pandey A, **Goru SK**, Malek V and Gaikwad AB (2015) "Insulin Sensitizing and Cardioprotective Effects of Esculetin and Telmisartan combination by Attenuating Ang II Mediated Vascular Reactivity and Cardiac Fibrosis". *European Journal of Pharmacology.* 765:591-7. [Impact Factor: 2.896]
12. Pandey A, **Goru SK**, Kadakol A, Malek V, and Gaikwad AB (2015) "Differential Regulation of ACE2 and NF-κB by Ang II Receptor Subtypes in Type 2 Diabetic Kidney". *Biochimie.* 118: 71-81. [Impact Factor: 3.112]
13. Kadakol A, Malek V, **Goru SK**, Pandey A, and Gaikwad AB (2015) "Esculetin Reverses Histone H2A/H2B Ubiquitination, H3 Dimethylation, Acetylation and Phosphorylation in preventing Type 2 Diabetic Cardiomyopathy". *Journal of Functional Foods.* 17:127–136. [Impact Factor: 3.144]
14. Kadakol A, Malek V, **Goru SK**, Pandey A, Bagal MB and Gaikwad AB (2015) "Esculetin Attenuates Alterations in Ang II and Acetylcholine Mediated Vascular Reactivity Associated with Hyperinsulinemia and Hyperglycemia". *Biochemical and Biophysical Research Communications.* 461(2), 342–347. [Impact Factor: 2.466]
15. **GS Kumar**, A Kulkarni, A Khurana, J Kaur, K Tikoo (2014) "Selenium nanoparticles involve HSP-70 and SIRT1 in preventing the progression of type 1 diabetic nephropathy" *Chemico Biological Interactions*. 223, 125-133. [Impact Factor: 3.143]
16. A Kulkarni, **GS Kumar**, J Kaur, K Tikoo (2014) "A comparative study of the toxicological aspects of vanadium pentoxide and vanadium oxide nanoparticles" *Inhalation Toxicology.* 26 (13), 772-788. [Impact Factor: 1.751]

Brief Biography of the Supervisor:

Dr. Gaikwad Anil Bhanudas is currently working as Head/Assistant Professor in Department of Pharmacy, at BITS Pilani, Pilani campus, Rajasthan. He did his Masters and PhD from Department of Pharmacology and Toxicology NIPER, SAS Nagar. He has first awarded Doctoral Sandwich Fellowship award from DAAD (German Academic Exchange Services) during his doctoral studies. He visited reputed overseas institutes as visiting scientist in Department of Medicine/Nephrology, Albert Einstein College of Medicine, Bronx, NY, USA and Nephrological Center, MedizinischePoliklinik, Ludwig-Maximilians-University, Munich, Germany, in the year of 2008 to 2009 and in 2010 respectively. His enthusiasm in research was recognized by various government agencies and accepted his research proposals. The title of the proposals and their respective sponsored Govt. agencies are; (1) "Esculetin a Therapeutic Intervention Targeted at Cardio-Renal Syndrome in Type 2 Diabetes" - SERB, DST Govt of India, Empowerment and Equity Opportunities for Excellence in Science; (2) "Understanding the Epigenetic Regulation in modulating Inflammatory Response mediated by NF-kB through AT1 or AT2 receptor in Type II diabetic nephropathy" - SERB, DST Govt of India, Fast Track for Young Scientist; (3) "Effect of Esculetin on Angiotensin II in rat thoracic aorta under hyperglycemia and hyperinsulinemic conditions" - UGC, Govt of India, Major Research Proposal; (4) "Role of Histone Ubiquitination& Angiotensin Converting Enzyme 2 in the Development of Renal Fibrosis under Type I diabetic Condition" - CSIR, Govt of India, EMR-II. Till the date, he has provided essential and novel evidences on histone post-translational modifications in the development of type 2 diabetic cardiomyopathy, type 1 and type 2 diabetic nephropathy. Dr. Gaikwad Anil Bhanudas is not only contributed in research but also in scientific writing by being one of the authors in several book chapters, which are published by Elsevier. His publications are the valuable contribution towards scientific community and in addition, he has more than 30 peer-reviewed research publications. Along with research achievement, he has proven himself for his administrative skills. At present he is guiding five PhD students and guided more than four M. Pharm students in the fulfillment of their dissertation.

