Advanced Glycation End-Products Induce Podocyte Injury and Contribute to Diabetic Nephropathy

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A Thesis Submitted for the Award of

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in Biochemistry

By

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CERTIFICATE

(For Ph.D. Dissertation)

This is to certify that this thesis entitled **“Advanced Glycation End-Products Induce Podocyte Injury and Contribute to Diabetic Nephropathy”** submitted by **Mr. Ashish Kumar Singh,** bearing registration number **19LBPH05** in the partial fulfilment of the requirement for the award of Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences, is a bonafide work carried out by him under my supervision. This thesis is free from plagiarism and has not been submitted previously in part or in full to the University or any other University or Institution for the award of any degree or diploma.

A. Publications:

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DECLARATION

I, **Ashish Kumar Singh**, hereby declare that this thesis entitled **"Advanced Glycation End-Products Induce Podocyte Injury and Contribute to Diabetic Nephropathy"** submitted by me under the guidance and supervision of **Dr. P. Anil Kumar,** is original and independent research work. I also declare that it has not been submitted previously in part or in full to the University or any other University or Institution for the award of any degree or diploma.

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Dedicated

to

My family and friends

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ABBREVIATIONS

OXPHOS	Oxidative phosphorylation
NOGPs	Non-oxidative glucose pathways
ROS	Reactive oxygen species
PKC	Protein kinase C
AGEs	Advanced glycation end products
SDH	Sorbitol dehydrogenase
AR	Aldose reductase
GFPT1	Glutamine-fructose-6-phosphatase aminotransferase
GNPNAT1	Glucosamine-6-phosphate N-acetyltransferase 1
PGM3	Phosphoglucomutase 3
DAG	Diacylglycerol
DHAP	Dihydroxyacetone phosphate
RAGE	Receptor for AGE
α-DC	α-dicarbonyl compounds
MG	Methylglyoxal
GO	Glyoxal
3-DG	3-deoxyglucuson
ALEs	Advanced lipid peroxidation end products
CML	Nε-(carboxymethyl)-lysine
CEL	Nε-(carboxyethyl)-lysine
MG-H1	Nδ-(5-methyl-4-oxo-5-hydroimidazolinone-2-yl)-l-ornithine
MOLD	methylglyoxal-lysine dimer
DOLD	3-deoxyglucosone-lysine dimer
IHC	Immunohistochemistry
IF	Immunofluorescence
fl-RAGE	Full-length RAGE
DN-RAGE	Dominant negative RAGE
N-RAGE	N-truncated RAGE
sRAGE	Soluble/secretory RAGE
SR	Scavenger receptor

Endo-AGEs	Endogenous AGEs
HbA1c	Glycated haemoglobin
GA	Glycated albumin
Exogenous AGEs	Exo-AGEs
LMW	Low molecular weight
HMW	High-molecular-weight
PEPT1	A peptide transporter 1
ECM	Extracellular matrix
NF-κB	Nuclear factor-κB
CREB	cAMP-response-element-binding protein
DR	Diabetic Retinopathy
DN	Diabetic Nephropathy
DC	Diabetic Cardiomyopathy
TGF-β	Transforming growth factor-β
MCP-1	Monocyte chemoattractant protein-1
VCAM-1	Vascular cell adhesion molecule-1
LDL	Low density lipoprotein
GFB	Glomerular filtration barrier
GBM	Glomerular basement membrane
SD	Slit-diaphragm
BMI	Body mass index
BP	Blood pressure
FBG	Fasting blood glucose
PBS	Phosphate buffer saline
AGI	Advanced glycation index
UACR	Urine albumin-creatinine ratio
GFR	Glomerular filtration rate
eGFR	Estimated glomerular filtration rate
ESKD	End-stage kidney disease
ND	Non-diabetic
PD	Pre-diabetic
DB	Diabetic

ADAM	A distintegrin and metalloprotease
NICD1	Notch intracellular domain
HES	Hairy/enhancer of split
HEY	Hairy/enhancer of split related with YRPW motif
DAPT	N-[N- (3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester
HPC	Human podocytes cells
EMT	Epithelial-mesenchymal transition
MET	Mesenchymal-epithelial transition
FSGS	Focal segmental glomerulosclerosis
TNBS	2,4,6-Trinitrobenzene Sulfonic Acid
JAG1	Jagged 1
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
qRT-PCR	Real-Time Quantitative Reverse Transcription
DAB	3,3′-Diaminobenzidine
WT1	Wilms' tumor suppressor gene1
DPX	Dibutylphthalate Polystyrene Xylene
BCA	Bicinchoninic acid assay
E-CAD	E-cadherin
N-CAD	N-cadherin
PAS	Periodic Acid-Schiff stain
H&E	Hematoxylin and eosin stain
MT	Masson’s Trichrome stain
TEM	Transmission Electron Microscopy
Col-IV	Collagen IV
FN	Fibronectin
α-SMA	α-smooth muscle actin

Chapter-I

Introduction

Chapter-I

Introduction

Hypothesis and objectives

Introduction

Saccharides (Sugar) are commonly found in many foods, such as fruits, vegetables, grains, etc. There are two main types of sugar: natural sugar, which is found in whole foods and added sugar, which is added to foods during processing and preparation. When we consume sugar, it is broken down into glucose, which is then used by the cells to convert it into ATP via a series of metabolic pathways. ATP provides energy to the body that is needed to carry out various physiological processes. During starvation, glucose is generated through the glycogenolysis and gluconeogenesis pathway by the liver **(Figure 1.1)** [1]. Blood glucose levels are tightly regulated by hormones such as insulin, glucagon, Growth hormone, and IGF-1. Insulin helps to lower blood glucose levels by promoting the uptake of glucose into cells, while glucagon helps to raise blood glucose levels by releasing glucose from the liver

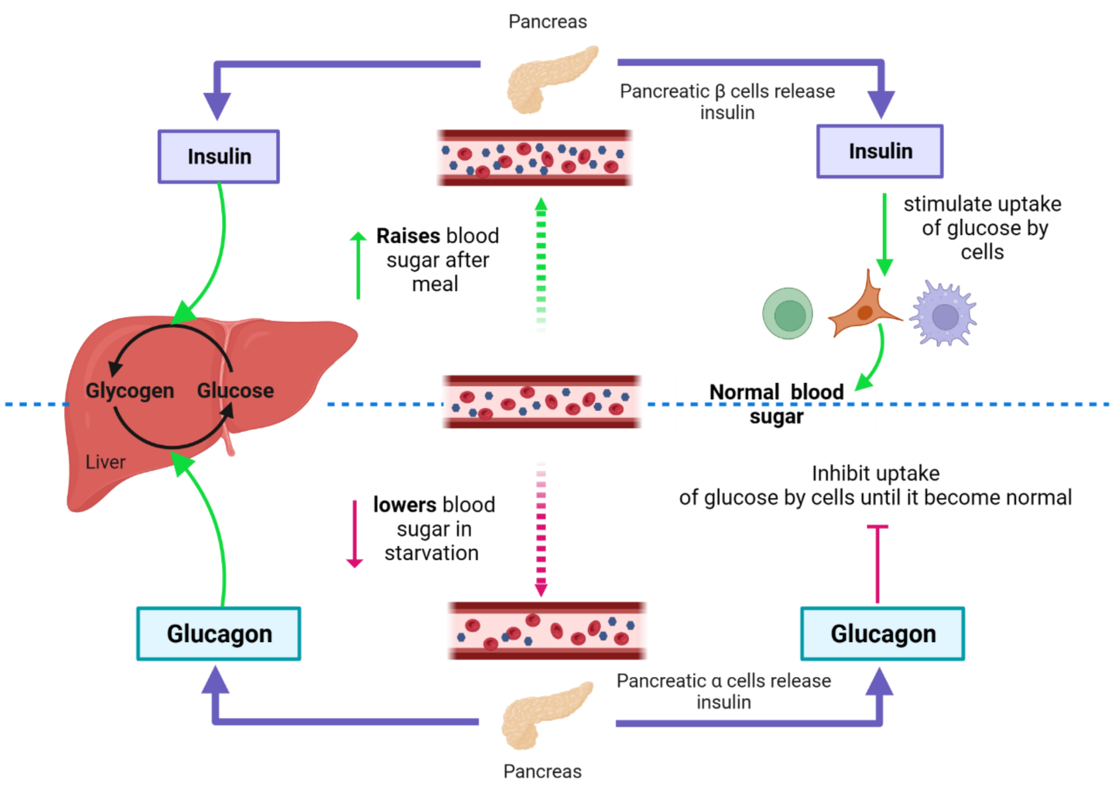

Figure 1.1: Regulation of blood sugar level. Diagram showing the mechanism of blood sugar level regulation by the pancreatic hormone insulin and glucagon. Insulin induces the uptake of glucose by cells and glycogenesis, in contrast, glucagon inhibits glucose utilization and induces glycogenolysis.

(Figure 1.1) [2, 3]. Maintaining optimal blood sugar levels (100mg/dl) is crucial for maintaining normal health. The clinical condition represented by excess glucose (<100mg/dl) in the blood is known as hyperglycemia and the overall state of the syndrome is referred to as diabetes mellitus (type 2). Modern life style and food like overcooked, fried food and cool drinks (contain more glucose) are the important players in obesity and diabetes. Chronic hyperglycemia inhibits glucose utilization followed by glucose toxicity which leads to irreversible cell damage. However, consuming too much added sugar is presented with health problems such as obesity, type 2 diabetes, and heart disease.

1.1 The deleterious effects of hyperglycemia

In normoglycemic condition, glucose oxidized via glycolysis, TCA cycle, and oxidative phosphorylation (OXPHOS) to generate ATP and provide energy for different physiological pathways. However, in hyperglycemia (diabetes), an excess flux of glucose in the blood causes an imbalance in glycolysis and OXPHOS followed by mitochondrial dysfunction [4]. This imbalance causes gradual inhibition of the main metabolic steps and enhances different glucose metabolic pathway perturbations called non-oxidative glucose pathways (NOGPs). In diabetes, NOGPs are the dominating pathway to cause increased oxidative stress (OS) via generating more amount of reactive oxygen species (ROS) [5, 6]. The four main NOGPs enhanced by hyperglycemia are the polyol pathway, hexosamine biosynthetic (HB) pathway, protein kinase C (PKC) Pathway, and advanced glycation end products (AGEs) pathway (**Figure 1.2)**. A brief explanation of these NOGPs is as follows.

- *The Polyol Pathway*

In normoglycemia, cellular glucose is predominantly converted into glucose-6-phosphate by hexokinase and enters glycolysis to generate ATP but very small (~3%) inter into polyol

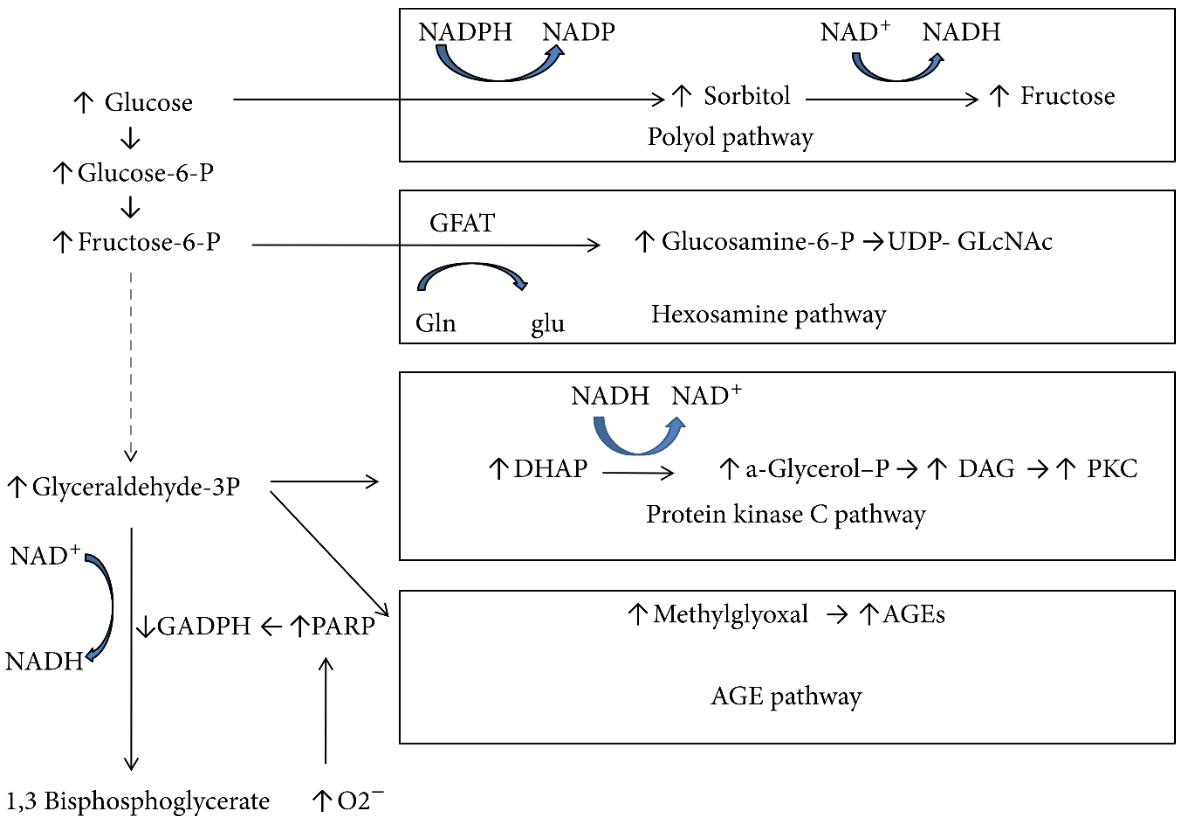

Figure 1.2: Non-oxidative glucose pathways (NOGPs). Diagram showing the activation of four metabolic pathway perturbations during hyperglycemic conditions and their involvement in ROS production. NADPH: Nicotinamide-adenine dinucleotide phosphate; GFAT: Glutamine-fructose-6-phosphate transaminase; DHAP: Dihydroxyacetone phosphate; UDP-GlcNAc: UDP-N-acetylglucosamine; PARP: Poly-ADP-ribose polymerase; DAG: diacylglycerol; GAPDH: Glyceraldehyde-3 phosphate dehydrogenase [13].

pathway. In hyperglycemia, saturation of hexokinase leads to an increase in the flux of glucose through the polyol pathway (25-30% of overall glucose metabolism) [7-9]. In the polyol pathway glucose is converted into fructose via a well-regulated two-step reaction. In the first step, glucose is reduced into sorbitol by a rate-limiting enzyme called aldose reductase (AR) and it requires NADPH as a cofactor [6, 10, 11]. The catalytic activity of AR increased in diabetes in comparison to normal conditions. The second step is the conversion of sorbitol into fructose catalyzed by a nicotinamide adenine dinucleotide (NAD) dependent enzyme sorbitol dehydrogenase (SDH) [5, 12]. The increased flux via the polyol pathway increases the ratio of NADH/NAD, which alters cellular redox status. The Mammalian sirtuin (protein deacetylase) using NAD as a cofactor is a major protein affected by the NAD reduction which participates in various functions in energy homeostasis, DNA repair, and cell survival [14]. The role of the polyol pathway has been extensively studied in diabetic vascular complications including DKD [15]. The accumulation of sorbitol detected in the renal glomeruli in people with diabetes is due to an increase in the activity of AR [16].

- *The hexosamine biosynthetic (HB) Pathway*

The HB Pathway is a type of glucose metabolic pathway used to generate UDP-GlcNAc (a nucleotide sugar) which is post-translationally added to proteins through the process called O- and N-GlcNAcylation [17, 18]. These proteins are involved in different functions like oxidative stress, nutrient sensing, epigenetic modifications, and apoptosis [19-22]. In this pathway, initially, glucose is converted into fructose-6-phosphate [23]. In the HB pathway, glutamine combined with fructose-6-phosphate results in glucosamine-6-phosphate (glucosamine-6-P), this reaction is catalysed by a rate-limiting enzyme glutamine-fructose-6-phosphatase aminotransferase (GFPT1) [24]. Immediately, glucosamine-6-phosphate N-acetyltransferase 1 (GNPNAT1) converts the glucosamine-6-phosphate into N-acetylglucosamine-6-phosphate, which is further converted to N-acetylglucosamine-1-phosphate by phosphoglucomutase 3 (PGM3). Later on UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1) leads to the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) which acts as a substrate for O-linked glycosylation of the target proteins [25, 26]. Under normoglycemic conditions, the HB pathway accounts for only 2–5% of total glucose metabolism but in hyperglycemic conditions, enhances the HB pathway by increasing the influx of fructose-6-P and contributes to oxidative stress [27]. More production of UDP-GlcNAc leads to the upregulation of transcriptional factors, (TGF-β1, NF-κB) causes oxidative stress [28-30].

- *The Protein Kinase C (PKC) Pathway*

PKC is a protein kinase belonging to the serine/threonine-related family that controls various intracellular signal transduction pathways [31]. The PKC pathway is activated by a well-studied second messenger named diacylglycerol (DAG). DAG play important in the regulation of various function like vascular permeability, growth factor signaling, calcium signaling, and endothelial activation [5, 32, 33]. Two main pathway for the production of DAG are (1) *de novo* pathway in which dihydroxyacetone phosphate (DHAP) is converted into glycerol-3-phosphate and produce DAG [34]. (2) The phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) via ligand-stimulated hydrolysis converted into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) of by phospholipase C (PLC) [35-37]. In hyperglycemia, an increased influx of glycolytic intermediates like dihydroxyacetone [34] and DHAP leads to more production of DAG followed by the upregulated PKC activity. PKC Activation comes up with several pathogenic results, by affecting the expression of endothelial nitric oxide synthase (e-NOS) an MAPK pathway, vascular endothelial growth factor (VEGF) [38] Connective tissue growth factor (CTGF) and transforming growth factor-β (TGF-β) [39], and plasminogen activator inhibitor-1 (PAI-1) [40]. All these play an important role in role in vascular disorders. More importantly, increased PKC activity has been associated with activation of NF-κB [41] and NAD(P)H oxidase [5] which are highly responsible for hyperglycemia-induced oxidative stress and inflammation.

- *The AGE-RAGE Pathway*

AGE signaling is another important NOGP whereby glucose can activate oxidative stress. AGEs are the non-enzymatic products of reactions between glucose and metabolites with proteins and other macromolecules. AGEs reflect its effect through the receptor for AGE (RAGE). The AGE-RAGE system is more active in diabetic condition which are involved in the onset and progression of diabetic complications and also other diseases like cancer, neurological disorder, atherosclerosis, etc. In this thesis work, we have explained AGEs and their effect on diabetic nephropathy in detail.

1.2 Advanced Glycation End-Products (AGEs)

1.2.1 Glycation and its intermediate products

The concept of Maillard reaction and glycation was first discovered in 1912 by L.C. Maillard. In this experiment, he observed a characteristic yellow-brown color upon heating a mixture of amino acids and reducing sugars which are responsible for the “browning” of foods (Maillard article). Later in the 1970s, the maillard reaction was recognized as a slow process *in vivo* condition, and the process was termed glycation [42, 43]. Glycation is a non-enzymatic reaction that occurs between the carboxyl group of reducing sugars like glucose or fructose and amino groups in any macromolecule like proteins, lipids, and nucleic acids [44]. The process of glycation is subdivided into three stages; early, intermediate, and late. In an early stage, the reactive carbonyl group of glucose reversibly attached with a free amino group of any macromolecule to form Schiff`s base (unstable compound) which immediately converted into amadori product (stable product) through amadori rearrangement [45] **(Figure 1.3)**. In an intermediate stage, Amadori products undergo successive displacements of the carbonyl group along the carbon skeleton of reducing sugar, which results in the formation of a variety of α-dicarbonyl compounds (α-DC) like Methylglyoxal (MG), glyoxal (GO), 3-deoxyglucuson (3-DG) and fructosamine via dehydration, oxidation. These dicarbonyl compounds are more highly reactive than reducing sugars. These dicarbonyl compounds are the first stable intermediates of glycation termed “early glycation products” 16037245. In the late stage, they irreversibly react with the new macromolecule and form their cross-linked products called AGEs via oxidation, dehydration, and cyclization reactions [44, 46] **(Figure 1.3)**. The early glycation products are generated by both oxidative and non-oxidative reactions (auto-oxidation, rearrange in functional groups, and hydrolysis) [47].

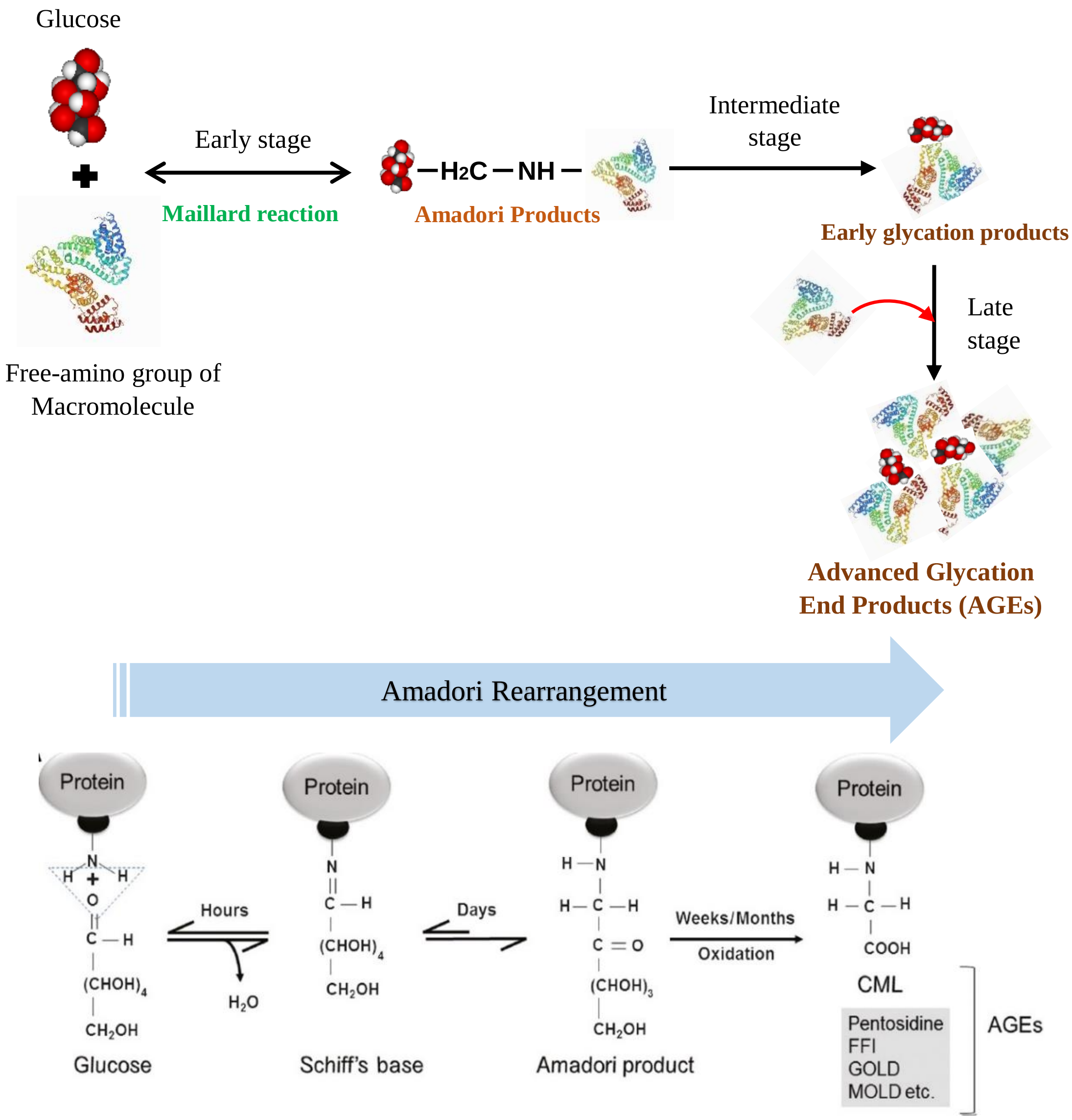

Figure 1.3: Glycation and AGEs formation. The diagram repesenting the stages (early, intermediate and late) during glycation reaction and down panel showing chemical rection, formation of schiff`s base, amadori product in the amadori rearrangment and the formation of final product AGEs (adapted from [48]).

1.2.2 Advanced product of glycation

AGEs are the insoluble, yellow-brown, heterogeneous compounds and advanced products of glycation formed by both exogenous and endogenous mechanisms. This heterogeneity of AGEs depends upon the type of reducing sugar and macromolecule involved in it. These AGEs are formed by the classical and alternative pathways. The classical pathway 1) the Maillard reaction including the formation of Schiff's base and amadori product which is later converted into early and advanced glycation product. The individual product of this pathway participates in AGEs formation via oxidative degradation or autoxidation. AGEs are formed by the autoxidation of glucose, fructose, glyceraldehyde-6-phosphate, etc. called Wolff pathway [49], through Schiff bases called Namiki pathway [50] through amadori product called Hodge pathway [51]. 2) The Polyol pathway also takes part in the generation of AGEs via the reduction of glucose into sorbitol followed by conversion into fructose [52]. In alternative pathway 1) Lipid peroxidation in which polyunsaturated fatty acids are oxidized into reactive carbonyl species (malondialdehyde, and 3-DG) and converted into the well-characterized advanced lipid peroxidation end products (ALEs) [53]. 2) Ketone bodies are converted into acetol and dicarbonyl compounds to generate AGEs [54, 55] **(Figure 1.4).** Some of the common AGEs are as follows: CML, Nε-(carboxymethyl)-lysine; Pentosidine; CEL, Nε-(carboxyethyl)-lysine; Argypyrimidine; CEA, Nδ-(carboxyethyl)-arginine; CMA, Nδ-

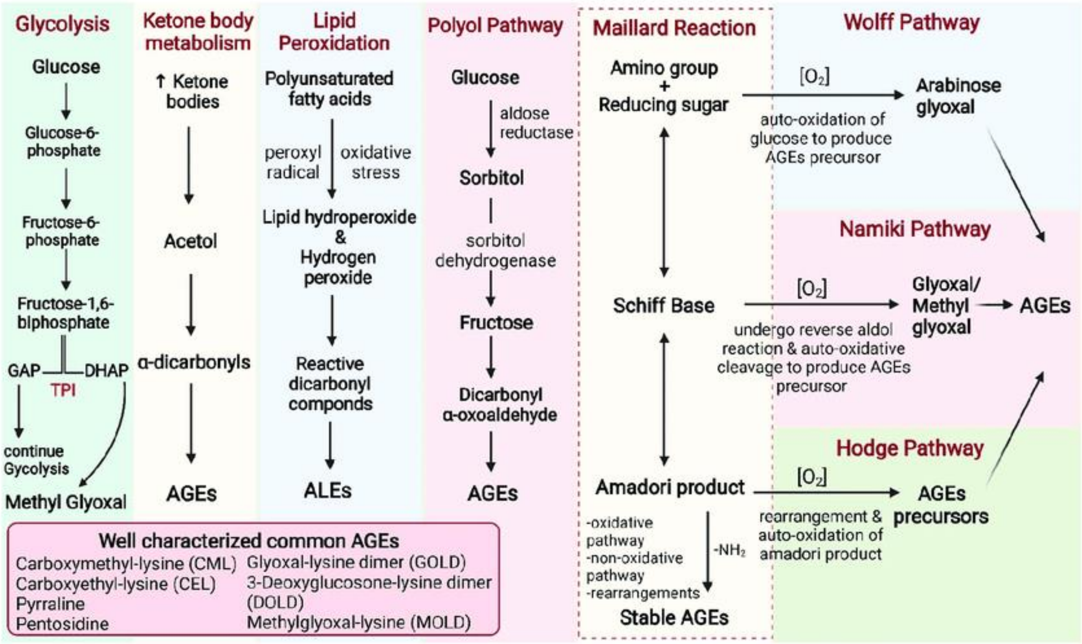

Figure 1.4: Pathways of AGEs formation. AGEs are generated by the different pathways such as Wolff pathway (autooxidation of glucose), Namiki pathway (autooxidation of the Schiff base) or Hodge pathway (non-oxidative cleavage of Amadori product), Polyol pathway, and Ketone body metabolism. These pathway generate AGEs via intermediate called reactive dicarbonyls. Lipid peroxidation pathway generate another class of AGEs that is advanced lipid peroxidation end products (ALEs). Dihydroxyacetone phosphate (DHAP) from glycolysis take part in AGEs formation via endogenous generation of methylglyoxal.

(carboxymethyl)-arginine; MG-H1, (Nδ-(5-methyl-4-oxo-5-hydroimidazolinone-2-yl)-l-ornithine); GLAP, glyceraldehyde derived pyridinium compound; GOLD (glyoxal-lysine dimer), Pyrraline; MOLD, (methylglyoxal-lysine dimer); Tetrahydropyrimidine; DOLD (3-deoxyglucosone-lysine dimer). Due to heterogeneity AGEs, AGEs are categorized into groups in several ways like sources, Precursors, Fluorescent, cross-linked/Non-cross-linked nature **(Table 1)** molecular weight, etc.

Sources	Molecular weight	Fluorescence nature	Linking nature	Example of AGEs
Endogenous	Low (LMW AGEs)	Fluorescent	Cross-linked	Pentosidine, Pentodilysine, Crossline, AGE-XI
			Non cross-linked	Argpyrimidine
Exogenous	High (HMW AGEs)	Non-Fluorescent	Cross-linked	GOLD, MOLD, DOLD
			Non cross-linked	CML, CEL, Pyrraline, Imidazolones

Table 1: Classification of AGEs. Table representing the ways of classification of AGEs based on their different properties with examples.

The heterogeneous nature of AGEs affects their detection and measurement and every methodology has advantages and disadvantages in the detection. The AGEs are detected and measured in both endo and exogenously by using a different technique like spectrofluorometric [56], LC/MS (liquid chromatography-mass spectrometry) [57], IB (Immunoblotting) [58], GC-MS (gas chromatography-mass spectrometry) [59], IHC (Immunohistochemistry) [60], HPLC (High-performance liquid chromatography) [61], IF (Immunofluorescence) [58], enzyme-linked immunosorbent assay (ELISA) [62]. CML, CEL, and pentosidine are well-studied AGEs. CML was first identified and described by Ahmed as the most abundant Endo-AGEs [63, 64]. It is a non-fluorescent and non-crosslinked exists in free, peptide, and protein adducts.

CML adduct used as a major epitope of the development of Monoclonal and polyclonal anti-AGE antibodies [64].

1.2.3 Receptors for AGEs

The receptor for AGEs (RAGE) is expressed in both developmental as well as the adult stage. It is constitutively expressed during embryonic development and inducibly in adults [65, 66]. In the adult, RAGE is represented in low concentration by a wide range of cells like vascular endothelial cells, epithelial cells, cardiomyocytes, neurons, neutrophils, macrophages, lymphocytes, and dendritic cells [65, 67]. RAGE is a type of pattern-recognition receptor (PRR) with a multi-ligand nature belonging to the immunoglobulin superfamily [68]. Due to its multi-ligand nature, it is represented by tissue ranging from micro to microvasculature organs. The RAGE gene present within the major histocompatibility complex class III region on chromosome 6 consists of 394 amino acids [69]. For the first time, RAGE was reported as a receptor of AGEs [70] but later, researchers found a broad range of other ligands likes S100/calgranulin family [71, 72] amphoterin (HMGB1) [73], β-amyloid & serum amyloid A [74], Calprotectin [75] **(Table 2)**. RAGE mRNA undergoes tissue-dependent alternate splicing which generates different splice variants (~20 in humans) and only full-length RAGE (fl-RAGE), soluble-RAGE (sRAGE), and endogenous secretory RAGE

RAGE ligands	Pathophysiologic impact
AGEs	Oxidant stress, inflammation, vascular stiffness.
Amyloid-β peptide & β-sheet fibrils	Amyloidosis
Mac-1	Inflammatory cell migration
Amphoterin	Inflammation, tumorigenesis
S100/calgranulins	Inflammation, tumorigenesis, vascular stiffness

Table 2: RAGE Ligands. Ligands for RAGE and their role in the different pathophysiological conditions.

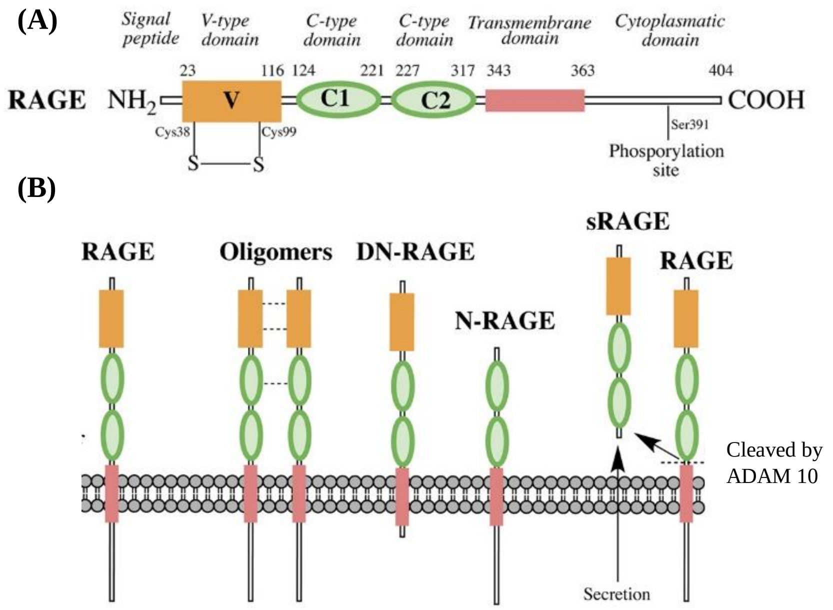

Figure 1.5: RAGE and its splice variants. (A) Structural detail of full-length RAGE, having five domain namely variable (V-type), constant (C1&C2-type), transmembrane and cytoplasmic domains with a disulfide bridge (Cys38-Cys99) in the V domain. **(B)** Representing the RAGE isoforms, from left Full-length RAGE (fl-RAGE) and its oligomer, dominant negative RAGE (DN-RAGE), N-truncated RAGE (N-RAGE), and soluble/secretory RAGE (sRAGE) which is a cleaved product of fl-RAGE by ADAM10. Figure adapted from [76].

(esRAGE) are detectable at protein level [77, 78]. Human fl-RAGE cleaved by the membrane metalloproteinase ADAM10, and extracellular domain released as a soluble isoform [79, 80] **(Figure 1.5B)**. sRAGE competitively bind AGEs inhibit their interaction with mRAGE induces activation of downstream genes including pro-inflammatory genes. RAGE consists of extracellular (V domain), hydrophobic (C domain), transmembrane, and a cytosolic tail [81] **(Figure 1.5A)**. The truncated C-terminal type (esRAGE), which contains only the extracellular segment and in opposite, truncated N-terminal type consists of the transmembrane and the cytosolic tail region. There are several other membrane bound AGE-receptors rather than RAGE belongs to the AGE-receptor complex (AGE-R1, AGE-R2, AGE-R3) [82, 83] and few from the scavenger receptor (SR) family (SR-A, SR-B, SR-BI, SR-E) [84-87]. They help in the removal of AGEs bind through endocytosis and degradation to maintain AGEs homeostasis

[88]. These receptors express on different cell in response chronic condition like aging, diabetes and hyperlipidemia [89].

1.2.4 Sources of AGEs

The AGEs are a family of heterogeneous compounds generated through a non-enzymatic glycation. The two main source of AGEs to human body are: 1) Endogenous and 2) Exogenous or Dietary AGEs. The formation of AGEs affected by the number of factors like degree of hyperglycemia, temperature, PH, the nature of the carbonyl substrates, extent of oxidative stress and condition of cooking etc.

Endogenous AGEs (Endo-AGEs):

Endo-AGEs are the non-toxic product of normal metabolic process formed in all type of tissues and body fluids. In normal physiological condition, AGEs are formed with slow rate by the both classical and alternative pathway (discussed above) and participate in normal aging process [90]. Formation of Endo-AGEs are much faster with intercellular sugars like glucose-6-phosphate and fructose then the glucose [91]. The diversity of Endo-AGEs in circulation is less due to the less physiological temperature. Glycated haemoglobin (HbA1c) is the first identified endogenous glycation product in which the glucose attached with valine residue at N-terminal of haemoglobin resulted into N-a-fructosylvaline [92]. The condition like diabetes, cancer and other vascular diseases enhanced its formation followed by its accumulation. There are several factors involved in promoting Endo-AGEs formation including exogenous factors (cigarette smoking, intake of high fat diets, cool drinks, spices, prolonged alcohol consumption), physiological status of cell etc [93]. Endo-AGEs in circulation enhanced glycation of plasma protein (Glycated haemoglobin, HbA1c and Glycated albumin, GA) and its accumulation which participate in several diseases [92].

Exogenous AGEs (Exo-AGEs) or dietary AGEs:

AGEs are naturally present in animals and plants origin foods, however the production and concentration of AGEs enhanced during cooking them. All Exo-AGEs are toxic in nature which mostly come through the foods, beverages and cigarette smoke. Exo-AGEs are the main culprit for the heterogeneous nature of AGEs and its amount are much more then Endo-AGEs [93-95]. Its formation influenced by several factor likes temperature (food browning), water content (dry-heat cooking), pH status and way of cooking (time of cooking, grilling and frying or broiling). In modern environment, the processing of food is based on 'Dry heat technology' and this including grilling of food on high flame (300-500F), frying in used oil, roasting, baking, barbecuing till browning [96]. The overcooked or high temperature cooked food at consist of more AGEs then food cooked with more water and low temperature or uncooked food [97]. Various databases and article conformed that presence of AGEs in in a broad range of food products like bakery & milk product, dry-heat cooked meat, fried food and beverages which contain highest concentration of like CML, CEL and MG-H1 [97, 98] **(Figure 1.6)**. Cigarette smoke is also responsible for the generation and accumulation of AGEs in the blood circulation and tissues of smokers. The reactive glycation products are present in the extract of tobacco leaves which get enhance in vivo and in vitro by tobacco smoke in time and concentration dependent [99].

1.2.5 AGEs absorption, clearance and accumulation

Absorption of AGEs: Both Exo and Endo-AGEs are involved in the generation of circulatory AGEs. For the normal body function AGEs should get degraded and excreted out though urine and faces. The maximum limit of dietary AGEs intake should be 15000 AGEs kU/day [93]. Researchers indicated that only 10-30% of Exo-AGEs are absorbed into the blood circulation [100, 101]. Dietary AGEs consist of low molecular weight (LMW, <5 kDa) AGEs bound with single/di/tri amino acid and high-molecular-weight (HMW) AGEs bound with peptide, proteins and lipid [102]. LMW fractions of AGEs are absorbed faster than the HMW fraction. Direct heat cooking modified the proteins in food reduces the degradation of HMW AGEs [103]. After oral administration it partially degraded by the gut proteases to get absorbed [104] and their bioavailability depends on type of food, gut environment, and traveling time in the gut. Single amino acid bound CML or CEL are absorbed through simple diffusion [105]. Dipeptide form of CML, CEL, pyrraline and MG-H1 are absorbed mostly into intestine through

Figure 1.6: Sources of Exo-AGEs. A pictorial representation of food product with high amount of AGEs in modern life style. **(A)** Overcooked meet product by grilling, roasting; shawarma, kabab, fish fry, french fry and sandwiches. **(B)** Bakery product; cookies. **(C)** Peanut and peanut butter. **(D)** Tobacco products **(E)** Malted Beverages.

a peptide transporter 1 (PEPT1) [105, 106]. The free form of pentosidine (in brewed coffee) are absorbed faster than the protein-bound (in bakery products) [107].

Clearance of AGEs: The absorbed AGEs are cleared through urine which depend on the renal function. The absorbed LMW AGEs (CML, CEL) are quickly filtered through kidney and excreted in urine [108]. The patients with renal disease has shown low level (5 %) excretion of the absorbed AGEs as compared to healthy subjects (30 %). In Forester's study, diet with low glycation products like fructoselysin, pyrraline and pentosidine shown that the urinary excretion of free fructoselysin, pyrraline and pentosidine was reduced by 90%, 50% and 60% respectively [107]. The unabsorbed HMW AGEs in gastrointestinal tract reaches into the colon where they are metabolized by the microbiome and excreted into faeces. In animal and human study reported that the 20 to 50% of ingested protein bound or HMW CML were excreted into faeces and suggesting a strong interaction between AGEs and the colonic microbiome [109, 110]. The studies shows strong correlation between dietary intake of AGEs and their presence in faecal excretion [110, 111]. The Endo-AGEs are degraded by the extracellular proteolysis and via a receptor mediated initialization. The AGEs-receptor (AGER1) is crucial for the receptor mediated degradation of endo-AGEs in cells like macrophages, kuffer, mesangial, and endothelial cells etc. [112-114]. These cells generate LMW soluble peptides release into the circulation and excreted by the kidney through urine [114].

Accumulation of AGEs: Helen Vlassara and her group for the first suggested that the pool of AGEs in human body could derived from both *in vivo* and *in vitro* (dietary) [101], later they defined as endogenous and exogenous AGEs, respectively. AGEs accumulated mainly due to the imbalance between the formation, absorbance and clearance of AGEs. In diabetes, high glucose accelerate the formation of endo-AGEs which is overload for the clearance leads to intra and intercellular accumulation. As discussed earlier, dietary exposure consists of heterogeneous AGEs which make it very less available for absorption and digestion. In urbanization, more intake of caloric and sugar rich food adding more AGEs in body. The overload of Exo and Endo AGEs leading it to accumulate in micro and microvasculature system which causes serious biological effect on the cell and the organ system.

1.3 Biological effect of AGEs

The AGEs can contribute in different diseases via two important mechanisms: Receptor independent and dependent. The receptor independent effect of AGEs were observed intracellularly where it cross-linked the extracellular matrix (ECM) protein, react with lipid of plasma membrane and permanently alter the cellular structure. The long lived matrix protein like collagen, fibronectin, and laminin undergoes glycation and formation of AGEs. The AGE-AGE intermolecular cross-linking of type I collagen and elastin causes altered the ECM structure and adding stiffness to it which can “trap” local macromolecules [115-118]. Glycation leads to an increased synthesis of different type of matrix collagen (III, α3 (IV), V, VI), laminin, and fibronectin in ECM, due to an upregulation of a transforming growth factor-β intermediate [119-121]. AGEs alters the formation of ECM by disruption of the binding between the noncollagenous domain (NC-1) and the helix-rich domain on type IV collagen in the basement membrane [122, 123]. Glycation of laminin and collagens reduces there interaction, decreased polymer elongation and disrupts binding of heparan sulfate proteoglycan finely results in inhibited adhesion ECM to endothelial cells [124, 125]. Last but not the least, lipids are also equally targeted for the glycation and contribute in AGEs formation. Studies were found that increased lipid-linked AGEs in samples from persons with and without diabetes [126]. Glycated LDL like apolipoprotein B (ApoB) suppresses the uptake and clearance of LDL by the reduced production of nitric oxide (NO) in endothelial cells [126-129].

The receptor dependent pathological effect of AGEs is inducing by interaction between AGEs and RAGE. RAGE is a multi-ligand receptor present at different cell type like monocytes, endothelial, macrophage, epithelial, muscle cell, adipocytes and others [74, 130-134]. Glycation of intracellular proteins disturbed the cellular properties which are important in vascular homeostasis [135]. Increased AGE formation on intracellular proteins is mainly due to the excess production of natural sugars like glucose-6-phosphate, fructose, and glyceraldehyde-3-phopshate [136, 137]. Intracellular AGE formation significantly increases the production of reactive oxygen species (ROS) and oxidative stress [138, 139]. Intracellular AGEs and oxidative stress activates central transcription factors (TFs) like nuclear factor-κB (NF-κB) [74], early growth response (EGR)-1 [140], cAMP-response-element-binding protein (CREB)-1 [141], and activator protein (AP)-1 [142]. Later these TFs induces various signaling pathways like mitogen-activated protein kinase (MAPK), JAK-STAT signaling, CDC42, RAC1 and other Ras family, SRC1, SMAD signaling and phosphoinositide 3-kinase [74, 143-145]. The AGE-RAGE signaling contribute in different disease condition and leads to organ damage [146-148]. Activation of NF-κB by AGE-RAGE axis enhances the expression of pro-inflammatory cytokines (IL-6, TNFα, TGF-β) and vascular adhesion molecules (VCAM-1, ICAM-1, endothelin-1) causes inflammation and finely lead to vascular stiffness [149].

1.4 AGEs in pathology of various diseases

AGEs get accumulated with the rising age and due to modern lifestyle including more intake of sugar add fast food, overcooked food, smoking as well as less physical activity in both adult and the young population increases the risk of chronic diseases. As previously discussed, AGEs exert its effect either by binding to RAGE or cross-linked the long-lived protein of intra or intercellular region. They are activated different kind of signaling pathway which leads to the primary complication like ROS production followed by oxidative stress, inflammation, vascular stiffness. Later AGEs and AGEs-induces primary complications arises in chronic diseases like diabetic complications (retinopathy, cataract formation, neuropathy, nephropathy, cardiomyopathy), neurodegenerative disorders, cancer, aging and age-related diseases which are briefly discussed below.

1.4.1 AGEs in diabetic complications

Diabetic Retinopathy (DR): DR is a major diabetic complication which causes blindness in diabetes patient with ages 30 -70 years. DR is distinguish by the retina lesions due to angiogenesis, vascular occlusion, pericytes loss, and vascular permeability [150, 151]. In chronic hyperglycemia, AGEs act as a crucial player in the progression of diabetic retinopathy [152]. Human and animal studies revealed that accumulation of AGEs in retinal cells causes its dysfunction during diabetic retinopathy [153, 154]. Accumulation of AGEs create premature vascular occlusion (closure) in the retinal endothelial microcirculation [155]. AGEs enhance the expression of intracellular cell adhesion molecules (ICAM) in retinal capillary endothelial cells lead to more leukocyte adherence causes diabetic retinal microvascular leukostasis [156, 157]. AGEs exposed retinal cells increases the expression of vascular endothelial cell growth factor (VEGF) via IL-6 secretion which stimulates angiogenesis and neovascularisation implicated in the pathogenesis of proliferative retinopathy [158, 159]. Evidences suggested that high circulatory AGEs enhanced the expression of RAGE mRNA in retinal cells of type 2 DM [160]. AGEs causes oxidative stress via the activation of PKC and NF-κB, and inflammation via secretion of cytokines (IL-α, IL-β, and IL-6) [51].

Diabetic Cataract: AGEs formed by the glycation of the eye lens protein is crucial in the diabetic cataract formation which is the leading cause of blindness in developing countries [161-163]. The lens protein formed high-molecular-weight aggregates due to the irreversible glycation which causes scattering of light and impaired vision [164]. AGE-RAGE interaction generated high amount of superoxide radicles lead to oxidative stress in lens by reducing it antioxidant capacity [165-167].