Brief Biography of the Candidate:

Mr. Goru Santosh Kumar graduated in pharmacy from Andhra University, Andhra pradesh, India in the year 2011. He received his MS degree in Regulatory Toxicology from National Institute of Pharmaceutical Education and Research, Mohali, India in 2013. In the same year, he has joined with Dr. Gaikwad Anil Bhanudas group, BITS-Pilani, Pilani Campus to pursue his doctoral research work. His area of interest is epigenetics and novel molecular mechanisms involved in diabetes and diabetes related diseases. He has published 13 research and 3 review articles in peer-reviewed journals.

Role of Histone Ubiquitination and Angiotensin Converting Enzyme 2 in the Development of Renal Fibrosis under Type I Diabetic Condition

THESIS

Submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

Mr. Goru Santosh Kumar

Under the Supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2017

Chapter 7
Conclusions

7. Conclusions:

- Even though there is a long standing relation between histone methylation and ECM gene expression in renal tissue under hyperglycaemic condition, the underlying mechanisms are still not clear. Our results showing the epigenetic regulation of HMTs SET7/9 and SUV39H1 through histone H2AK119Ub and H2BK120Ub. Further, our study leads us to the conclusion that histone H2AK119Ub and H2BK120Ub orchestrate diabetic renal fibrosis by regulating active (H3K4Me2) and repressive (H3K9Me2) chromatin marks through modulating the expression of their respective HMTs, SET7/9 and SUV39H1 (Figure 32). These results suggest that the epigenetic regulation of SET7/9 and SUV39H1 by histone H2AK119Ub and H2BK120Ub, may be a novel mechanism involved in the development of renal fibrosis in diabetes.
- Aspirin treatment significantly inhibited the renal fibrosis in diabetic animals. In our study, we observed increased expression of Mysm1, a histone H2A specific deubiquitinase in glomeruli isolated from diabetic animals. Further, we also observed the decreased expression of H2AK119Ub in glomeruli of diabetic animals. In addition, these changes resulted in the increased expression of HMT Set7 in glomeruli of diabetic animals. Set7, mainly involves in the active gene transcription and also involves in the development of renal fibrosis. Moreover, all these changes were significantly reversed by higher dose of aspirin treatment. In conclusion, these results clearly indicating that, aspirin prevents renal fibrosis in diabetic animals through a novel mechanism, which involves Mysm1, H2AK119Ub and Set7 (Figure 33). However, additional research is required to know the complete molecular and epigenetic mechanisms of aspirin involved in the progression of DN.
- ACE2, the enzyme which degrades pathological peptide, Ang II and genereates protective Ang 1-7 was found to be inactivated and downregulated in diabetic renal diseases. However, several studies indicating the protective role of DIZE, the ACE2 activator in various disease pathologies, the exact mechanism is still remained enigmatic. In our study, DIZE, significantly restored the reduced ACE2 levels in glomeruli isolated from diabetic animals. On the contrary, recent studies questions the mechanism of DIZE on direct activation of ACE2 whereas, they lack the

explanation over ACE2 activation on chronic DIZE administration. In addition, recent studies indicates that AT2 receptors and AT2 receptor activation involves in the activation and expression of ACE2 enzyme. Moreover, in our study, chronic DIZE administration increased AT2 receptor and mRNA expression in diabetic kidney. Further, DIZE treatment decreased the levels of Ang II and increased the levels of Ang 1-7, indicating the increased ACE2 activation in diabetic kidney. Interestingly, we observed the downregulation of Mas1 receptor expression in diabetic kidney and did not restore even after the DIZE treatment. Surprisingly, all the protective actions of DIZE were blocked by the AT2 blocker. Based on these results, we concluded that, DIZE mediates its reno-protective actions through ACE2/Ang 1-7/AT2 axis (Figure 34).

Chapter 8

Future prospective