Diabetic Neuropathy: Neuropathy is one of the major diabetic complication affect peripheral nervous system which distinguish by segmental demyelination and axonal degradation [168]. Human and animal studies revealed that the localization of AGEs in the peripheral nerve [169]. The formation and accumulation of AGEs causes damage peripheral nerve directly by the modification in neural protein or indirectly by RAGE [170]. Glyoxalase I (GLO1) is a key and highly conserved enzyme in the glyoxalase pathway and which detoxify the effect of reactive dicarbonyls and its accumulation [171, 172]. The expression and activity of GLO1 is influences by more AGEs production leads to damage in the sensory neuron of peripheral nervous system [173]. AGEs accumulation in the diabetic individual induces and modification in P0 protein and axonal cytoskeletal proteins causes fibril loss and hindrance in axonal transport respectively [174, 175]. *In vitro* experimentation proved that AGEs exposure to neuronal cells and Schwann cells induces apoptotic cell death [174]. AGEs induced oxidative stress modify the Na+/K+ ATPase which causes loss in neural conductivity [176]. It is reported that AGEs induces vasoconstriction by quenching nitric oxide (NO) 1991829, and reduction in the expression of NO synthase which stimulate hypoxia in the peripheral nerve [177]. The binding of AGEs to RAGE induces transcription factors like NF-κB and activator protein-1 (AP-1) which activates vascular cell adhesion molecule-1 and cytokines stimulates oxidative stress and inflammation in vascular endothelial cells [178].

Diabetic Cardiomyopathy (DC): DC is well known cause of heart failure in diabetic individuals. It is distinguish by the hypertrophy and fibrosis in myocardial cells, which leads to diastolic dysfunction. AGEs may contribute to the development of DC either via modifying properties of ECM proteins followed by formation of cross-linking or causing intracellular interaction with AGE receptors [179]. More HbA1c level in myocardium related with diastolic dysfunction [180]. Experimental studies revealed that activated RAGE reduces the PARP-γ activation causes myocardial fibrosis [181]. Evidences suggested that AGEs induced RAGE activate mitochondrial membrane potential (MMP) depolarization causes cardiomyocytes dysfunction. Activated RAGE upregulate transforming growth factor-β (TGF-β) which increase the synthesis of collagen types (III, α3(IV), V, VI) , laminin, and fibronectin in the ECM causes myocardial fibrosis [121]. Human RAGE overexpression transgenic mice model, indicate that the reduction in the level Ca^{2+} concentration which interfere in the myocardial contractibility [182]. Crosslinking of ECM protein disturbs the flexibility characteristic of matrix proteins causes stiffness in vasculature and reduction in contractibility of myocardial cells [183].

Atherosclerosis: It is a major long-term complication of diabetes, characterized by the plaque deposition in the inner wall of arteries causes reduced or block in the blood flow and finally end up with myocardial infarction/cardiomyopathy. Plaque is a sticky material builds up inside the arteries composed of cholesterol, fat, blood cells and other substances. AGEs accumulation causes atherosclerotic lesions by the induction of inflammation, lipid/protein modifications, and endothelial dysfunction [184]. In particular, AGEs shows pro-apoptotic effect on endothelial cells and endothelial progenitor cells which may implicated in endothelial dysfunction [185, 186]. Circulatory AGEs stimulated various adherent protein like monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1) and vascular cell adhesion molecule-1 (VCAM-1) which allow to attract and adhere several inflammatory cells in vasculature [187-189]. AGEs interfere in vascular dilation, endothelial regeneration, and platelet adhesion by inhibiting the expression of Nitric oxide synthase in endothelial cells [190, 191]. AGEs modified low density lipoprotein (LDL) activates a toll-like receptor 4 (TLR4) followed by the inducing proinflammatory cytokine which promotes atherogenesis [192]. In vascular smooth muscle cells (SMCs), AGEs induced RAGE activates protein tyrosine phosphatases which causes cell proliferation and chemotactic migration which implicated in atherosclerosis [193].

1.4.2 AGEs in neurodegenerative disorders

Alzheimer’s disease (AD) and Parkinson’s disease (PD) are the most common neurodegenerative disorders which primarily due the aggregation of different protein. Evidences suggested that the glycation play a crucial role in these aggregations. AGEs generates by the glycation of β-amyloid protein (Aβ) take part in the formation of β-amyloid plaques and neurofibrillary tangles (NFTs) leading cause of AD [194, 195]. Diabetic patients with AD shows more dance β-amyloid plaques and higher RAGE expression then AD patients 21900634. Aβ-AGEs induces RAGE activates amyloid precursor protein (APP) which increases the level of β-amyloid [196]. Aβ-AGEs induces RAGE also activates glycogen synthase kinase-3 (GSK-3) causes pathologies of AD [197]. Gene like α-synuclein (α-syn), pink 1 and parkin are the crucial for the development of PD. Glycation of α-syn causes aggregation followed by Lewy bodies (LB) in the pathogenesis of PD [198]. The α-syn-AGEs aggregate induces α-syn oligomerization and its stabilization which is more toxic then α-syn aggregates [198]. α-syn oligomers altering membrane permeability by formation of pore in membrane can cause a loss of cell homeostasis and neuronal cell dysfunction.

1.4.3 AGEs in aging

Evidences suggested that accumulation of AGEs accelerate during progression of age in multiple species [199]. Progressive deposition of various type of cross-linked collagen associated with vascular diseases and their importance in aging [200-202]. In human population based study revealed that the more plasma CML level significantly correlated with high risk of mortality in older adults [203]. Since, AGEs are the potential biomarker in diabetes, it also crucial in o a potential driver of aging. Glycation of long-lived protein like collagen, crystallins and elastin accumulated in form of AGEs over the years [204-206].

1.5 Diabetic Nephropathy (DN) and Kidney

Nephropathy is presented with stages of progressive proteinuria (microalbuminuria-macroalbuminuria-overt proteinuria) and culminates in end-stage renal disease. Pre-eminent glomerular changes during nephropathy include mesangial expansion basement membranes thickening, hypertrophy and loss of podocytes [207, 208]. As mentioned above, podocytes are instrumental in the glomerular filtration, and to ensure protein-free primary urine, podocyte loss considered as a hallmark of nephropathy. Nephron, the structural and functional unit of the kidney and each having ~1 million nephrons [209]. Each nephron contain two part: a glomerulus and tubular part **(Figure 1.6)**. The glomerulus help in the filtration of blood thus the formation of urine, while the tubular part help in the selective reabsorption of minerals and secretion of protein-free ultra-filtrated urine. Glomerulus consist of glomerular filtration barrier (GFB) which composed of luminal side capillary endothelium layer, inner glomerular basement membrane (GBM) layers and podocytes towards urinary space. **(Figure 1.7)** [210].

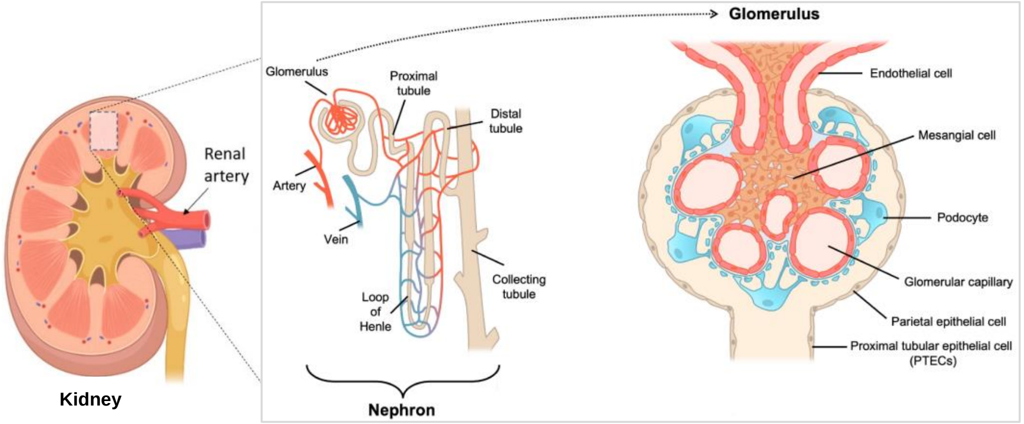

Figure 1.7: Enteral structure of kidney and nephron The pictorial representation of nephron consist of glomerulus and tubular region. The glomerulus is a filtration unit composed of endothelial cells of blood capillaries, mesangial cells, podocytes and epithelial cells. (figure was adopted and modified from these artical [211, 212]).

A large body of evidence suggests podocytes are vitally important for the normal function of GFB and in turn kidney function. Podocytes are the specialized terminally differentiated visceral epithelial cells with a complex cell structure contain huge cell body and foot processes (primary and secondary) [213] **(Figure 1.8)**. Foot-processes help podocytes adhere firmly to the basement membrane and provide epithelial coverage to the glomerular capillaries [214]. Interdigitating adjacent foot-processes formed a zipper-like structure slit-diaphragm (SD), crucial in establishing the size-selective permeability of the GFB, Thus. podocytes allow only water and small molecules to filter into urinary space and retain large molecules including proteins in the plasma [215]. Therefore, an injury to podocytes expected to impair the efficiency of glomerular filtration and is evidenced by proteinuria. The extent of damage to the podocytes dictates the magnitude of proteinuria [216].

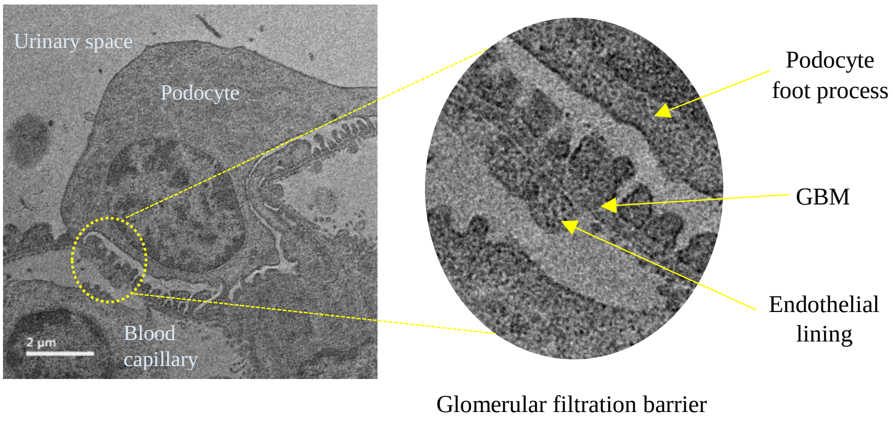

Figure 1.8: Glomerular filtration barrier. A transmission electron microscopic images showing podocyte wrapped around blood capillary and urinary space. The zoomed area showing the glomerular filtration barrier consist of podocyte foot process, glomerular basement membrane (GBM), endothelial lining.

1.6 The Role of AGEs in the pathology of DN

Both, clinical and experimental studies suggest that AGEs participated in the pathogenesis of nephropathy particularly in diabetic subjects [217]. In an animal study, AGEs administrated non-diabetic rats shows proteinuria and degenerative changes in glomerulus which indicated the crucial role of AGEs in the pathogenesis of renal damage [218]. Podocytes, the prominent cells that constitute glomerular filtration barrier express RAGE and AGE-RAGE axis activation cause podocyte injury [219]. Human population study suggested that the CML/serum protein level and more circulating AGE in patients with proteinuria are correlated with the severity of renal damage [219]. The extremity of DN correlates with the AGE induced

RAGE expression in glomerular and tubular compartments. A decrease in glomerular filtration rate is a hallmark in the extremity of DN. Study suggest that an AGE-RAGE axis activates TGF-β-induced epithelial-mesenchymal transition of normal epithelial cells [220]. Moreover, restricted intake of dietary AGEs in patients with diabetes or kidney disease as well as in healthy subjects also reduces markers of oxidative stress and inflammation [221]. The contribution of AGEs to podocyte EMT was enigmatic until a few years ago. Kumar et al. (2016) demonstrated that podocyte express RAGE and glucose-derived AGEs (particularly CML) binds to RAGE and induce podocyte EMT via a zinc finger transcription factor called ZEB2 [219]. Although endogenous AGEs are implicated in organ dysfunction, the impact of food-derived dietary AGEs on renal biology is not known. The extent of dietary AGEs accumulation in the glomerular region and the consequence of AGEs deposition of podocyte function remains enigmatic.

Hypothesis and objectives

Renal physiology is significantly affected by both intracellular and extracellular AGEs. Diabetic individual shows excess AGEs accumulation and these glycotoxins adversely affect and contribute to the pathology of chronic kidney disease. Recent studies claimed that the association of AGEs accumulation with the pathogenesis of DN, however, the precise mechanisms of AGE toxicity on the molecular basis of the disease is largely unknown. In this doctorial study we have worked on hypothesis that how AGE are associated with injury of podocytes and contributes to the pathogenesis of DN. To study was carried out with following objective:

Objective

1. To investigate the association of AGEs with podocyte injury and glomerulosclerosis in diabetic patients.

2. To investigate the role of AGEs in re-activation of Notch Signaling and EMT in podocytes.

Chapter-II

Materials and Methods

Chapter-II: Materials and Methods

2.1 Materials

2.1.1 Reagents

2.1.2 Antibodies

2.2 Methods

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2.2.22 Nephroseq analysis

2.2.23 Statistical analysis

Materials and Methods:

2.1 Materials:

2.1.1 Reagents

Fetal bovine serum, RPMI 1640, TRIzol reagent, cyclohexylbenzamide (FPS-ZM1) (#553030), N-[N- (3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester (DAPT) (#D5942), N-Benzyl-4-chloro-N-, phalloidin fluorescein isothiocyanate labeled (P5282) and glutaraldehyde (#G5882) were purchased from Sigma-Aldrich (St Louis, Missouri). ProLong Diamond Antifade Mountant (#P36961) was purchased from MPL Technologies. Alexa Fluor 488 or 594 conjugated anti-mouse or anti-rabbit IgG was brought from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). Mouse/Rabbit PolyDetector DAB HRP Brown Detection Kit was purchased from Bio SB (Santa Barbara, California). Dual colour Protein marker, cDNA kit, and quantitative reverse transcription-PCR (qRT-PCR) reagents were brought from Bio-Rad (Hercules, California). Scrambled RNA and small interfering RNA (siRNA) specific to receptor for AGEs (RAGE) and Notch1 were purchased from Kaneka Eurogentec (Belgium).

2.1.2 Antibodies

Anti-AGE antibody (generated in-house in NIN, Hyderabad). Anti-RAGE (#3611), anti-activated Notch1 (#8925), anti-WT-1 (#212951), anti-SIRT1 (#110304), anti-histone H2B (#177430), cleaved caspase 3 (#2302) and Bax (#32503) were obtained from Abcam (Cambridge, Massachusetts). Anti-β-actin (#4970), anti-Notch1 (#3608), anti-E-cadherin (#3195), anti-N-cadherin (#13116), and anti-α-SMA (#19245) were obtained from Cell Signaling Technology (Danvers, Massachusetts). Anti-JAG1 (#PAB807Hu01), Anti-fibronectin (#PAA037Hu01), anti-Col4 (#PAA180Hu01), and anti-vimentin (#PAB040Hu01) were purchased from Cloud-Clone (Houston, Texas). Anti-podocin (#JB51-33) and anti-nephrin (#NBP1-77303) were obtained from Novus Biologicals (Minneapolis, Minnesota).

Anti-α-SMA (#ab5694) was purchased from Abcam (Cambridge, MA).Anti-Hes1 (SC166410) and anti-Zeb2 (#PA520980) were purchased from Santa Cruz Biotechnology (Dallas, Texas) and Thermo Fisher Scientific (Waltham, Massachusetts), respectively. The Goat anti-mouse Alexa Fluor^{TM} 647 crossed-adsorbed (A32728) and Goat anti-rabbit Alexa Fluor^{TM} 488 crossed-adsorbed (A27034) was purchased from Invitrigen.

2.2 METHODS:

2.2.1 Study population and recruitment of subjects

Initially we recruited 1085 study subjects from outpatients attending diabetes specialties centres in Guntur and Vijayawada (Andhra Pradesh, India). All study subjects falls under age range (20 to 80 years) with normal and abnormal kidney function. The subjects were voluntary participated in the study after explaining the nature of study but out of which 172 subjects were refuse to participate. We were use several demanded criteria for the enrolment of subject participants **(Table 3).** After application of all inclusion and exclusion criteria 300 subjects were screened for the further study. These subjects are distributed into three study groups **1)** Non-Diabetic (ND) **2)** Pre-Diabetic (PD) **3)** Diabetic Nephropathy (DN) **(Figure 2.1)**.

Inclusion criteria	**Exclusion criteria**
Age group	Age group
Health status	Haematuria, Non-Diabetic Glomerulopathy
	Hypertension, Obesity
	Other Diabetic complications
	Any medication, supplementation

Table 3: Inclusion and exclusion criteria for study subjects.

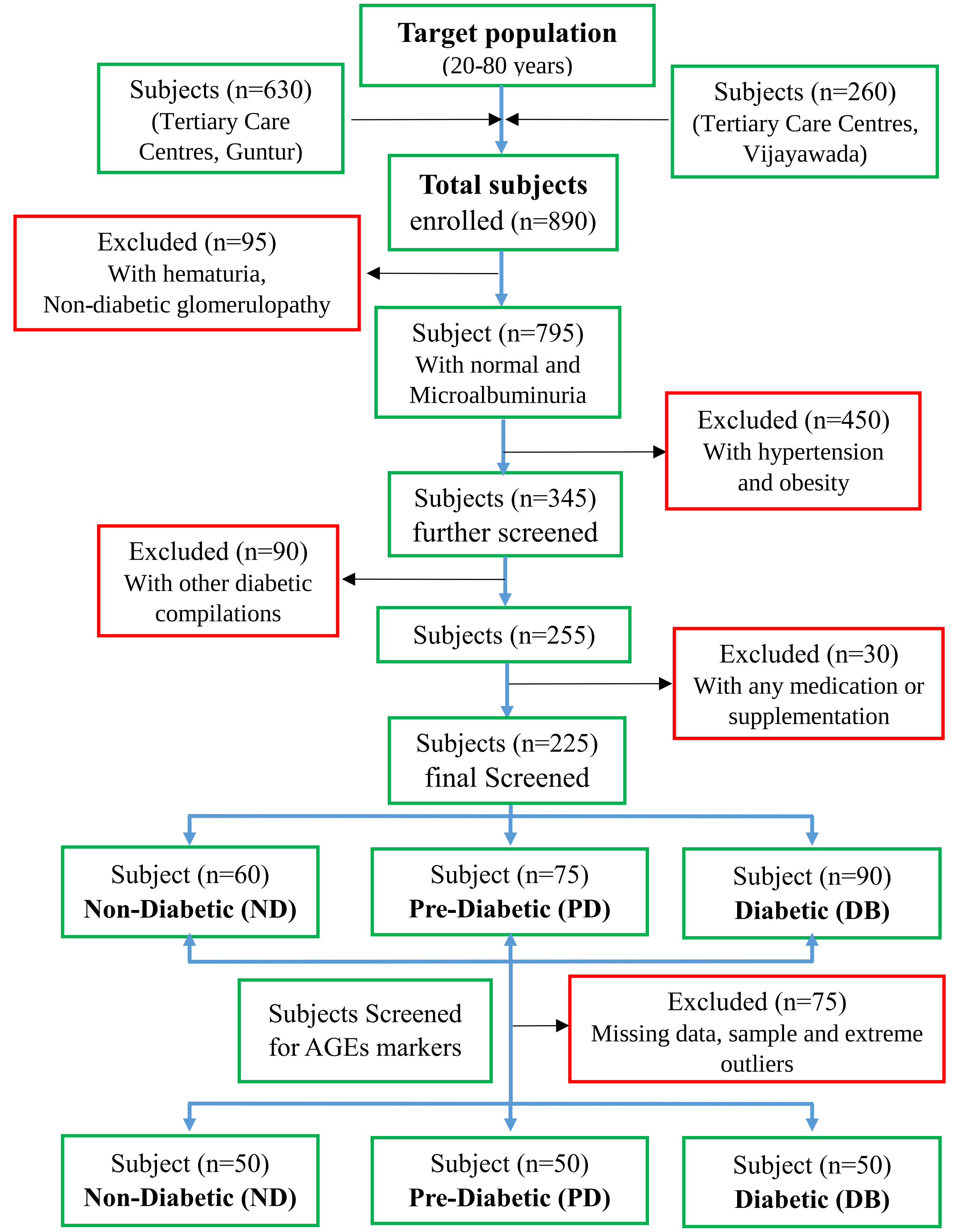

Figure 2.1: Recruitment of study subjects. Flow chart representing the recrutment and screeing strategy to recruit study subjects.

The completely filled Informed consent form was taken from each study subject which enrolled in the study. This study was carried out following "The Code of Ethics of the World Medical

Association (Declaration of Helsinki)" and approved by the Institutional Review Board of Guntur General Hospital, Guntur (GMC/IEC/120/2018).

2.2.2 Examination of clinical parameters

The anthropometric assessments such as height, weight and waist were measured by the staff for the study subjects. Body mass index (BMI) was determined by using the this formula: weight (kg)/ height (m^2). Blood pressure (BP) was estimated in triplicate through Omron digital oscillometer (#HEM7120). Fasting blood glucose (FBG) level was evaluated in blood using a Roche glucometer (Accu-Chek Aviva, R. Diagnostics GmbH, Germany). The whole blood samples (12h fasting and post-prandial) were collected in heparin tubes and immediately centrifuged at 3,500 rpm, 4°C for 20 min to separate plasma and RBC. The AGEs parameters like HbA1c and GA % were analysed in whole blood. HbA1c % were calculated by using an automated boronate affinity based D10 analyzer (Bio-Rad#12010405). GA % were measured by using Lucica GA-L kit (AK pharma, Tokyo) and AU2700 chemistry analyser (Beckman Coulter System Co. Switzerland).

2.2.3 Serum and Urine AGEs detection

The detection of AGEs in serum and urine sample of study subjects by using the protocol which described by Sampathkumar et al. [222]. In brief, the serum and urine sample were sequentially diluted to 1:5, 1:10, 1:20,1:40 &1:50 times in 1X phosphate buffer saline (PBS) and intrinsic AGE-specific fluorescence (λex:370nm and λem:440 nm) was estimated using spectrofluorometer (JASCO-FP-4500). The fluorescence intensity of each dilution is directly proportional to concentration of AGEs, and the increase in fluorescence intensity at each addition of serum/urine sample were plotted on linear regression graph. The slop of curve fitted regression line was considered advanced glycation index (AGI) and represented as arbitrary units (100 units= 1 units of AGI).

2.2.4 Renal filtration parameter (UACR, GFR, eGFR)

The on spot collected urine was used to determine renal filtration status by urine albumin-creatinine ratio (UACR), glomerular filtration rate (GFR) and estimated glomerular filtration rate (eGFR). UACR were measured in both human and mice urine sample by using the commercially available kit for urinary albumin (#COD11573) and creatinine (#COD11502) from Biosystems (Barcelona, Spain). GFR was calculated in mice of different experimental groups by using ELISA based FIT-GFR Test Kit (BioPal, Worcester, USA) for Inulin in serum and the following formula used to calculate GFR calculated (GFR = ((I)/(B/b))/KW (I = amount of inulin delivered by the bolus injection, B = y-intercept, b = decay constant, x = time, and KW = kilo weight of the animal). Estimated Glomerular Filtration Rate (eGFR) of each study subject were calculated by using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation: $\mathrm{GFR} = 141 \times \min(\mathrm{Scr}/\kappa, 1)^{\alpha} \times \max(\mathrm{Scr}/\kappa, 1)^{-1.209} \times 0.993^{\mathrm{Age}} \times 1.018$ [if female] $\times 1.159$ [if black] where Scr = serum creatinine (mg/dL), $\kappa = 0.7$ if females & 0.9 if males, $\alpha = -0.329$ if females & -0.411 if males, min = minimum of Scr /κ or 1, and max = maximum of Scr /κ or 1 [223, 224].

2.2.5 Exogenous AGEs preparation

The preparation of glucose-derived AGEs were performed by using earlier protocol with minor changes [225, 226]. Briefly, we prepared three group of solutions in phosphate buffer of pH 7.6 (1 mM sodium azide in 0.4M phosphate solution) namely only D-glucose (90 mg/mL), only bovine serum albumin (BSA) (100 mg/mL) and solution of D-glucose and BSA. All three group of solutions were prepared in sterile condition and incubated at 37°C for 3 weeks. Dialysis were done for D-glucose-BSA group against phosphate buffer to eliminate the unreacted D-glucose. Formation of glucose-derived AGEs was confirmed (λex: 370 nm and λem: 400–500 nm) and by western blotting with AGE-specific antibody. Extent of glycation of BSA in D-glucose-BSA group was observed using non-tryptophan AGE fluorescence and by measuring free amino groups using 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay (#BC86) according to manufacturer protocol. Briefly, A different concentration of D-glucose-BSA and only BSA (50–200 µg/mL) samples were prepared in reaction buffer (0.1M NaHCO3) followed by the addition of 250 µL of 0.01% TNBS in each sample and incubated for 2hr. reaction were stopped by using mixture of 10% SDS and 1N HCL and absorbance measured at 335 nm. The conformation of AGEs formation in D-glucose-BSA were done by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie stain and immunoblotting using AGE antibody.

2.2.6 Human patient samples

The human kidney biopsies sample were obtained without patient identifiers from pathology lab of Guntur Medical College. Diabetic subject with nephropathy (n = 12) considered as a DN group, in contrast to ND group (n = 12) having non-diabetic subjects. The non-affected part of the kidney tissue was utilized for histological examinations. These kidney biopsy sample where sectioned and used for the different staining studies.

2.2.7 Animal experimentation

C57 black/6J male mice (6–8 weeks old, 30±5 g) were used in this study. These mice were randomly distributed into four groups, viz control (CTL), AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 treatment groups (n=6). The CTL group were injected with an equal volume of phosphate buffer (vehicle), whereas the experimental group injected with intraperitoneal injected with AGEs (10 mg/kg body weight), AGEs+inhibitors DAPT (10 mg/kg body weight), and AGEs +FPS-ZM1 (1 mg/kg body weight) per day for 4 weeks. To check the proteinuria level in mice group, urine was collected each week. After experimental period, we collected 24hr urine to check GFR and UACR. Urine samples which was collected during period loaded on SDS-PAGE followed by silver staining to visualize the proteins level in urine. The perfused kidney where use for other study described below.

All the human and animal study where performed under the Institutional Animal Ethics Committee of University of Hyderabad.

2.2.8 Slide preparation and histological analysis

The kidneys were extracted from mice and the cortex portion was isolated after being perfused with either saline or PBS, followed by 4% paraformaldehyde perfusion. The tissues were fixed with 4% paraformaldehyde for a period of 24 to 48 hours. Afterward, excess fixative was removed by washing the tissues with tap water for 1 to 2 hours. Subsequently, tissues were passed through a series of alcohol gradient changes (70%, 80%, 95% ×1 time and 100% × 3 time, 1hr each) for dehydration. The tissues were then deparaffinised with xylene (2 × 3 time, 1hr each) and then immersed in paraffin with three changes, 1 hour each, and finally embedded in a paraffin block, allowing it to cool under 4°C for 24 hours. Later 3-5µm thick paraffin sections were made by Leica microtome and placed into a 40°C water bath, followed by the transfer of sections on to pre-coated glass slides. The slide were allow to dry for overnight at 37°C. The slides were subjected to different staining like Periodic-acid Schiff Base (PAS), Masson's trichrome (MT) to find glomerulosclerosis, and Haematoxylin and Eosin (H&E) to check altered morphology as described earlier 30387200.

2.2.9 Immunohistochemistry (IHC) and staining

IHC is a staining technique used to detect the expression of various protein in tissue section slides. We have used three different staining protocol namely: Immunofluorescence (Alexa Fluor 2° antibody used as stain), 3,3′-Diaminobenzidine (DAB) staining and specific WT1 staining for podocytes. IHC steps as follow; 1) Deparaffinization: The slides were heated at 70°C for 45min to remove extra paraffin and allowed to come to room temperature. Next, slides were kept in xylene (3 times × 5 min each) to remove paraffin. 2) Rehydration: In this step slides were incubated in different % of ethanol as follow 100% (3 times × 5 min each), 96% (2 times × 3 min each), and 70% for 3 min continuation with ddH2O washing (3 times × 5 min each). 3) Endogenous peroxidase blocking: In tissue sections high peroxidase activity were blocked by incubating with 3% peroxidase solution in methanol for 20 min followed by Phosphate buffer saline (PBS) washing (3 times × 5 min each). 4) Antigen retrieval: To unmask the antigen epitope slides were boiled in 10mM citrate buffer pH-6.0 for 12 min with every 3min interval and allow it to cool down followed with PBS washing (3 times × 5 min each). 5) Permeabilization: In this step slides were incubated with 0.4% Triton X-100 in PBS for 10 min followed with PBS washing (3 times × 5 min each). 6) Blocking: The slides were blocked by incubating with 3% BSA in PBS for 1hr followed by gentle washing with PBS to just remove BSA 7) Primary antibody (1° Ab) incubation: The slides where incubated with 1° Ab prepared in 3% BSA in the range of 1:100 to 1:200 (depend on Ab specificity) followed with PBS washing (3 times × 5 min each). 8) Secondary antibody (2°Ab) incubation: After cleaning, slides were incubated with Alexa Fluor 2°Ab (1:200 in 3% BSA) and PolyDetector HRP 2°Ab for 1hr in Immunofluorescence and DAB staining respectively followed with PBS washing (3 times × 5 min each). 9) Mounting and Imaging: In case of immunofluorescence DAPI mount was used to mount slides and covered with coverslip. In case of after 2°Ab slide were incubated DAB substrate for till browning and washed with PBS (3 times × 5 min each). Next, slides were allow for dehydration steps by using ethanol (90%, 100%, 100% × 5 min each) and dry for 2min follow with mounting with DPX (Dibutylphthalate Polystyrene Xylene) mountant. Imaging were done by using with leica microscope trinocular.

WT1 staining is type specific staining for podocytes shared steps (1-3) with IHC. The antigen retrieval were done like IHC by using ddH2O with unmasking solution (Vector H-3300) in place of citrate buffer. The slides were blocked by incubation with 1-3 drop of avidin (Vector Sp-2001) depend on the size of section in humidified chamber for 15min followed by gentle wash with PBS for 5min. Next, incubated with ant-mouse IgG (1:50 in 1X PBS) for 1hr at RT. Later slides were incubated with 1°Ab (WT-1NBP2-44607; 1:100 in skimmed milk) at

4°C for 6-8hr. Next, Slides were incubated with 2°Ab (anti- mouse IgG1B-406603 for Biolegend; 1:100 in skimmed milk) for 30min in humidified chamber. Each step were followed by washing with PBS (3 times × 5 min each). In next step, slide were incubated with few drop of ABC solution (Avidin / Biotin Complex; Vector PK-6100) for 30 min in humidified chamber followed by 1 time PBS and tris for 5min. Colour were developed by using pre-heated DAB solution (Tris buffer+DAB+Nikel chloide) at 37°C for 3 min followed by counterstaining with methyl green for 6min. The slide were quickly decoloured by rehydeating with with inceasing concentration of ethanol 2 time 96%, 2 time 100% and Xylene each for 2 min and mounded with VectaMount followed by imaging with leica microscope trinocular.

2.2.10 Morphological analysis and TEM imaging

Renal cortex from experimental mice kidney and human biopsy tissue samples were fixed by using glutaraldehyde (2.5% in 1X PBS) for 24hr at 4ºC. Samples were washed with 1X PBS (3 x 10 min), followed by fixation with 1% osmium tetroxide in PBS for 2hr at 4ºC and washing with distilled water (3 x 15 mins). Thin sections (60 nm) were mounted on 200 mesh copper grids and allowed to air dry for 2 hrs to overnight. Later, these copper grids were stained with 3% aqueous solution of uranyl acetate followed by 3% solution of lead citrate. Images were taken by using a JEOL JEM-1400 Electron Microscope (Japan).

2.2.11 Culturing of human podocytes

The conditionally immortalized human podocytes cells (HPC), was acquired from Prof. Moin Saleem (University of Bristol, Bristol, UK). The culturing of these cells were done by using previously described protocol [227]. Briefly: HPC were allow to propagate under permissive temperature 33°C with 5% CO_2 in medium (RPMI-1640 + 10% FBS + 1% of mixture of penicillin-streptomycin + 1% insulin-transferrin-selenium solution). When the cells reached approximately 60% confluence (For immunoblotting) and 30-40% confluence (For immunofluorescence), they were transferred to 37°C to induce non-permissive conditions for

10-13 days. This step was necessary to inhibit the temperature-sensitive SV40 antigen and promote its differentiation. After reviving each vial of frizzed cells, the 3 to 5 passage number cells were used for experiment. The 13th day differentiated podocytes were treated with AGEs in different concentration (25–200 µg/mL) for different time (0–72 hours) with or without chemical inhibitors: DAPT (5 µg/mL) and FPS-ZM1 (1 µg/mL). After completion of treatment cells were used for different experiment.

2.2.12 Lysate preparation and Protein estimation

After the completion of treatment time, the petri-dish with podocyte cells were washed with ice-cold PBS. The cells were scraped with 1ml PBS and collected in centrifuge tube followed by harvesting it at 6000 rpm for 5 min. Next for the cell lysis, RIPA buffer (150mM NaCl, 1% NP-40, 0.1% SDS, 2 µg/mL aprotinin, 1mM PMSF) was added into the cell pallet and kept it for rotation at 50 rpm for 30 min on rotospin. These tubes were centrifuged at 13,500 rpm for 25 min and the supernatant were collected in a fresh tubes. The cell and glomerular lysates were used for the estimation of total protein in the lysate by standard bicinchoninic acid assay (BCA) and intensity measured at 562nm. Excel calculations were done to know the protein concentration µg/µl in each sample followed by the sample preparation for the SDS-PAGE.

2.2.13 siRNA interference and Transfection

Transfections of siRNA (against RAGE, Notch1 and Non-silencing control) were carried out using Lipofectamine RNAiMax Reagent (Life Technologies) as per the manufacturer's instructions. After sixteen hours of transfection, the HPC cells were exposed to AGEs for 48 hours and processed for mRNA and western blotting.

2.2.14 SDS-PAGE and western blotting

The different % of SDS-PAGE gel (8-15%) were casted which depend on the molecular weight of protein. An equal amount of pre-wormed protein samples were loaded on gel. The gel was run in reducing condition at 70V till stacking region and shifted to 90V for 2hr. Later, gel was transferred to nitrocellulose membranes by electroblotting in transfer buffer at 50V for 90 min. The membranes were soaked in blocking buffer 5% non-fat milk for 1h at room temperature. Subsequently, blots were incubated with primary antibodies (1:1000 or 1:500) for 6-8hr at 4°C followed by washing with 1X TBST (3 time x 5 min). Next, blots were incubated with secondary antibodies (1:10000, anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase) incubation for 2hr at room temperature followed by washing with 1X TBST (5 time x 5 min). Blots were developed using the ECL substrate (#1705060, Bio-rad, Hercules, CA, USA) and visualized under Bio-Rad Versa Doc 5000 MFP.

2.2.15 Coomassie brilliant blue and Silver staining

The protein/AGEs in urine or serum sample were run on 10% SDS-PAGE gel and visualized by the coomassie brilliant blue (CBB) and the silver staining (depending on the amount of protein). The SDS-PAGE gel were stained by the CBB solution for 1hr at RT. Later, gel was distained with distaining solution (2 time x 1hr) until protein bands are visualized. Silver staining were used to detect less amount of protein (0.05-0.2 ng) in sample. Silver staining were done in following steps: 1) immediately after running, gel were washed with water and dipped into fixative solution (50ml methanol, 40ml water, 10ml glacial acetic acid and 100ul of formaldehyde) for 1hr; 2) gel were submerged in 50% ethanol for 40 min; 3) later gel where incubate with sensitizing solution (10 mg thio sulphate in in 50ml distilled water) for 1 min to sensitize the protein followed by washing with distilled water (3 time x 30sec); 3) next gel were submerge in staining solution (100mg silver nitrite, 26µl of formaldehyde in 50ml distilled water) for 30 min followed by washing with distilled water (3 time x 30sec); 4) gel were dipped in developing solution (3g of sodium carbonate, 36µl of formaldehyde 50ml distilled) for 30 sec to 1 min to develop band and stop immediately with few drops of glacial acetic acid.

2.2.16 mRNA isolation and cDNA preparation

Total RNA was isolated by the Trizol method as manufacturer's protocol, Sigma. Briefly, cells were collected and washed with ice-cold PBS. Trizol was added to the cell pellets and incubated for 5min at room temperature. 0.2mL of chloroform was added and mixed vigorously. Samples were spun down at 12,000 rpm for 15min at 4ºC and collected the upper transparent layer into a fresh tube. RNA was precipitated by adding half of the trizol volume of propanol to the samples, mixed gently and left it for 10min at room temperature. Samples were then spun down at 10,000 rpm for 20min at 4ºC. RNA pellets were washed with 75% ethanol. Then, the pellets were dried at 37ºC for 30min and resuspended in DEPC-treated water. The total RNA concentration was estimated by nanodrop and one microgram of RNA was used as an input for cDNA synthesis.

2.2.17 Real-Time Quantitative Reverse Transcription (qRT-PCR)

The total transcripts were extracted by using Trizol reagent (Invitrogen, Waltham, MA, USA) and isolated using an RNA isolation kit (Qiagen, Hilden, Germany). We examined the RNA quality on 2% agarose gels and quantification performed by NanoDrop (#ND2000LAPTOP, USA). Next, 1 μg of total RNA was reverse transcribed using the cDNA synthesis kit (Thermo Fisher Scientific). qPCR analysis was performed by QuantStudio 3 system (Applied Biosystem) with the SYBR Green (Kappa biosystems, USA) Master Mix using three-step standard cycling conditions with sequence-specific primers. To ensure a single PCR product was amplified, we examined the melting curve for each primer. mRNA expression of each gene was normalized using the expression of β-actin as a housekeeping gene.

2.2.18 Immunofluorescence

HPC were cultured on coverslips to reach 80% confluent, fixed with 4% paraformaldehyde. Then, cells were permeabilized in with 0.25% Triton X-100 in PBS. Cells were blocked with 5% BSA in PBST for 1 hr at room temperature. Primary antibody was prepared with 3% BSA in PBS and incubated in a humidified chamber at 4ºC for overnight. Followed by primary antibody incubation, cells were washed several times with PBST and incubated with the secondary antibody prepared in 5% BSA for 2 hr at room temperature in dark. Then, the cells were washed with PBS several times and mounted on a glass slide using DAPI with anti-fade and left slides for 12 hr at room temperature. Imaging was done in Leica trinocular fluorescent microscope under 60x oil objective.

2.2.19 Migration assay

The phenomenon of EMT in podocyte cells was assessed using a "wound-healing" migration assay. A confluent monolayer of podocytes in 6-well plates was wounded with 10 µl pipette tip following two perpendicular diameters, giving rise to two acellular clear areas per 47 well. After washing with PBS, podocyte cells were treated with AGEs, AGEs+DAPT, AGEs+FPS-ZM1 and incubated for 12h, 24h, 48h and 72h. Photographs were taken at different times of incubation for AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 along with CTL (after 0h, 12h, 24h, 48h, and 72h). The extent of migration at these time points was determined by image analysis (ImageJ software) in both control and treated wells.

2.2.20 Albumin influx assay

The Albumin influx assay was conducted in accordance with previously outlined methods [228]. In brief, HPC were cultured on collagen-coated transwell filters in a 6-well plate and allowed to undergo differentiation for a period of 12 days. Subsequently, the cells were treated with or without AGEs, AGEs + DAPT and AGEs + FPS-ZM1 to 48hr. Following this, the media was aspirated, and the cells were rinsed twice with a solution containing 1mM MgCl2 and 1mM CaCl2 to preserve cadherin junctions. In the lower chamber, 2 ml of RPMI 1640 medium supplemented with 40 mg/ml BSA was add, whereas in the upper chamber, only RPMI 1640 was added. Protein content in the medium collected from the upper chamber at various time points was quantified.

2.2.21 Phalloidin staining

Phalloidin staining were used to stain for the F-actin to observe the arrangement of stress fibres in differentiated podocytes as described earlier [229]. In summary, the cells were fixed with 4% paraformaldehyde at RT for 15 minutes. After washing, they were permeabilized for 15 minutes using 0.3% Triton X-100 in PBS, followed by blocking with 5% BSA. Next, the cells were treated with rhodamine-phalloidin (Invitrogen Corp., CarlsbadCA, USA) for 15 minutes at RT to stain F-actin. Confocal microscopy was employed to examine the slides. Fifty cells per group were assessed to determine the ratio of cells retaining distinct F-actin fibres.

2.2.22 Nephroseq analysis

Nephroseq is a web-based systems biology search engine. It provide platform to renal research community to analyze gene expression and data mining. Nephroseq contains around 2600 samples from 5 renal tissues across 16 chronic kidney diseases. There are two tools that can be used for data interrogation. First tool allows the retrieval of tissue specific gene expression. The second tool allows advanced analysis linking it to various systems biology tools. In our case we studied expression of several marker including AGEs-RAGE signaling.

2.2.23 Statistical analysis

All data are given as mean±SD. The statistical significance between two groups was done by using Student’s t-test and p value ($p \leq 0.05$) taken as significant. All graph and their statistical analyses were carried by using GraphPad Prism V 8.0.1 (244). Correlation analysis between two parameters were carried out by using Pearson`s correlation coefficient with R program (V 4.0.3).

Chapter-III

To investigate the association of AGEs with podocyte injury and glomerulosclerosis in diabetic patients

Chapter-III: To investigate the association of AGEs with podocyte injury and glomerulosclerosis in diabetic patients.

Contents

3.1 Introduction:

Diabetes has long been a growing epidemic, and Asia accounts for 60% of the world's diabetic population [219, 230]. The increased prevalence of diabetes led to a surge in macro and microvascular complications such as visual impairment, coronary heart disease, stroke, neuropathy, and diabetic nephropathy (DN). DN is a chronic disease that accounts for 44% of new end-stage kidney disease (ESKD) cases, with 6% attributed to type I and 38% attributed to type II diabetes [231]. It was projected that an increase of 3 million DN cases over 20 years [231]. DN clinical manifestations include glomerular transient hyperfiltration, proteinuria, kidney hypertrophy, fibrosis, and decreased glomerular filtration rate (GFR) [232]. In healthy individual, GFR decreases with the increasing age. The early DN stage, a patient shows hyperfiltration, represented by a rise in GFR and occasional microalbuminuria (ratio of urine albumin to creatinine ≥ 30 mg/g) [232]. The DN's progressive stage is represented by a gradual decline of the GFR, persistence of microalbuminuria, and subsequent macroalbuminuria (ratio of urine albumin to creatinine ≥ 300 mg/g). The advanced DN stage is characterized by severe proteinuria and chronic kidney insufficiency that ultimately manifest in ESKD. Both albuminuria and impaired GFR are the strongest predictors of progression to ESKD in patients with diabetes. Biomarkers play an essential role in the early detection of DN and its progression to ESKD, whereas microalbuminuria is one of the predominant markers [233].

Microalbuminuria also indicates generalized endothelial dysfunction and suggests kidney involvement with cardiovascular and cerebral impairment [234]. Microalbuminuria is considered an early stage of, rather than a predictor of, DN and subsequent kidney impairment. Furthermore, microalbuminuria reflects not only glomerular injury but also tubular lesions [233]. Although microalbuminuria is accepted to indicate potential renal damage, it is neither an accurately predict progression of diabetic kidney disease nor reliably be used to track response to therapy [235]. Regression from microalbuminuria to normoalbuminuria is more likely than progression toward overt proteinuria [236]. There is a growing need for a more dependable and reliable biomarker for both predicting and tracking DN. Among the myriad hemodynamic, metabolic, and inflammatory factors that participate in DN's pathogenesis, persistent hyperglycemia is predominant. It is noteworthy that a strong relationship between poor glycemic control and DN exists [237, 238].

Prolonged hyperglycemia ensures the formation of AGEs in the kidney and other sites of diabetic complications [218]. AGEs comprise heterogeneous compounds formed during a series of nonenzymatic glycation reactions between the amino group of proteins, lipids, and nucleotides with reducing sugars [48, 239, 240]. DN patients with macroalbuminuria and patients on hemodialysis had significantly higher serum AGEs than those with microalbuminuria [241]. One of the extensively characterized AGEs is carboxymethyl-lysine (CML) and is being used as markers for in vivo formation of AGEs [44, 239, 242]. CML has been used as a biomarker for long-term protein damage. Elevated tissue CML concentrations are associated with kidney and retinal complications in patients with diabetes [243, 244]. In the case of DN, early screening and evaluation of the kidney injury may help assess the risk of ESKD and strategizing the therapeutic regimen. Although glycated hemoglobin (HbA1c) has proven to be a reliable prognostic marker in the general diabetic population, it may not be valid in patients with diabetes and chronic kidney disease [245]. It is debated whether HbA1c corresponds to the same mean glucose concentrations in people with ESKD [245, 246]. Further, HbA1c is influenced by several factors, including the RBCs' lifespan, administration of erythropoietin, uremic environment, and blood transfusions [245, 247, 248]. In contrast, glycated albumin [249] is suggested as a preferred marker for assessing glycemic control in advanced chronic kidney disease only [245].

According to UK prospective diabetes study, intensive blood-glucose control in patients with type II diabetes reduces microvascular complications, particularly in patients with a diabetic kidney disease whose cardiovascular risk increases with worsening proteinuria [247, 250]. Therefore, a biomarker that could predict impaired kidney function in patients with poor glycemic control and microalbuminuria would help manage DN effectively. Accumulation of serum AGEs in DN is due to increased accumulation and decreased elimination by the kidney [241]. We examined serum and glomerular AGEs association with glomerular injury and macroalbuminuria in patients with DN. Our study identified glomerular CML levels correlate significantly with epithelial-mesenchymal transition (EMT) of glomerular podocytes and glomerulosclerosis in patients with DN.

3.2 Results

3.2.1 The poor glycaemic control and AGEs indicators (GA and HbA1c) are associated with the loss of kidney function in diabetic subjects.

The clinical parameters of non-diabetic (ND), pre-diabetic (PD) and diabetic (DB) subjects are given in **(Table 4)**. The mean age of 90 patients was 50 ± 11 years, BMI 28.9 ± 5.3, fasting blood sugar (FBS) 171.3 ± 37.4, duration of diabetes 10 ± 3.4, Serum creatinine 2.9 ± 0.9 **(Table 4)**. We were examined the FBS and albuminuria level in study subject and found that their level are high in DB in compare to ND and PD **(Figure 3.1A-B)**. As we known that estimated GFR (eGFR) defines kidney's function [251]. So, we measured eGFR level and found gradual decrease for ND, PD to DB subject **(Figure 3.1C)**. In diabetes condition, the poor glycaemic control is also determine by the excess amadori poducts like HbA1c and GA (Glycated Albumin) [252] which act as AGEs indicator [240, 253]. After estimation we were found increased level of HbA1c (10.2 ± 2.4) and GA (24.6 ± 4.2) in diabetic subject then PD, ND **(Figure 3.1D-E)**. Together data suggested that poor glycaemic control and excess AGEs indicator in diabetic subject associated with loss of kidney function.

Parameter (unit)	ND	PD	DB
Age (yrs)	48 ± 11	53 ± 12	50 ± 11
BMI (Kg/m²)	27.4 ± 4.3	28.1 ± 4.9	28.9 ± 5.3
Known duration of diabetes (yrs)	N/A	2 ± 0.6	10 ± 3.4
Known duration of proteinuria (yrs)	N/A	1.2 ± 0.9	2.5 ± 2.8
Fasting glucose (mg/dL)	90.8 ± 5.7	112.5 ± 6.6	171.3 ± 37.4
Systolic blood pressure (mm Hg)	127.8 ± 15.5	126.7 ± 15.2	127.8 ± 18.4
Diastolic blood pressure (mm Hg)	80.4 ± 6.2	79.2 ± 8	81.3 ± 7.9
Serum creatinine (mg/dL)	0.58 ± 0.3	0.89 ± 0.3	2.9 ± 0.9
Albumin (mg/24h)	16.7 ± 8.9	20.9 ± 12.4	111.4 ± 46.6
eGFR (ml/min/1.73m2)	103.2 ± 13.2	76.8 ± 11.9	45.30± 14.4
HbA1c (%)	5 ± 0.5	6 ± 0.2	10.2 ± 2.4
GA (%)	9.8 ± 2.3	15 ± 2.3	24.6 ± 4.2

Table 4: Clinical parameters of the study subjects. Table represent the clinical parameters chosen in study subjects. The number of study subjects in groups as Normal (n=60), Pre-diabetic (n=75) and Diabetes (n=90) but for parameter like eGFR, HbA1c, GA (n=50) in each group.Data presented as mean ± SD. $p < 0.001$.

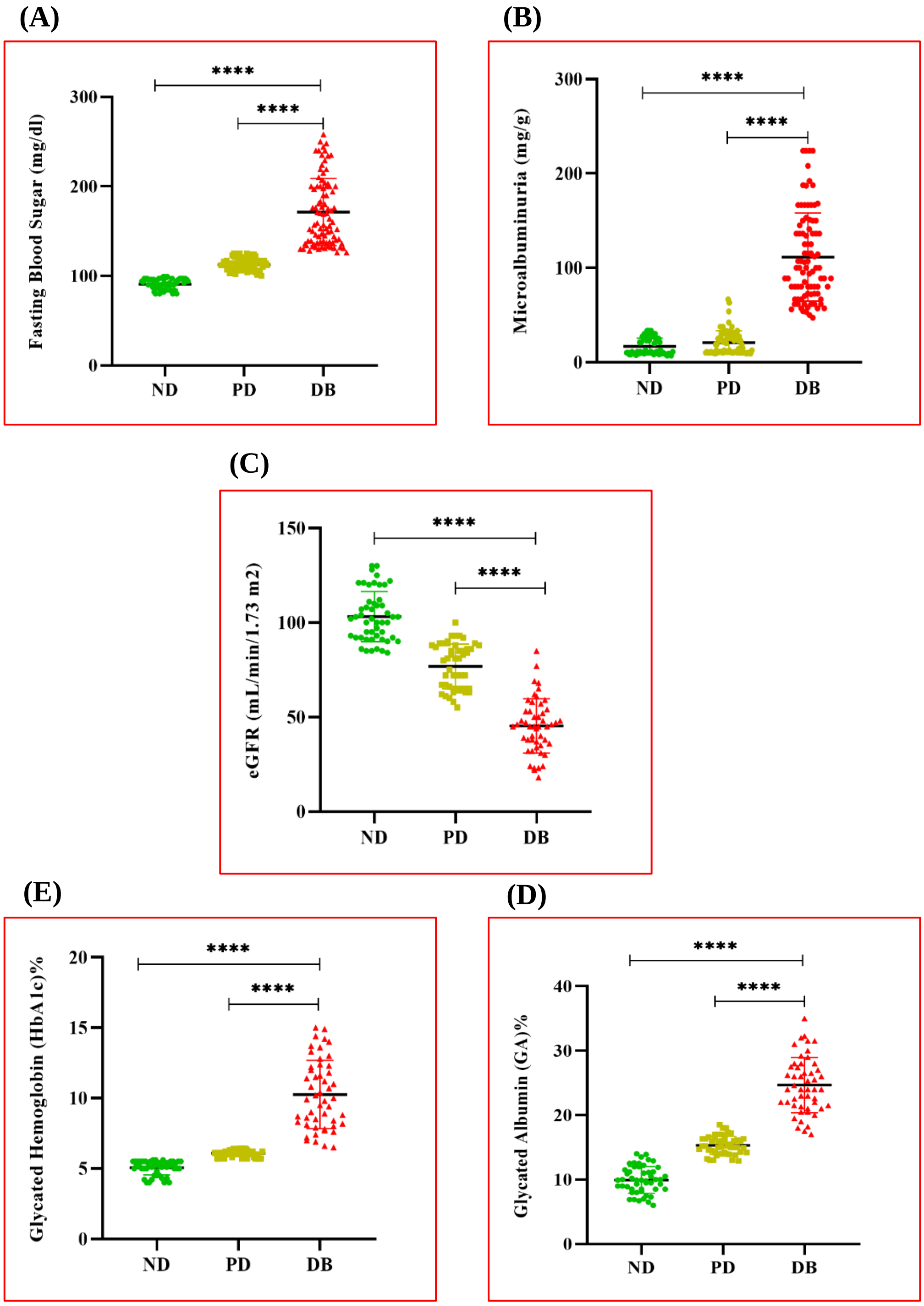

Figure 3.1: Analysis of glycaemic parameters in diabetic subjects. (A) Fasting Blood glucose **(B)** microalbuminuria status **(C)** eGFR, **(D)** HbA1c, and **(E)** GA was measured in ND, PD and DB groups. (n = 50) in each group. ****p < 0.0001. Data were expressed as the mean ± SD, and statistical significance is calculated by using the student t-test.

3.2.2 Accumulation of AGEs is associated with proteinuria and loss of kidney function.

Diabetes is presented with accumulation of AGEs in body fluids and tissues [240]. Therefore, we measured the serum and urinary AGEs by detecting the intrinsic AGE-specific fluorescence with fluorimeter (described in methodology). The slope of regression line of fluorescence intensity of study subjects was computed as AGE index (AGI) and presented as arbitrary units. Interestingly, we found significantly high AGI in DB then ND subjects **(Figure 3.2A-B)**. Increased level of CML were found in serum and urine of diabetic patients [254]. Further, the extent of AGEs in serum sample by Coomassie Blue Staining of SDS-PAGE and immunoblotting using an anti-CML antibody **(Figure 3.2C-D)**.

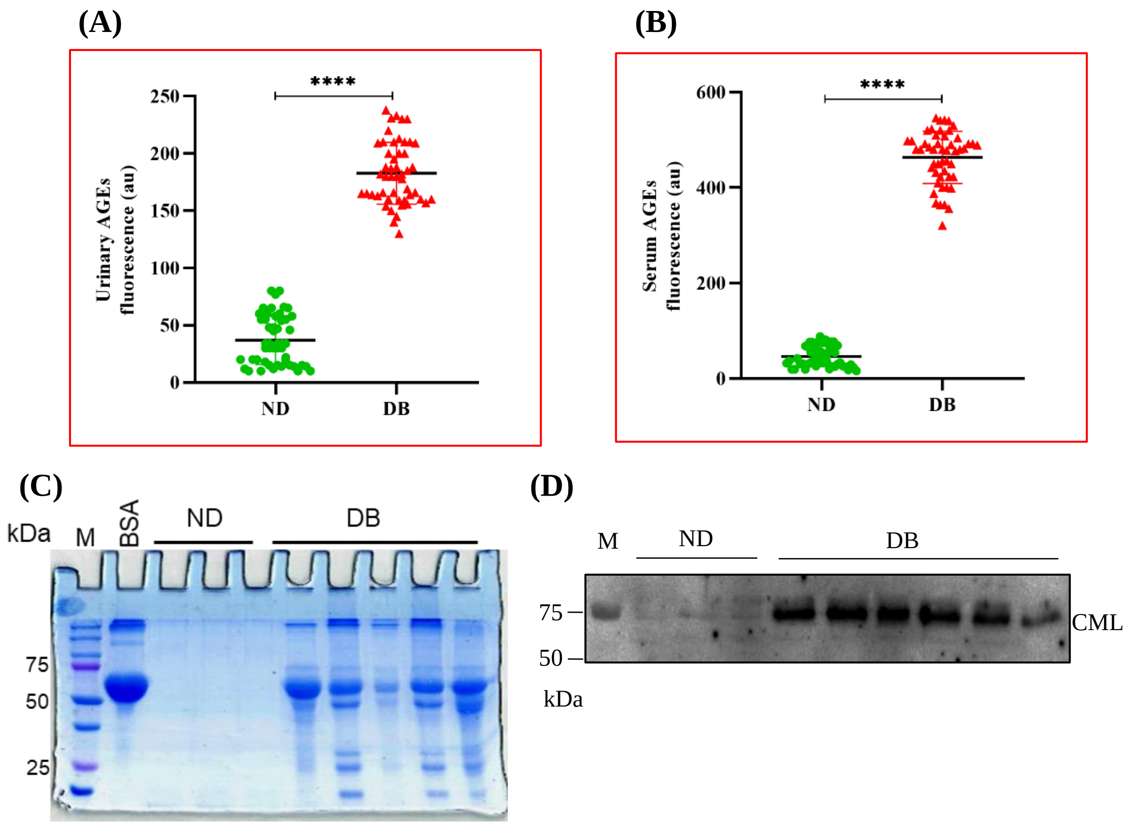

Figure 3.2: Elevated AGEs in serum and urine of diabetic subjects. (A) Urinary AGEs fluorescence **(B)** Serum AGEs in (ND), (PD) and (DB) groups. ****p < 0.0001, (n = 50). Data are presented as the mean ± SD, and student t-test were used to determine statistical significance between groups. **(C)** Coomassie brilliant blue staining of SDS-PAGE gel showing pattern of AGEs in serum sample of ND (n=3) and DB (n=5) group **(D)** Western blot analysis of CML in ND and DB groups.

Together data suggested that presence of excess AGEs in serum and urine associated with proteinuria with loss of kidney function.

3.2.3 Glomerular CML correlated with decreased podocin expression and podocyte foot process effacement.

The elevated level and its accumulation of CML were found in diabetic kidneys and glomeruli [255]. In diabetic patients, we found that the accumulation of CML in glomerulus **(Figure 3.3A)**. According to an earlier study, accumulation of CML in glomeruli was proportional with decreased podocyte count [48]. Therefore, we stained glomerular sections from DB subject with anti-podocin and found decreased expression of podocin **(Figure 3.3B)**. Stastiscal analysis shows inverse correlation between glomerular CML staining with decreased expression of podocin **(Figure 3.3C)**. Depletion of podocin leads to podocyte foot-process effacement [256, 257]. TEM analysis data show podocyte foot-process effacement in DB subject **(Figure 3.3D)**. Above data suggest that excess AGEs, particularly CML related with decreased podocin expression and podocyte foot-process effacement in DB subjects with nephropathy.

3.2.4 Excess of glomular CML is associated with loss of E-cad and podocyte EMT.

Since excess glomerular CML correlates with decreased podocin number, we next ascertained the mechanisms of podocyte depletion in diabetic patients. An earlier study from our group reported that podocytes undergo EMT in mice administered with CML [219]. Therefore, we investigated whether EMT occurs or not in glomeruli from DN patients. We performed immunostaining for E-cadherin (E-CAD, a bonafide marker of epithelial phenotype) and found that its expression was significantly decreased in DN patients **(Figure 3.4A)**. A negative correlation was observed between decreased E-CAD expression and the accumulation of glomerular CML staining **(Figure 3.4B)**.

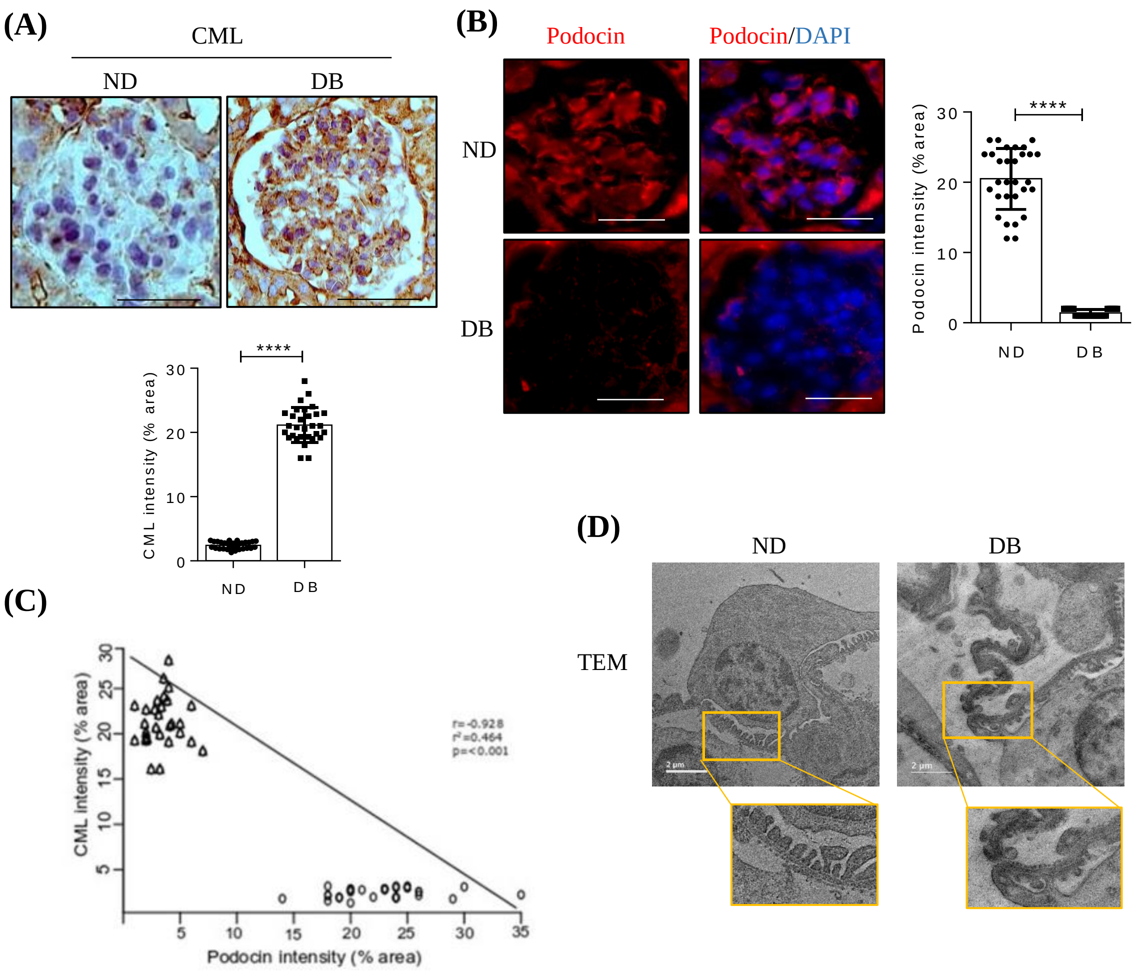

Figure 3.3: Glomerular CML is associated with podocyte injury. **(A)** DAB staining of glomerulus section with anti-CML Ab shows more CML in DB groups then ND (Left). Dot plot represent the quantification of % area per field from glomerulus. **(B)** Immunofluorescence analysis of podocin in the glomerulus of ND and DB section (Left). Dot plot represent the quantification of % stained area per field from glomerulus. Scale bar = 20μm. In dot plot each data point represents the average value of 6 glomeruli. ****P < 0.0001. The intensity of glomerular expression of CML and podocin was quantified using ImageJ. **(C)** Correlation analysis of CML vs. podocin staining intensity in the glomerulus of ND and DB groups. Correlation is significant at the 0.001 level (2-tailed). **(D)** TEM images showing the foot process effacement of podocyte (zoomed area) in ND and DB biopsies. Scale bars indicate 2μm.

The data derived from Nephroseq also corroborated with our observation that in DN, decreased expression of epithelial marker (E-CAD/CDH1), (Occludin /OCLN), and increased expression of mesenchymal marker (N-cadherin/CDH2) and transcription factors that elicit EMT

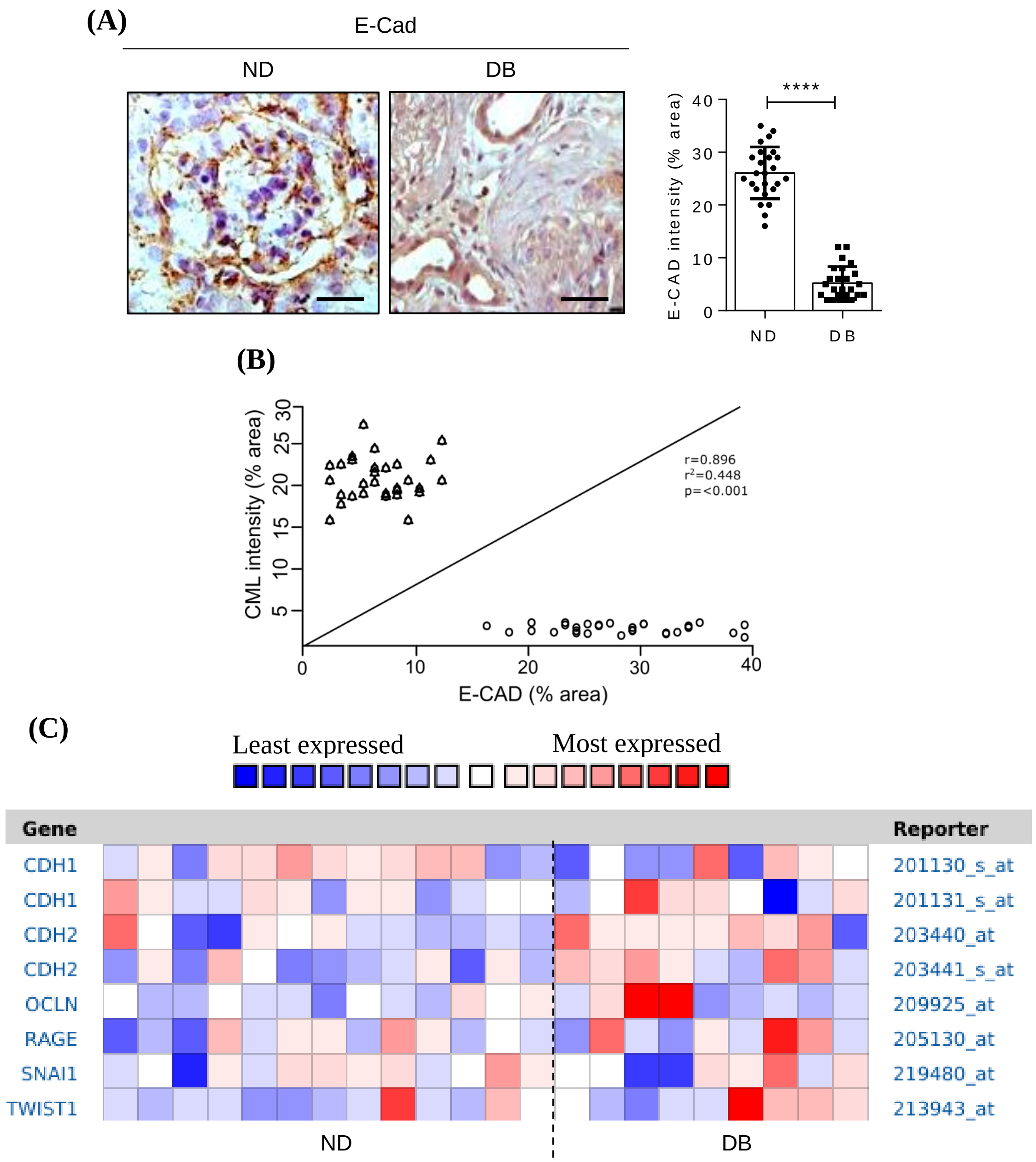

Figure 3.4: Glomerular CML associated with decreased epithelial markers. **(A)** IHC analysis of E-cad (CDH1) in the kidney biopsies sections from ND and DB groups (Left). Scale bar = 20µm. The stained area (%) per field from glomerulus was quantified, and each data point represents the average value of 6 glomeruli from ND and DB both the groups (Right). ****P < 0.0001. The intensity of glomerular expression of E-cad was quantified using ImageJ. **(B)** Correlation between CML vs. E-cad expression in the glomeruli from ND and DB biopsy samples. Correlation is significant at the 0.001 level (2-tailed). **(C)** Nephroseq analysis comparing CDH1, CDH2 (N-cad), RAGE, OCLN (Occludin), SNAI (Snail) and TWIST expression levels in non-diabetic (n = 13) vs. diabetic individuals (n = 9) data set from Woroniecka diabetic glomerulus dataset. Data are the mean ± SD, and statistical significance is calculated by using the student t-test.

phenomenon such as SNAI1 and TWST1 (**Figure 3.4C**). Nephroseq data also revealed the upregulation of receptors for AGE (RAGE) in DN patients **(Figure 3.4C)**. H&E staining and

TEM imaging revealed detached podocytes in glomerular space (arrow mark) of DN patients **(Figure 3.5A-B)**. Together the data suggest that excess glomerular CML associated with E-cadherin loss and podocyte EMT in DB subject with nephropathy, which might be responsible for the observed detached phenotype.

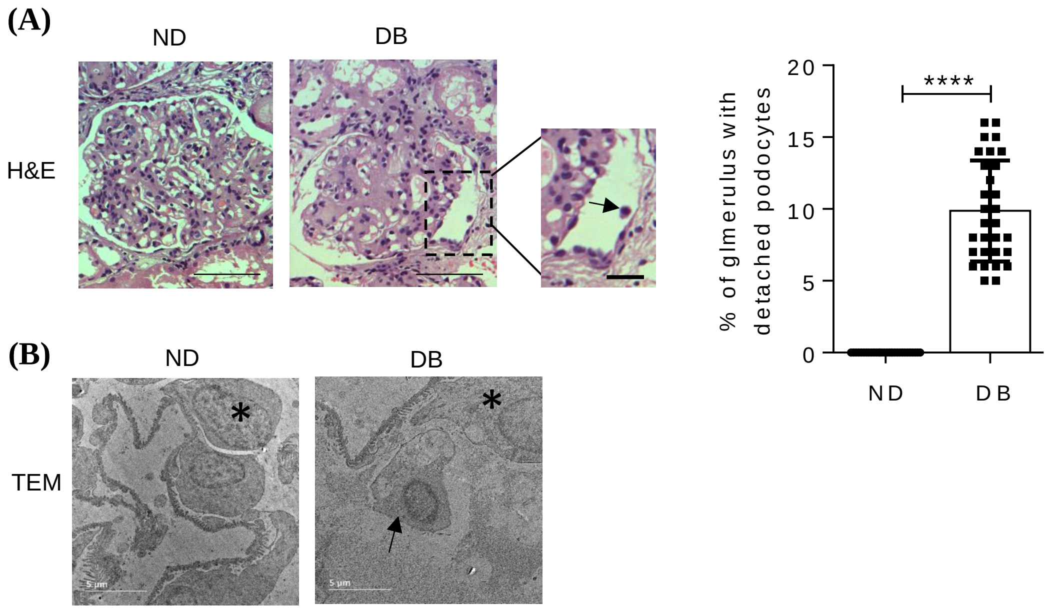

Figure 3.5: AGEs contributes to podocyte injury. (A) H&E staining in glomeruli from ND and DB kidney biopsy samples. A zoomed image representing the detached podocyte (black arrow). Scale bar = 20µm. Detached podocytes were quantified (%), and each data point represents the average value of 6 glomeruli from n = 30 kidney biopsy samples in ND and DB groups. ****P < 0.0001. (Right) **(B)** TEM images of podocyte from ND vs. DB showing the position of podocyte. The attached and detached podocytes from GBM indicated by asterisks and the black arrow.

3.2.5 Glomerular AGEs and loss of podocytes are associated with glomerulosclerosis.

It demonstrated the correlation of decreased podocyte count with the onset of proteinuria and glomerulosclerosis [219]. Since these podocytes counteract the outward forces of glomerular pressure and help maintain the capillary loop’s shape, depletion of podocytes leads to bulging of the GBM [258]. Additionally, the denuded GBM form a synechia attachment with the parietal epithelial cells and Bowman’s capsule, which is thought to ensure focal segmental glomerular sclerosis (FSGS). Since we observed decreased podocyte count in diabetic patients, we assessed the extent of fibrotic changes in the kidney sections. As anticipated, PAS and MT staining revealed significant fibrotic changes in the glomerular region concomitant with a high glomerular injury score **(Figure 3.6A)**. Expression of fibrotic markers such as α-SMA, Col 4, and fibronectin was upregulated in these injured glomeruli as evidenced by Immunostaining and quantification **(Figure 3.6B)**. Nephroseq analysis of DN patients also revealed elevated expression of fibrotic markers **(Figure 3.7)**. Both our experimental and Nephroseq data suggest

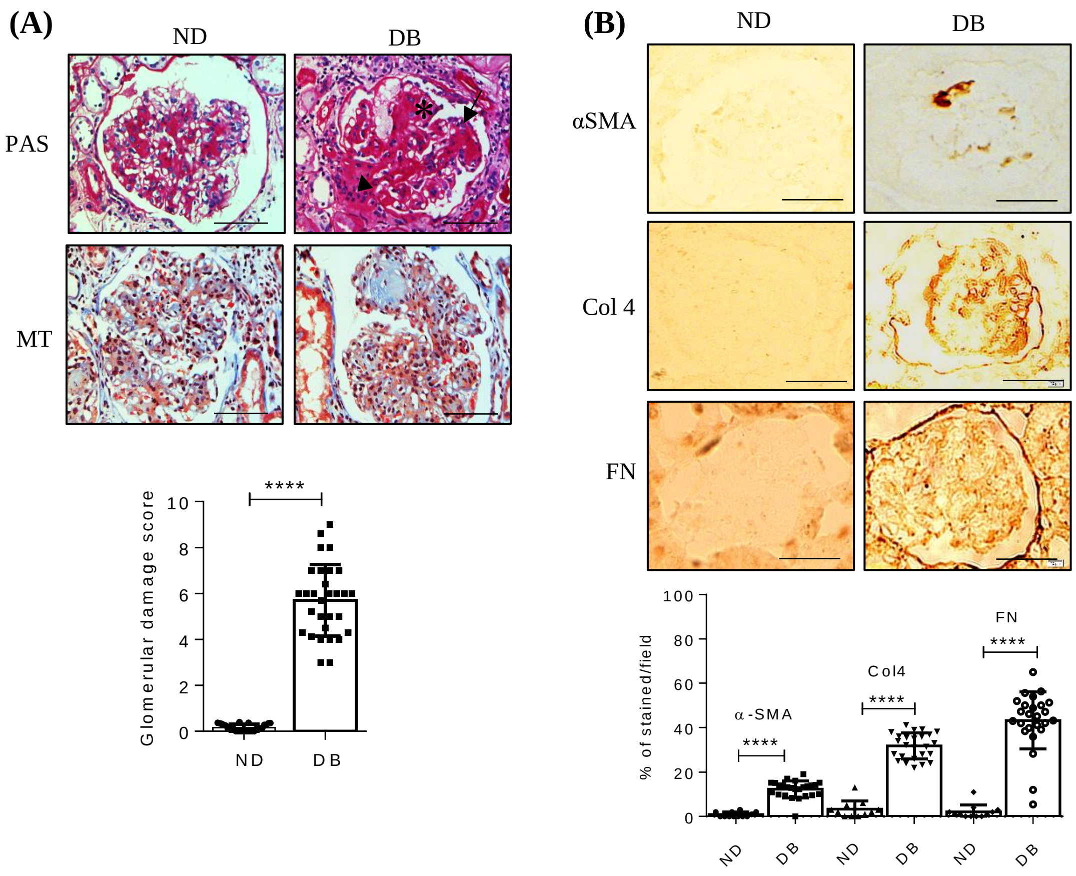

Figure 3.6: AGEs are associated with local fibrosis in the glomerulus. **(A)** PAS) and MT staining in glomeruli from ND and DB patients. Magnification = (x 630). Scale bar = 20µm. Glomerular damage score was derived from PAS-stained images by summing the glomerular capillary blockage (black asterisk), adhesion of glomerular tuft to Bowman's capsule (black arrowhead), and focal segmentation of glomerular tuft (black arrow). Each data point represents the average value of 6 glomeruli from n = 30 kidney biopsy samples from ND and DB groups. **(B)** Representative images of IHC analysis for Collagen 4, (Col4), α-smooth muscle actin (α-SMA), and fibronectin (FN) in glomerular sections from ND and DB biopsies. Scale bar = 50µm. The % of stained area per field from glomerulus was quantified and represented as a dot plot (right panel). Each data point represents the average value of 6 glomeruli from n = 25 kidney biopsy samples in ND and DB groups. The intensity of glomerular expression of each marker was quantified using ImageJ. ****P < 0.0001.

that AGE/RAGE activation is associated with glomerular fibrosis.

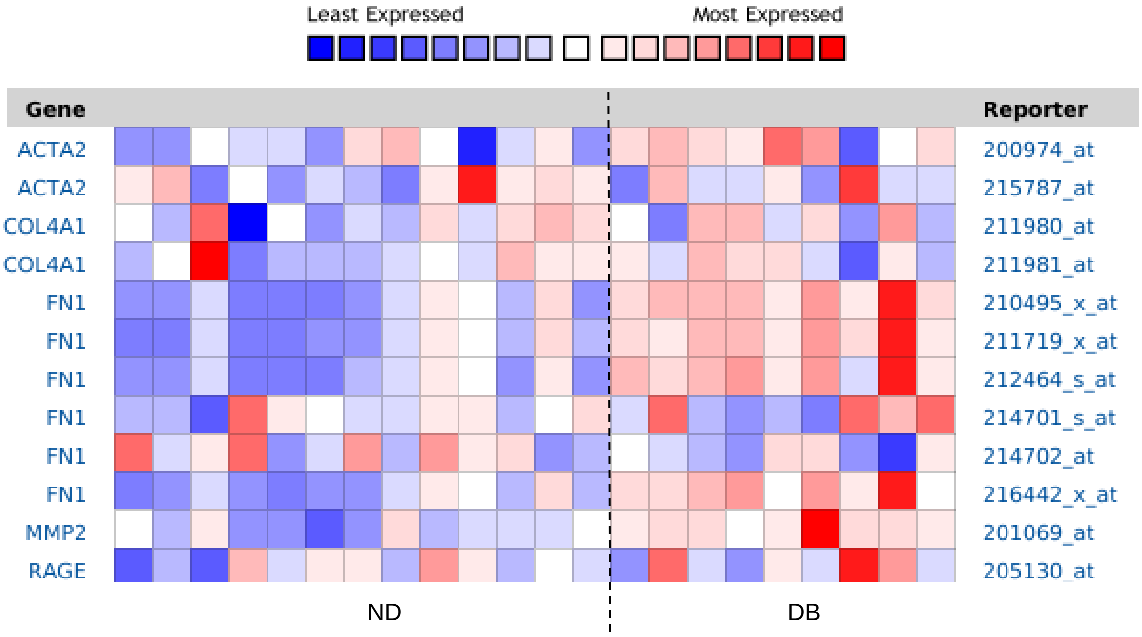

Figure 3.7: Nephroseq analysis. The data comparing ACTA2 (α-SMA), COL4A1, FN1, and MMP2 expression in ND (n = 13) vs. DB individuals (n = 9). Nephroseq data is acquired from Woroniecka diabetic glomerulus dataset.

3.3 Discussion:

The incidence of ESKD is increasing globally, and DN is one of the leading causes. Although eGFR and albuminuria reflect kidney function, these parameters are part of the diagnosis. Declined eGFR and albuminuria may not seldom predict the DN's extent when the serum creatinine levels have risen already. Therefore, a more effective indicator that can predict DN's progression is greatly warranted to deal with DN and, consequently, ESKD. In the present study, we found the HbA1c, GA, AGI index significantly associated with decreased kidney function. Both serum and urinary AGEs are significantly associated with adverse kidney function in these patients with DN. Elevated CML proportionate with reduced expression of podocyte-specific markers such as nephrin and podocin. Our study suggests that AGEs associate with EMT of podocytes and glomerulosclerosis in DN patients. Similarly, in vivo administration of AGEs resulted in podocyte EMT, glomerulosclerosis, and proteinuria. Inhibition of RAGE prevented AGEs induced adverse kidney effects both in vivo and in vitro, such as podocyte depletion, sclerosis, and proteinuria. Together, the data presented in our study demonstrate that AGEs may predict DN progression, particularly podocyte injury. Chronic elevation of blood glucose levels is an exacerbating factor that ensures the non-enzymatic glycation and formation of AGEs, which deposit irreversibly in several organs and blood vessels [259]. Serum levels of AGEs not only associate with the severity of diabetic complications, including retinopathy and nephropathy [260] but also predict mortality [261-264]. In addition to predicting the risk of diabetic complications, Luft et al. reported that circulating CML levels predict the risk of developing diabetes [265]. With each 100 ng/ml increment in CML the risk of developing diabetes increases by 35% in individuals with impaired fasting glucose [265]. While in the American cohort circulating CML levels were associated with insulin resistance (HOMA-IR), in the Japanese cohort, no association was found for CML despite AGEs were associated with the HOMA-IR index [93, 266].

AGEs elicit intracellular signaling via interaction with RAGE localized to endothelial cells, macrophages, and vascular smooth muscle cells. The dominant AGE epitope for binding to the RAGE is CML [267]. At the same time, CML modifications of proteins are predominant AGEs that accumulate in vivo [268]. Elevated serum CML levels were observed in patients with kidney failure [269]. Enhanced CML accumulation was observed in glomerular nodular lesions from patients with DN [270]. AGEs-RAGE interaction elicits cellular injury by producing reactive oxygen species, activating profibrotic and proinflammatory cascades [139, 240]. A recent report suggests that it may be necessary to evaluate glycemic control in patients with diabetes undergoing hemodialysis by combining several glycemic control indicators, including GA, HbA1c, and pre-dialysis blood glucose levels [271]. Infusion of AGEs into rats induced albuminuria, and histological changes like that occur during DN. Contrastingly, preventing AGEs formation improved proteinuria and preserved kidney function. DN is presented with reduced podocyte density.

The specific effect of AGEs on podocyte biology is being investigated recently. AGEs, particularly CML, induce EMT of podocytes by inducing transcription factor Zeb2 through activation of NF-kB signaling cascade [219]. At the same time, inhibition of NF-kB prevented CML-dependent induction of Zeb2 and loss of E-cadherin, which is crucial for maintaining epithelial morphology and cell-cell adhesion [219]. AGEs accumulate in glomeruli and elicit the expression of ECM components such as type IV collagen and laminin. AGEs provoke premature senescence of the kidney cells, particularly cells in the proximal tubule. These novel actions of AGEs in eliciting podocytopathy vis-a-vis the pathogenesis of proteinuria and DN could be adapted as prognostic markers to assess the glomeruli's irreversible damage during the progression of DN.

HbA1c is the most used marker for glycemic control, and it is also used to predict the morbidity of vascular complications. HbA1c reflects plasma glucose levels for the past 2–3 months due to erythrocytes' long lifespan. However, certain clinical conditions such as kidney anemia and hemolytic anemia during which lifespan of erythrocytes vis-a-vis HbA1c measurements are affected and underestimate glycemic control. Furthermore, low hemoglobin levels may result in falsely low HbA1c values that underestimate glycemic control in patients undergoing dialysis. Increased hemoglobin turnover might contribute to lower glycated hemoglobin in advanced CKD and may mislead the clinical judgment. On the other hand, erythropoietin treatment in anemic patients with kidney disease significantly alters the HbA1c levels [247]. Therefore, it is considered that over-reliance on HbA1c as the sole marker of glycemic control could lead to errors in assessing actual changes in glycemia [247].

In this setting, an additional assessment of glycemic control is required. AGI might represent a better glycemic control marker than HbA1c in diabetic patients with kidney insufficiency. In a recent study, Chitra et al. reported that plasma AGI was significantly ($P < 0.05$) associated with diabetic cataracts [272]. In a recent study, Farhan et al. reported an association between AGEs/soluble RAGE ratio and urinary albumin/serum creatinine ratio in type II diabetic patients [273]. Similar to our study, this study also suggests that serum AGEs can consider as predictors of vascular complications in uncontrolled type II diabetic patients. Therefore, markers that provide an index of long-term glycemic control are essential tools in DN patients' care, considering the increased incidence of DM and progression toward ESKD. In this study, we measured only one AGEs-CML.

The association of other AGEs with podocyte injury may be similar as we observed or maybe different, which needs to be investigated. Another limitation a relatively small sample size. Our subjects were abnormally hyperglycemic, and the data with diabetic patients with a good glycemic index may be different. A prolonged followup study with more patient numbers would make the present observation stronger. However, given the supportive findings from animal studies and biopsy samples, AGI's potential and measurement of individual AGEs could give a better index of progression of DN to ESKD. In summary, our study demonstrated that in proteinuria patients, glomerular CML levels correlate significantly with EMT of podocytes and glomerular injury. Administration of AGEs to mice provoked EMT of podocytes proved the cause and effect relationship between AGEs and glomerular injury. The findings suggest that CML could provoke podocyte injury and the risk of DN progression to ESKD. Therefore, serum and urinary AGI and CML might be considered a potential surrogate prognostic marker for DN.

Chapter-IV

To investigate the role of AGEs in re-activation of Notch signaling and EMT in podocytes

Chapter-IV

To investigate the role of AGEs in re-activation of Notch signaling and EMT in podocytes.

Contents

4.1 Introduction

Glomerular podocytes are the important cells which involved in the filtration of blood and regulation of glomerular permselectivity. Other than that podocyte releases vascular endothelial growth factor-a (VEGF-a) and platelet-derived growth factor (PDGF), which are necessary for maintaining the renal cells and their function [274-277]. Glomerular diseases exhibit early cellular changes characterized by podocyte injury and loss, proteinuria and glomerulosclerosis [278]. Studies have demonstrated a decline in the number of podocytes in diabetic nephropathy (DN) [279].

The discovery of intact podocytes in the urine of proteinuric patients led to the proposal that podocytes could detach from the underlying GBM [280]. It was suggested that the transition of podocytes from an epithelial state to a highly motile mesenchymal phenotype, or abnormalities in proteins such as integrins or dystroglycans that aid in podocyte adherence to the GBM, could be responsible for podocyte detachment and subsequent loss into the urine [281, 282]. The de-differentiation of podocytes and re-activation of signaling pathways that are typically active during embryonic development contribute to podocyte injury and the development of proteinuria [249, 283, 284].

Notch pathway is significantly conserved Signaling pathway among all metazoans which is a type of a short-range signaling between two adjacent cells [285-287]. Notch signaling crucial for cell-fate determination, differentiation, proliferation, organ development (at embryonic stage) and adult tissue homeostasis [288]. The Notch also play an important role in the nephrogenesis and podocyte fate determination [289, 290]. The Notch can signal both ways canonical (Notch-ligand dependent) [287] and non-canonical (through GPCR and enzyme linked receptor) [291, 292]. The Notch signaling consists of five ligands like Jagged (1, 2), Delta-like (1, 3, & 4,) and four membrane bound receptors (Notch 1–4). The notch receptor consists of extracellular region (EGF-like repets, NRR), a small transmembrane region, a cytosolic region (RAM, NLS, TAD and PEST domain) and three proteolytic cleavage sites S1, S2, S3 for trafficking and Signaling **(Figure 4.1)**.

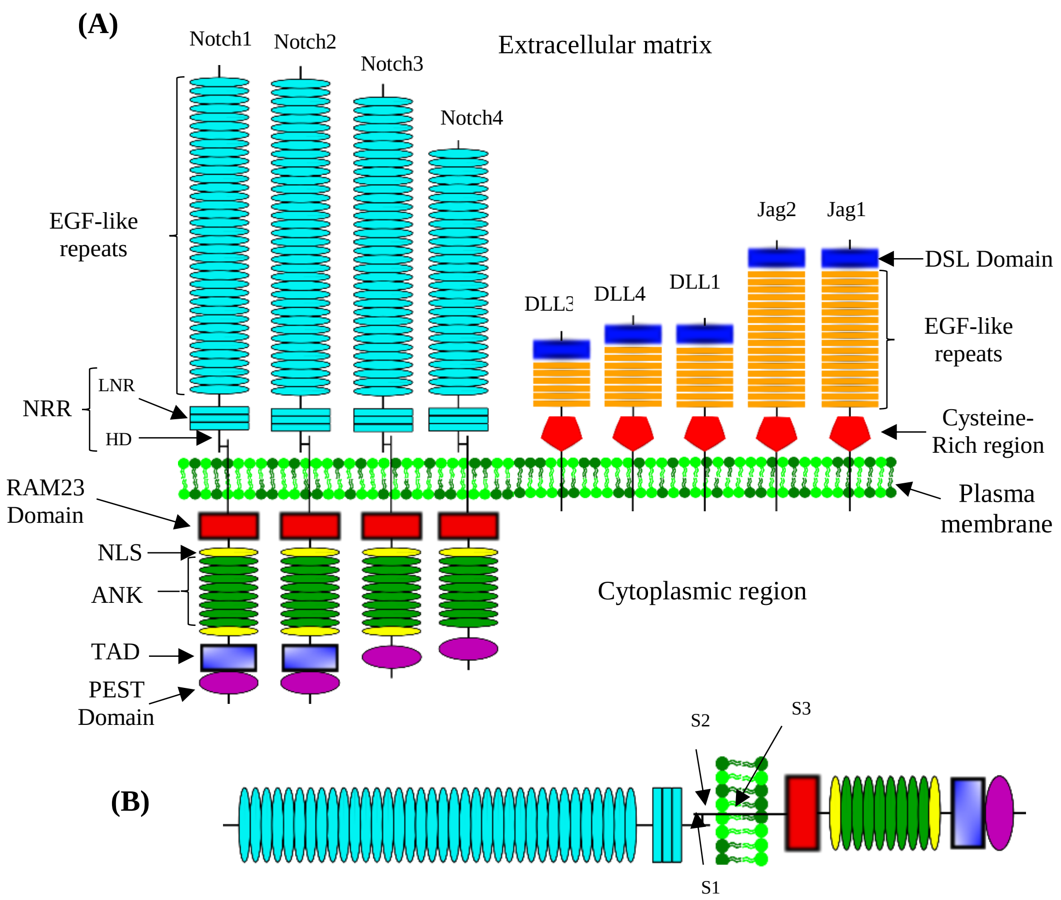

Figure 4.1: Structural details of ligands and its receptor of Notch signaling. (A) The structure of Notch receptors (Notch1-4) and ligands (Jag1,2 & DLL1,3,4). **(B)** Black arrows indicating the cleavage sites (S1–3) on the Notch receptor. Abbreviations: EGF, epidermal growth factor; NRR, negative regulatory region; NLS, nuclear localization sequence; LNR, cysteine-rich Lin12/Notch repeats; TAD, transcriptional activation domain; HD, heterodimerization domain; ANK, Ankyrin; DSL, Delta/Serrate/Lag-2; DLL, delta-like; Jag, jagged.

After getting external stimuli, ligand of signal sending cell interacts with the receptor on signal receiving cell. Most of the Notch goes through the S1 cleavage for transport to the cell surface [293]. Subsequently after binding, due to conformational changes Notch getting cleavaged at S2 and S3 sites by a distintegrin and metalloprotease (ADAM) and γ-secretase respectively. After cleavage cytosolic region of Notch 1 called Notch intracellular domain (NICD1) released into cytosol [294-296]. NICD transported into nucleus where it binds with transcription factor CSL together with other co-factors like mastermind-like protein and forms a ternary complex to activate gene transcription. The most conserved target transcription factors of NOTCH signaling are hairy/enhancer of split (HES) and hairy/enhancer of split related with YRPW motif (HEY) family **(Figure 4.2)** [297, 298]. Activated notch Signaling and others regulate organ development during embryogenesis via distinguish program called cell phage transitions namely epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) [299-303]. Notch Signaling has been found one of the major regulator in the induction of EMT [304, 305]. EMT is a process that results in multiple biochemical changes of an epithelial cell which is attached to a basement membrane to a mesenchymal cell phenotype **(Figure 4.3)**. This phenotype includes enhanced migratory property, invasiveness, resistance to apoptosis and increased production of ECM components [306]. EMT has been classified into three major types: **1) Type 1 EMT** that normally occurs during embryonic development; **2) Type 2 EMT** that occurs during tissue regeneration, wound healing and organ fibrosis; and **3) Type 3 EMT** that is associated with metastasis and cancer progression [307-309].

The Notch signaling are active during the early development, however it becomes inactive in both mice and human adult kidneys. Blockage of Notch signaling or knockout of its interacting genes shows podocyte depletion in the early development of mouse kidney [289, 310, 311]. Ectopic expression of Notch in podocytes is related to focal segmental glomerulosclerosis (FSGS) [312, 313] and apoptosis [314]. Recently, our lab and others group claimed that the re-activation of Notch signaling in podocytes from DN subjects [312, 314]. Despite this observation, the exact mechanism behind the re-activation of Notch signaling in podocytes during diabetic kidney disease remains to be

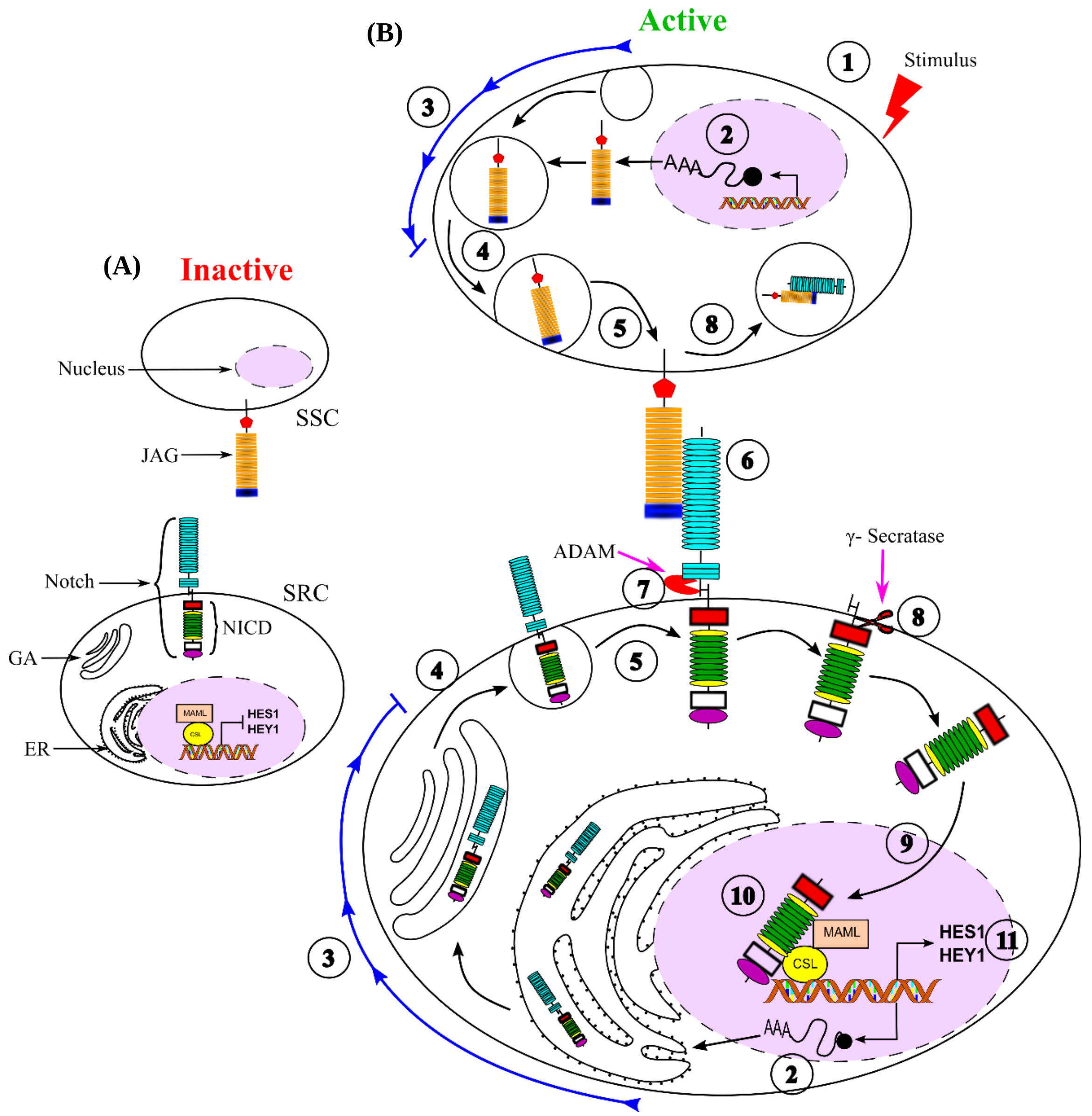

Figure 4.2: The canonical Notch signaling. (A) Representing the inactive state of Notch Signaling with no interaction between ligand (ex. JAG) and receptor (Notch). **(B)** Representing the active stage of Notch Signaling and the numbers indicate the sequential events in its activation as follow: **1)** Attack of external stimuli on SSC; **2)** Expression of both ligand and receptor on respective cell; **3)** Packing of ligand in membrane vesicle in SSC and transport of receptor from ER to GA in SRC; **4)** Transport of ligand and receptor on membrane; **5)** Representation of ligand and receptor onto the membrane; **6)** Interaction of ligand and receptor and conformational changes in receptor ; **7)** Cleavage of Notch at S2 site by ADAM; **8)** Cleavage of Notch at S3 site by γ-secretase; **9)** Translocation of NICD into the nucleus; **10)** Interaction of NICD with other proteins and ternary complex formation; **11)** Activation of downstream targets like Hes1 and Hey1. SSC (Signal Sinding Cell); SCR (Signal Receiving Cell); GA (Golgi Apparatus); ER; (Endoplasmic Reticulum); ADAM (A disintegrin and metalloprotease); NICD (Notch Intracellular Domain).

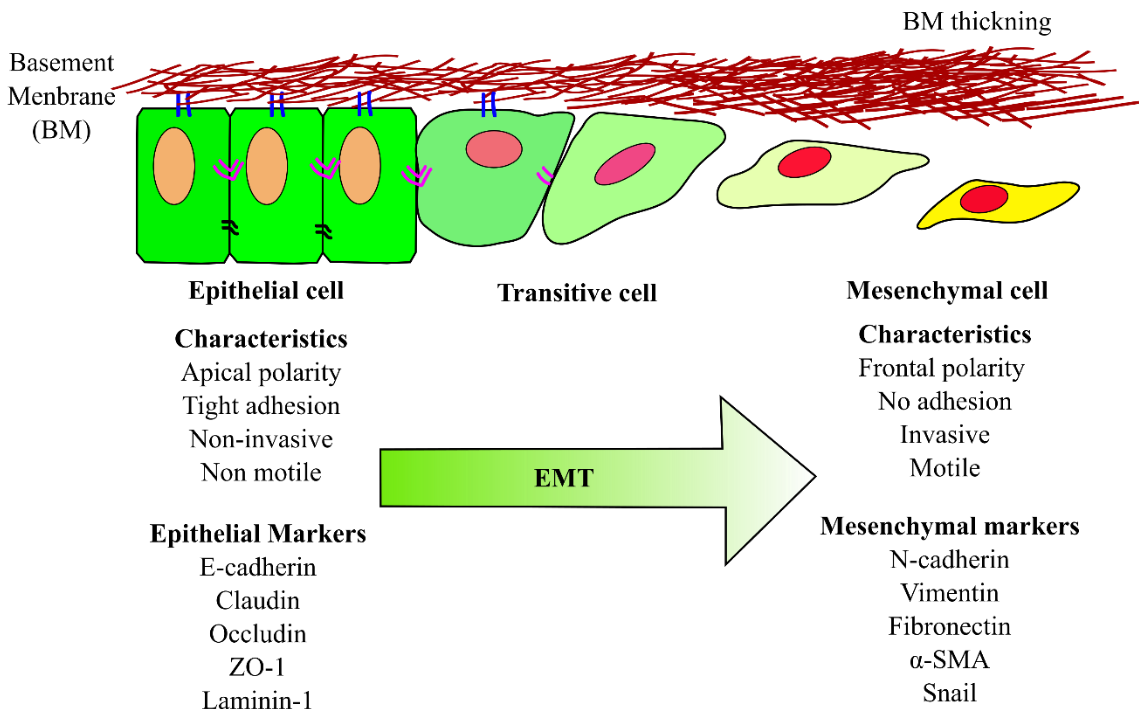

Figure 4.3: EMT markers. Schematic diagram representing the basic mechanism of the EMT and characteristics and the selective marker of cell undergoing EMT.

fully elucidate.Studies suggests that accumulation of AGEs play a crucial role in the pathogenesis of macrovascular and microvascular complications in diabetes. AGEs formed and accumulate in the glomerulus during hyperglycemia, as well as due to aging and uremia. In diabetic condition, accumulation of AGEs is associated with glomerular hyperfiltration, GBM thickening, glomerulosclerosis, and tubulointerstitial fibrosis, thus impaired renal function [48].

In this study, we examined whether AGEs stimulate Notch Signaling in human podocytes cells (HPC) because higher AGE levels and reactivated Notch Signaling temporally related to the pathogenesis of nephropathy. We found that AGEs causes re-activation of Notch Signaling in podocytes. Additionally, we also found that AGEs-induced altered podocyte structure (*in vitro)*, proteinuria and glomerulosclerosis (*in vivo)*.

Chapter-IV (Part-a)

To study the re-activation of Notch1 signaling by AGEs in Podocytes

4a.1 Results

4a.1.1 Preparation and conformation of AGEs.

Glucose-derived AGEs as exo-AGEs were prepared by incubating D-glucose and BSA as described in the 'materials and methods' section. We have observed thickening and browning in D-glucose-BSA sample during the 3 weeks incubation **(Figure 4a.1)**. We were measured the extent of AGE formation by non-tryptophan AGE fluorescence and found

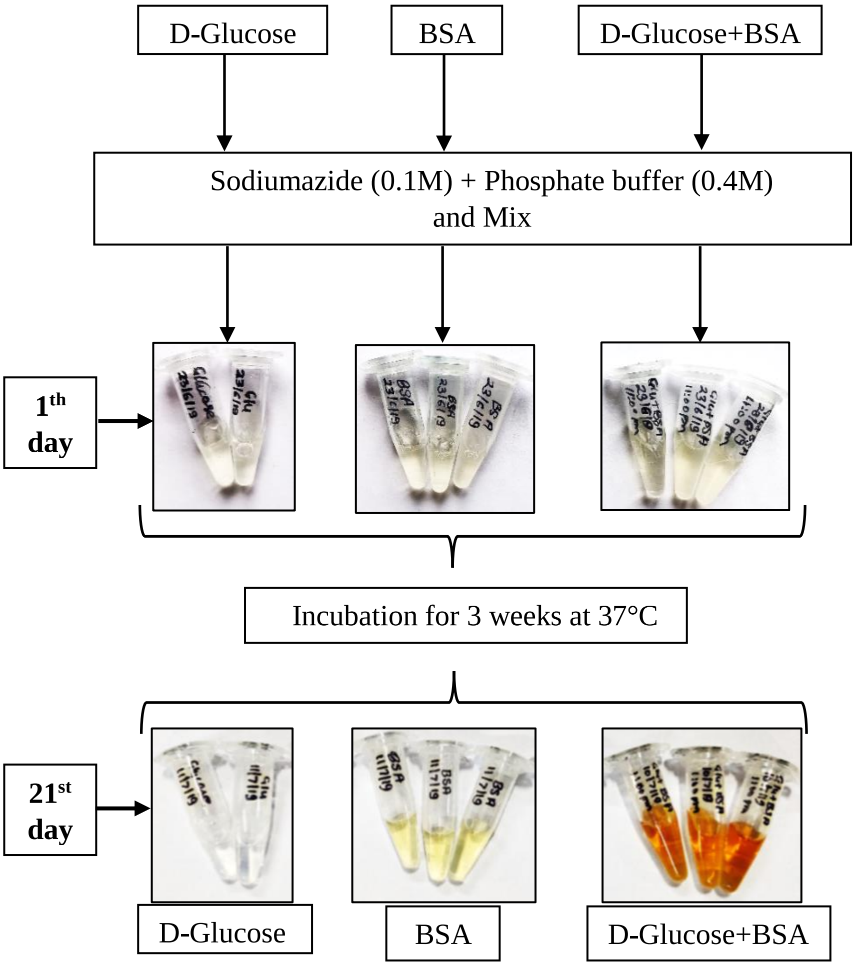

Figure 4a.1: Preparation of AGEs. Schematic representation of preparation of exogenous AGEs. D-Glucose, BSA as a control and mixture of D-Glucose+BSA indicating as AGEs from 1th to 21nd day.

maximum intensity of AGEs around 440nm **(Figure 4a.2A)**. Later, we assessed the degree of glycation by using 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) assay which measured the free amino groups of BSA and D-glucose-BSA sample. Here we have found that about a 50% less free amino groups in D-glucose-BSA sample then BSA alone **(Figure 4a.2B)**. These two experiments indicate the formation of AGEs in D-glucose-BSA sample. For further evaluation, we loaded these on SDS-PAGE followed by with coomassie blue staining **(Fig 4a.2C).** We were observed high-molecular-weight aggregates in D-glucose-BSA preparation. The conformation of AGEs in the D-glucose-BSA sample was done by immunoblotting with the anti-AGE antibody **(Fig 4a.2D)**. The data suggest AGEs were selectively formed in D-glucose-

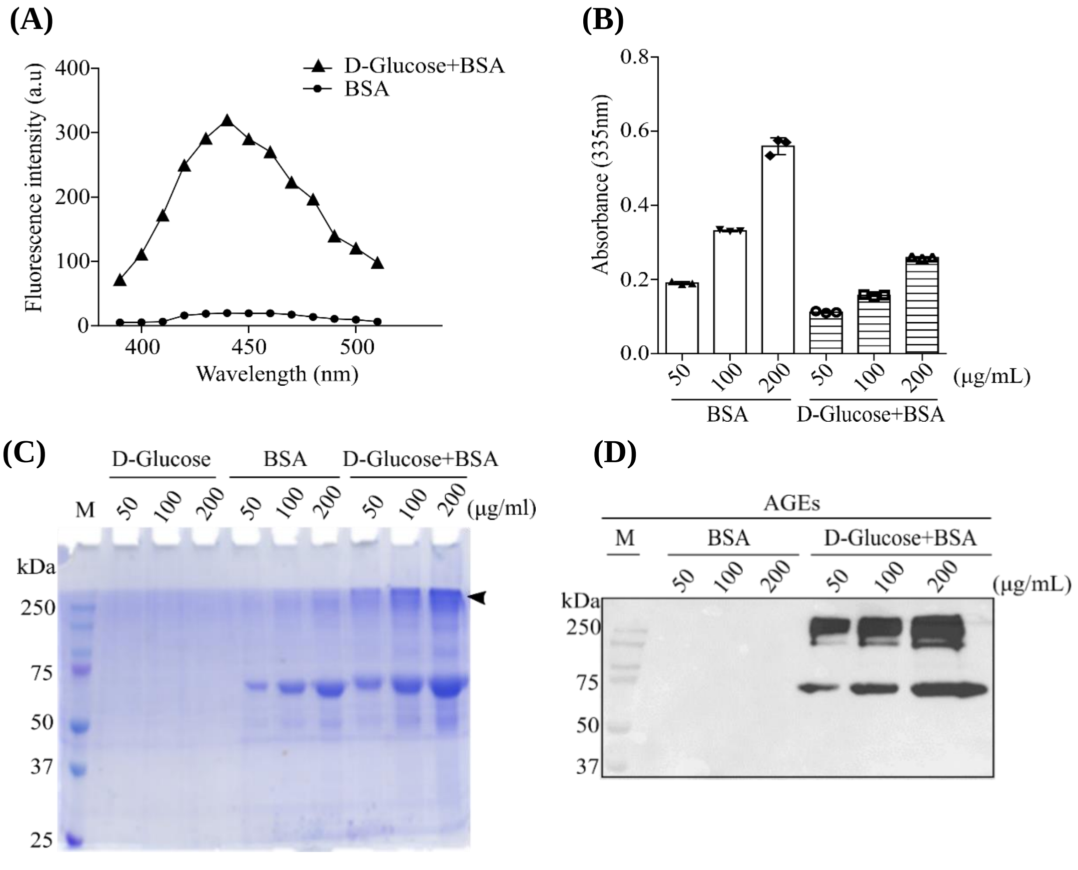

Figure 4a.2: Conformation of AGEs formation. (A) AGE fluorescence indicate the formation of AGEs in D-glucose+BSA solution (λex: 370 nm & λem: 400–500 nm). **(B)** TNBS assay quantified the free amines in different concentration of BSA and D-glucose+BSA (50–200 µg/mL). **(C)** SDS-PAGE with Coomassie staining shows the formation of AGEs in D-glucose+BSA solution. The high-molecular-weight aggregates of AGEs indicated by arrowhead. **(D)** Western blot indicates the formation of AGEs in D-glucose+BSA preparations. M stands for standard protein marker.

BSA preparation. After demonstrating the formation of glucose-derived AGEs in D-glucose-BSA sample, we referred those as AGEs and used for treating HPC and injecting into animals.

4a.1.2 AGEs induces notch Signaling in podocyte in vitro

To study the effect of AGEs on Notch signaling components, we treated HPC with AGEs **(Figure 4a.3A)** and BSA in a dose-dependent manner **(Figure 4a.3B).** We found that induced level of RAGE and NICD1 in AGEs treated HPC, but BSA alone failed to induce RAGE and NICD. Later we also observed that induced level of RAGE, JAG1, NICD1, and HES1 in AGEs treated HPC in time- dependent manner **(Figure 4a.3C).** As we know that γ-secretase cleaves Notch1 to release NICD1, we assessed the γ-secretase activity in AGEs treated HPC. We

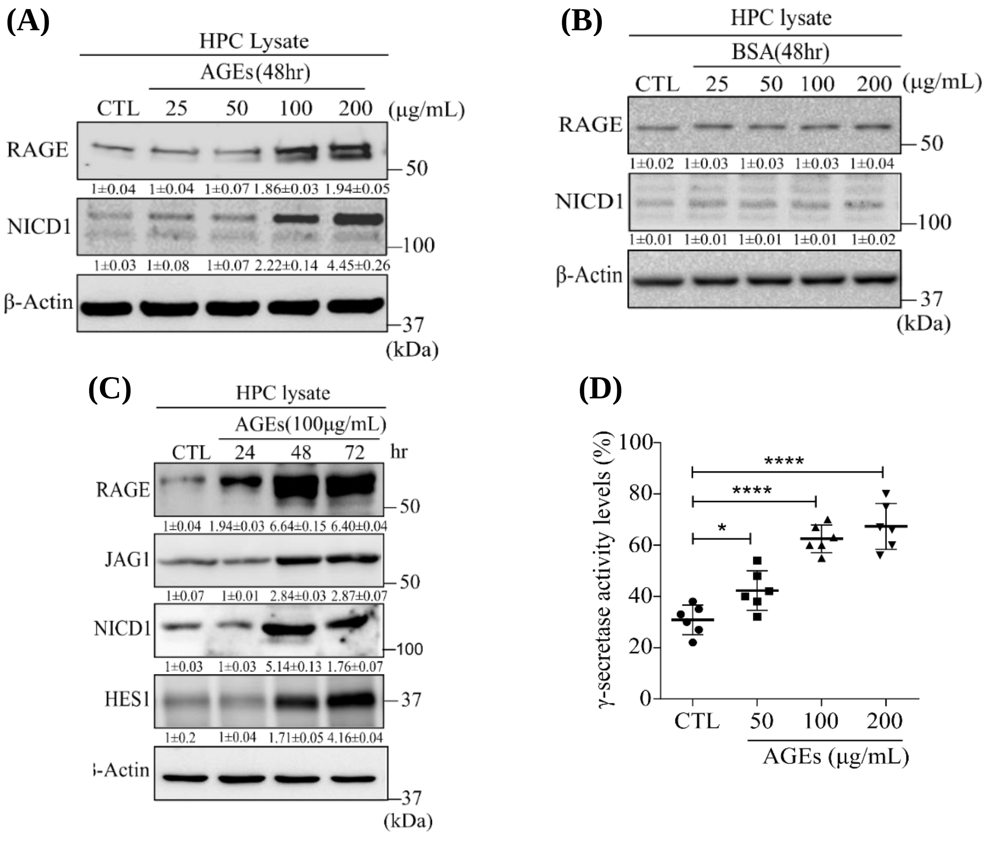

Figure 4a.3: AGEs reactivate Notch1 signaling in HPC. Western blot shows the expression of RAGE, NICD1, and β-actin in concentration -dependent (25–200 µg/mL, 48 hours) treated HPC with **(A)** AGEs **(B)** BSA alone. **(C)** Western blot shows the expression of RAGE, JAG1, NICD1, HES1, and β-actin in HPC treated with AGEs (100 µg/mL) for 24-72 hrs. **(A–C)** The value of fold expression (mean±SD) was presented after normalizing with β-actin (n=3). **(D)** γ-secretase activity measured in HPC treated with AGEs for 48 hrs(n=6). *P<0.05, ****P<0.0001.

observed a significant increase in γ-secretase activity with the different dose of AGE treatment **(Figure 4a.3D)**. Above data suggest that AGEs induced Notch 1 signaling in human podocytes.

4a.1.3 Both RAGE and γ-secretase are required for AGEs activated notch Signaling

To confirm the role of γ-secretase in AGE-induced Notch1 activation, we next treated the

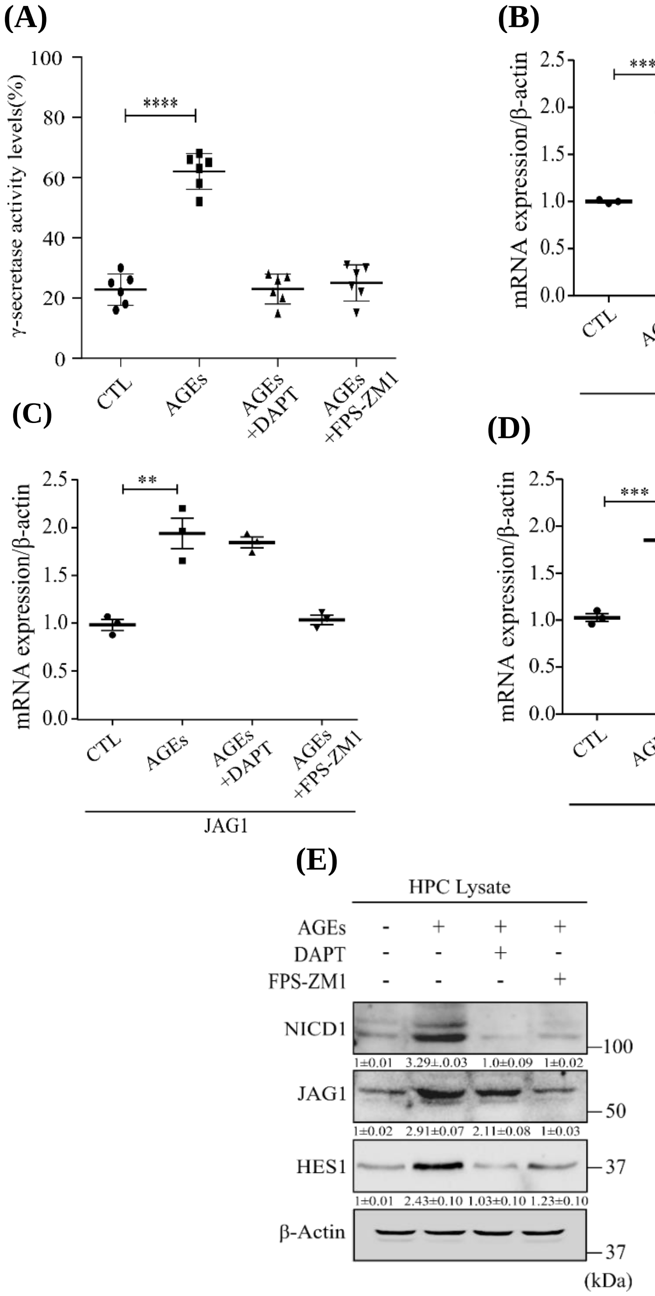

Figure 4a.4: AGEs induced Notch signaling require γ-secretase. We measured γ-secretase activity in HPC treated with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 for 48 hrs. n=6. The qRT-PCR analysis showing the expression of **(B)** NOTCH1, **(C)** JAG1, and **(D)** HES1. β-actin was used as an internal control. **P<0.01, ***P<0.0001, ****p<0.0001.

HPC with AGEs in the absence or presence of a γ-secretase inhibitor, DAPT. As anticipated, DAPT treatment to the AGE-exposed HPC decreased the γ-secretase activity **(Figure 4a.4A)**. Further, we noticed reduced γ-secretase activity in podocytes that were treated simultaneously with AGEs and FPS-ZM1, a RAGE inhibitor **(Figure 4a.4A).** The ability of AGEs to induce NOTCH1, JAG1, or HES1 is ameliorated in the presence of FPS-ZM1 as measured by qRT-PCR **(Figure 4a.4B–D)** and western blotting **(Figure 4a.4E).** Furthermore, DAPT also prevented HES1 expression in AGE-treated human podocytes as measured by qRT-PCR **(Figure 4a.4D)** and immunoblotting **(Figure 4a.4E)**. Interestingly, NICD1 levels were decreased following treatment with DAPT **(Figure 4a.4E)**. As anticipated, DAPT treatment does not affect the expression of both NOTCH1 **(Figure 4a.4B)** and its ligand JAG1 **(Figure 4a.4C).** We investigated for co-localization of NICD1 and HES1 in the nucleus in response to AGE treatment. As expected there is a predominant accumulation of both NICD1 and HES1 in the nucleus of podocytes as demonstrated by immunoblotting of nuclear fraction from

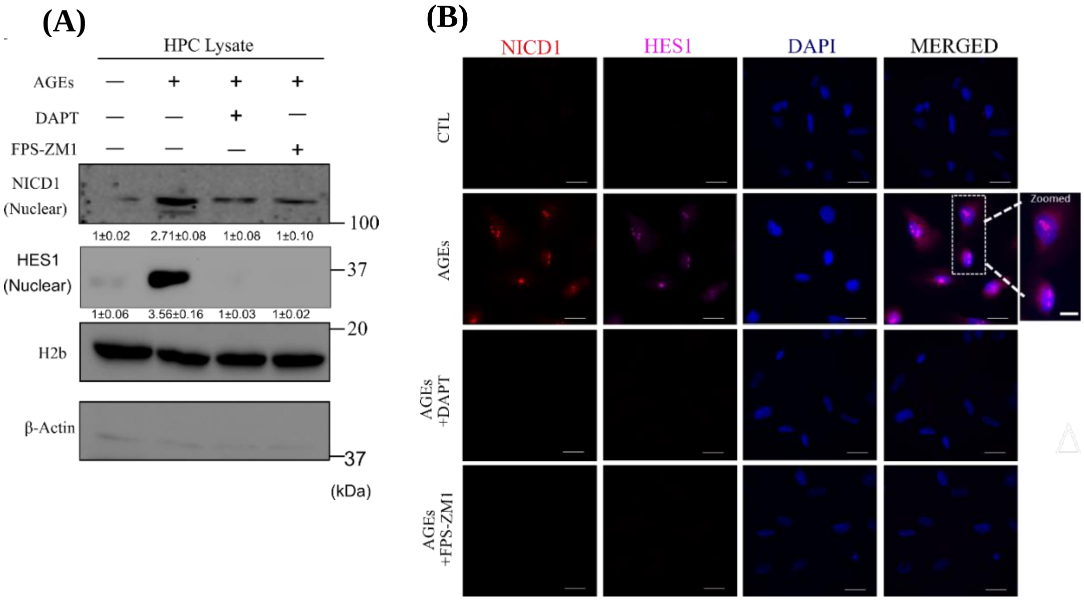

Figure 4a.5: RAGE inhibition prevent Notch signaling. (A) Immunoblots for NICD1, HES1 and histone H2B, the nuclear extract of HPC, treated with or without AGEs, AGEs+DAPT and AGEs+FPSZM1. The fold change values were presented with the expression of the respective genes after normalizing with H2B**. (B)** Immunofluorescence for the nuclear co-localization study of NICD1 (Cy3, red) and HES1 (Cy5, far-red) in HPC treated with or without (CTL) AGEs, AGEs+DAPT and AGEs+FPS-ZM1. Magnification ×630. Scale bar=20 µm.

podocytes exposed to AGEs **(Figure 4a.5A)** and with immunofluorescence **(Figure 4a.5B).** Next, we studied the essential role of RAGE and NOTCH1 in AGE-induced Notch1 signaling by siRNA-mediated knocking down of RAGE and NOTCH1 expression, respectively. Knockdown of RAGE expression resulted in blunting of AGE-induced expression of NOTCH1, NICD1, JAG1, and HES1 **(Figure 4a.6A)**, while knockdown of NOTCH1 expression resulted in blunting of AGE-induced expression of NICD1, JAG1, and HES1 expression **(Figure 4a.6A)**. Furthermore, AGE-induced γ-secretase activity is also ameliorated by the knocking down of RAGE and NOTCH1 expression, respectively **(Figure 4a.6B)**.

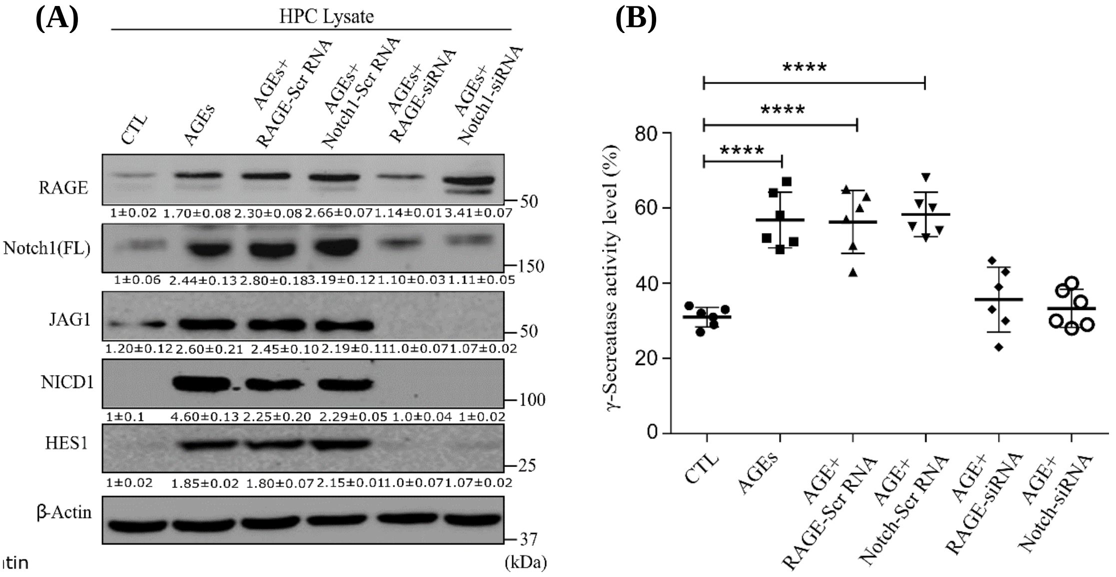

Figure 4a.6: NICD1 formation by AGEs require RAGE and Notch. (A) HPC cells transfected with specific siRNA targeting RAGE and Notch1were subjected to immunoblotting for Notch signaling components **(B)** γ-secretase activity in HPC in which RAGE and Notch1 expression was knockdown by siRNA and treated with or without AGEs. n=6. ****p<0.0001.

4a.1.4 AGE-induced Notch Signaling is abrogated by RAGE inhibitor in vivo.

A large body of evidence reported the accumulation of AGEs in glomeruli from patients with DN or experimental animals [315]. Therefore we investigated the expression of RAGE and components of Notch signaling in mice administered with AGEs for 4 weeks. We have observed enhanced RAGE expression in glomerular sections from AGE-administered mice

(Figure 4a.7A). Furthermore, elevated glomerular expression of NICD1, JAG1, and HES1 was observed in AGE-injected mice **(Figure 4a.7B-C)**, whereas co-administration of DAPT ameliorated AGE-induced NICD1 and HES1 expression **(Figure 4a.7B-C)**. Indeed simultaneous administration of RAGE inhibitor (FPS-ZM1) attenuated the AGE-induced expression of NICD1, JAG1, and HES1 **(Figure 4a.7B-C)**. Furthermore, the data suggest AGEs activate Notch Signaling in glomerular podocytes of AGEs administrated mice.

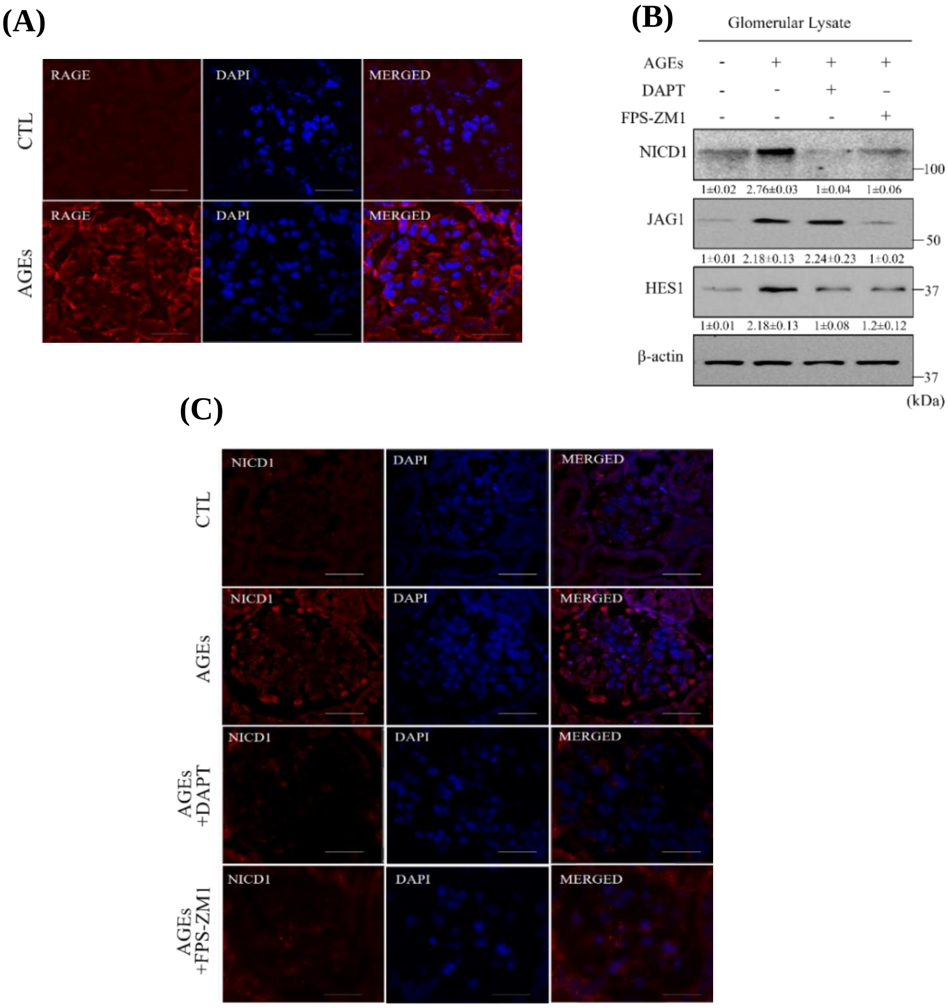

Figure 4a.7: ***In vivo*** **validation of Notch1 activation by AGEs**. **(A)** Immunostaining for RAGE expression in mice glomeruli treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6, each group). Magnification ×630. Scale bar=20 μm. **(B)** Immunoblotting analysis for NICD1, JAG1, HES1, and β-actin in mice glomerular lysates from with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 treated mice (n=6, each group). The fold change values were presented with the expression of the respective genes after normalizing with β-actin. **(C)** Double immunostaining with anti-NICD1 (Alexa 555) and anti-WT1 (Cy3, red) in glomerular sections from with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 treatment (n=6). Magnification ×630. Scale bar=20 μm.

4a.1.5 Activated Notch1 Signaling in subject with DN.

AGEs are elevated in plasma and accumulate in several tissues including the kidney in patients with diabetes. AGEs are considered a new therapeutic target in chronic kidney disease [316]. Elevated HbA1c and glycated albumin levels in subjects with DN were associated with decreased renal function as evidenced by increased UACR **(Table 5)**. Non-tryptophan AGE fluorescence also revealed the presence of AGEs in the serum from subjects with DN **(Figure 4a.8A)** and we also fund increased urinary protein levels compared with control subjects (**Figure 4a.8B)**. Notch signaling is inactive in the adult kidney, whereas activated Notch signaling is reported in glomerular diseases particularly in podocytes [314]. Therefore, to confirm our data that AGEs activate Notch1 signaling in humans, we analyzed glomerular NICD1 expression in people with DN. Immunohistochemical analysis of kidney from people with DN revealed increased expression of AGEs, RAGE, NICD1, and HES1 compared with non-diabetics (**Figure 4a.8C)**. Together the data suggest that activated Notch signaling in glomerular podocytes of subjects with DN.

Parameters	Control	DN
Age (yrs)	50±3.5	50±4.2
Duration of diabetes (yrs)	_	8.5±4
Fasting Glucose (mg/dL)	96±18	152±28
PP Glucose (mg/dL)	104±24	210±43
HbA1c (%)	5.2±0.7	8.4±1.1
GA (%)	13.8±2.5	27.6±7.3
UACR (mg/g)	2.4±0.8	228±64
eGFR (mL/min × 1.73 m2)	103±12	71±14

Table 5: Clinical characteristics of the study subjects in the DN group and non-DN group.

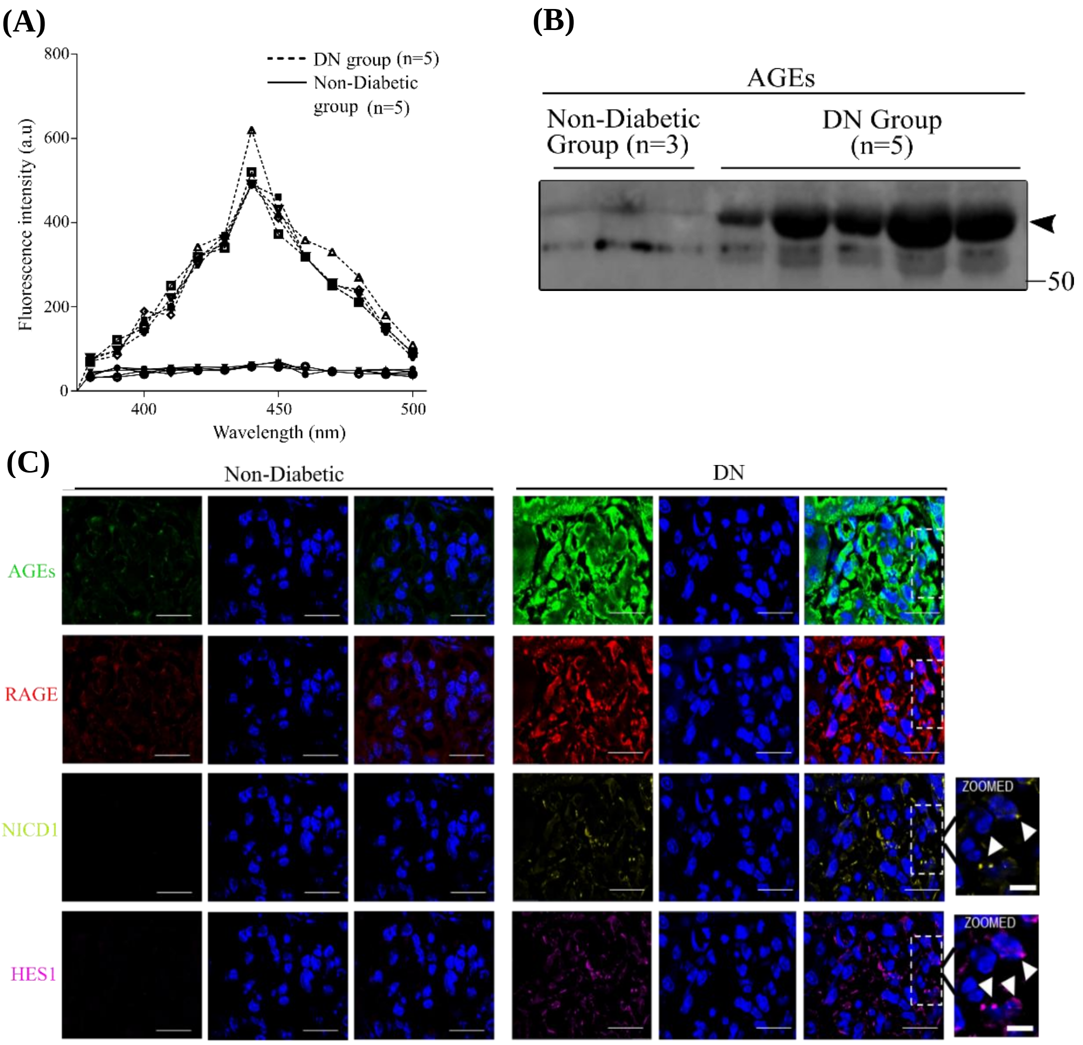

Figure 4A.8: Diabetic patients show activated Notch signaling. (A) Immunoblotting for AGEs in urine samples of DN (n=5) and non-diabetic group (n=3). The arrowhead indicates the positive staining for AGEs in the urine samples of a patient with DN. **(B)** IHC analysis of glomerular serial sections from patients with DN (n=16) and non-diabetic groups (n=10) for AGEs (DyLight488, green), RAGE (Cy3, red), NICD1 (Alexa Fluor 555), and HES1 (Cy5, far-red). Scale bar=20 μm.

Chapter-IV (Part-b)

To study the role of AGEs elevated Notch1 in the loss of podocyte and the induction of glomerulosclerosis

4b.1 Result

4b.1.1 AGEs-induced Notch1 signal stimulate podocyte EMT

Activation of Notch1 signaling was shown to enhance the migratory properties of podocytes via EMT [249]. Furthermore, in our earlier study, podocytes exposed to CML were shown to possess enhanced migration properties [219]. Therefore, we assessed the migratory property of HPC treated with AGEs in the presence and absence of DAPT and FPS-ZM1. Both

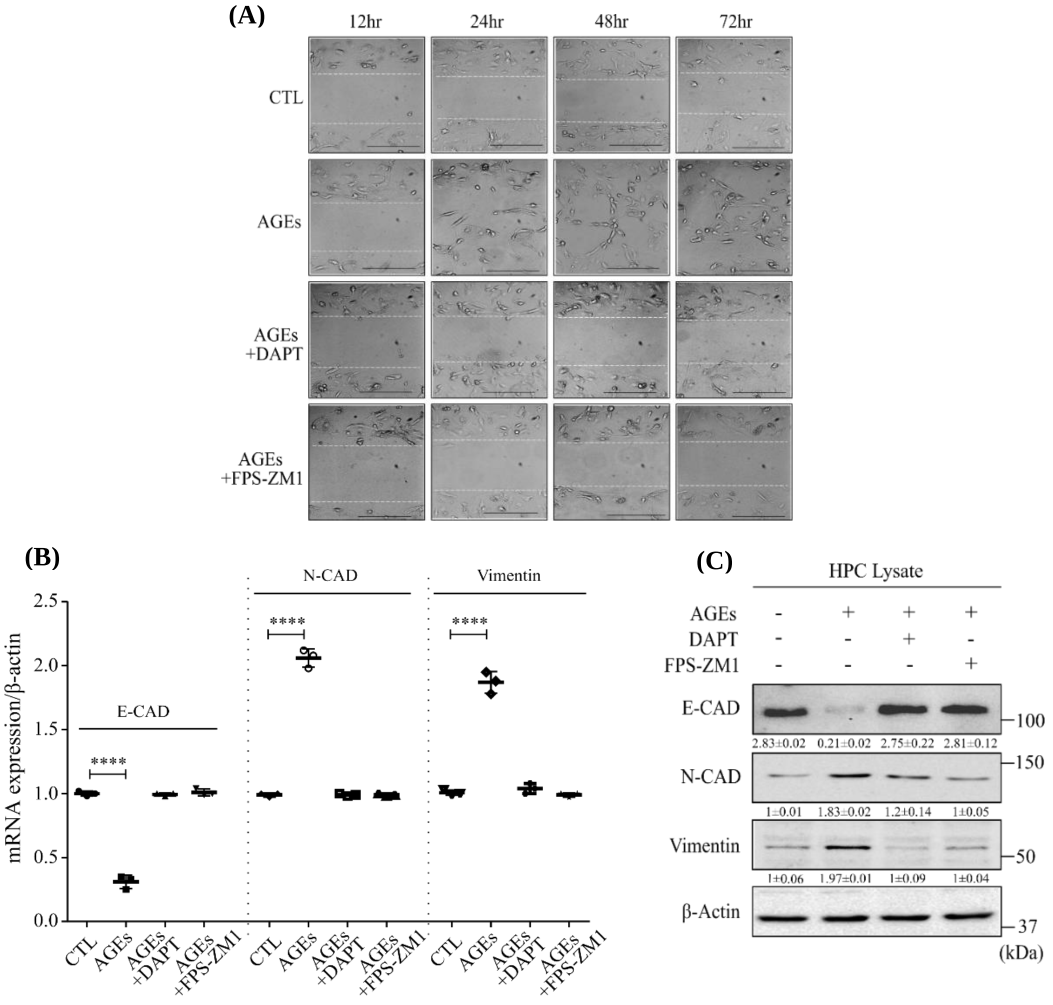

Figure 4b.1: Expression of EMT markers in AGEs-treated HPC. (A) Wound healing assay was performed to demonstrate the extent of motility of HPC. Images were captured at an indicated time interval (12-72hrs) using an Olympus inverted microscope. Scale bar = 100 µm. **(B)** Expression of E-CAD, N-CAD, and vimentin in HPC was analyzed by qRT-PCR. ****P<0.0001. **(C)** Immunoblotting analysis of expression of E-CAD, N-CAD and vimentin in HPC.

DAPT and FPS-ZM1 attenuated AGE-induced podocyte motility **(Figure 4b.1A)**. As we could see the enhanced migration of HPC treated with AGEs, we assessed the expression of epithelial marker (E-cad) and mesenchymal markers (N-cad and vimentin). Loss of E-cadherin and increased N-cadherin, that is, cadherin switch, are hallmark features of EMT. Exposure of podocytes to AGEs manifested in cadherin switch and increased expression of vimentin **(Figure 4b.1B-C),** whereas DAPT and FPS-ZM1 prevented AGE-induced cadherin switch and vimentin expression **(Figure 4b.1B-C)**. EMT is accompanied by dramatic changes in cytoskeleton remodelling [315]. Therefore, to assess the cytoskeletal abnormalities induced by AGEs, we stained podocytes with phalloidin, which specifically stains F-actin. DAPT and FPS-ZM1 prevented AGE-induced F-actin reorganization in human podocytes **(Figure 4b.2A)**. We

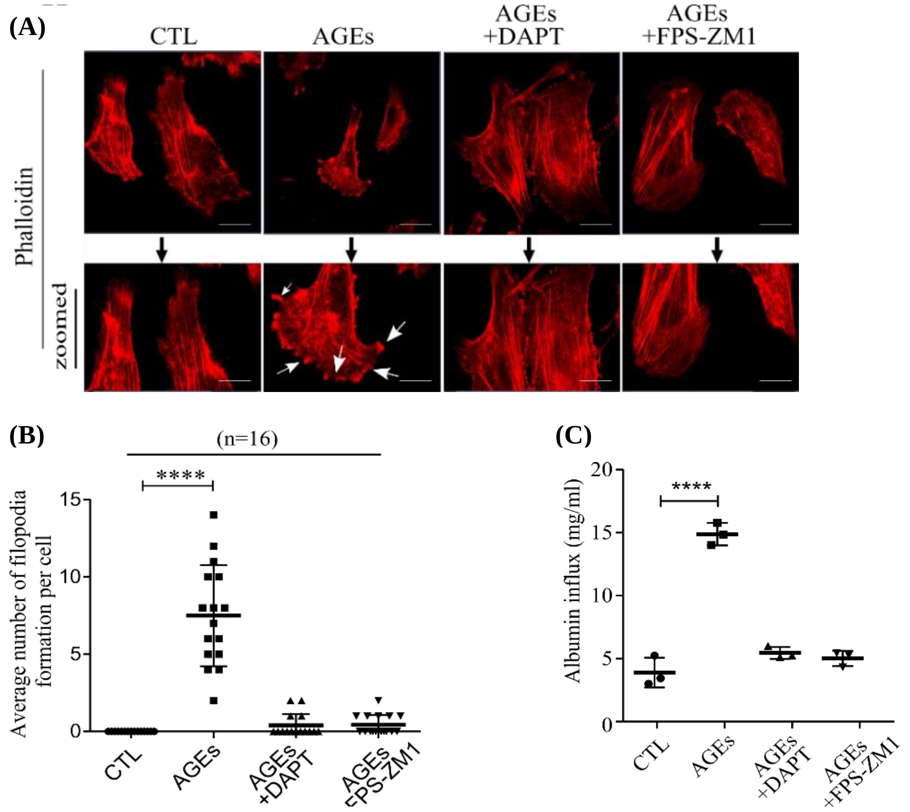

Figure 4b.2: AGEs alter podocyte cytoskeletal components. (A) Phalloidin staining of podocytes showing F-actin arrangement. The white arrow indicate filopodia formation. Scale bar=20 µm. **(B)** Quantification of the average number of filopodia formation observed from the phalloidin staining (n=16). ****P<0.0001. **(C)** AGEs alter podocyte permeability in vitro. Albumin permeability across the podocyte monolayer was determined after 48 hours of exposure to AGEs (n=3). ****P<0.0001.

measured an average number of filopodia per cell and found that DAPT and FPS-ZM1 prevented AGE-induced filopodia formation in human podocytes **(Figure 4b.2B)**. Due to the gain of mesenchymal nature podocyte might loss there filtration barrier function. To assess the functional consequence of podocyte EMT, we measured permselectivity of podocyte by albumin influx assay. Exposure of podocyte monolayer to AGEs resulted in increased permeability to albumin, whereas DAPT or FPS-ZM1 prevented AGE-induced albumin leakage across podocyte monolayer **(Figure 4b.2C)**. All these data suggest that HPC undergo EMT on treatment with AGEs, and Notch activation is required for EMT of AGE-treated podocytes in vitro.

4b.1.2 AGEs reactivated Notch signaling promote EMT *in vivo*.

In earlier research, my supervisor demonstrated that AGEs induces EMT in glomerular podocytes in mice model [317]. In this study, we assessed the involvement of Notch in EMT elicited by AGEs **(Figure 4b.3A).** This result were supported by the Nephroseq analysis and suggests that there is a strong correlation in the expression of RAGE, Notch1, Hes1, and EMT marker (vimentin) in non-diabetics versus DN mouse kidney data **(Figure 4b.3B).** Together the data suggest that activated Notch signaling in mice model with DN is concomitant with EMT of glomerular podocytes.

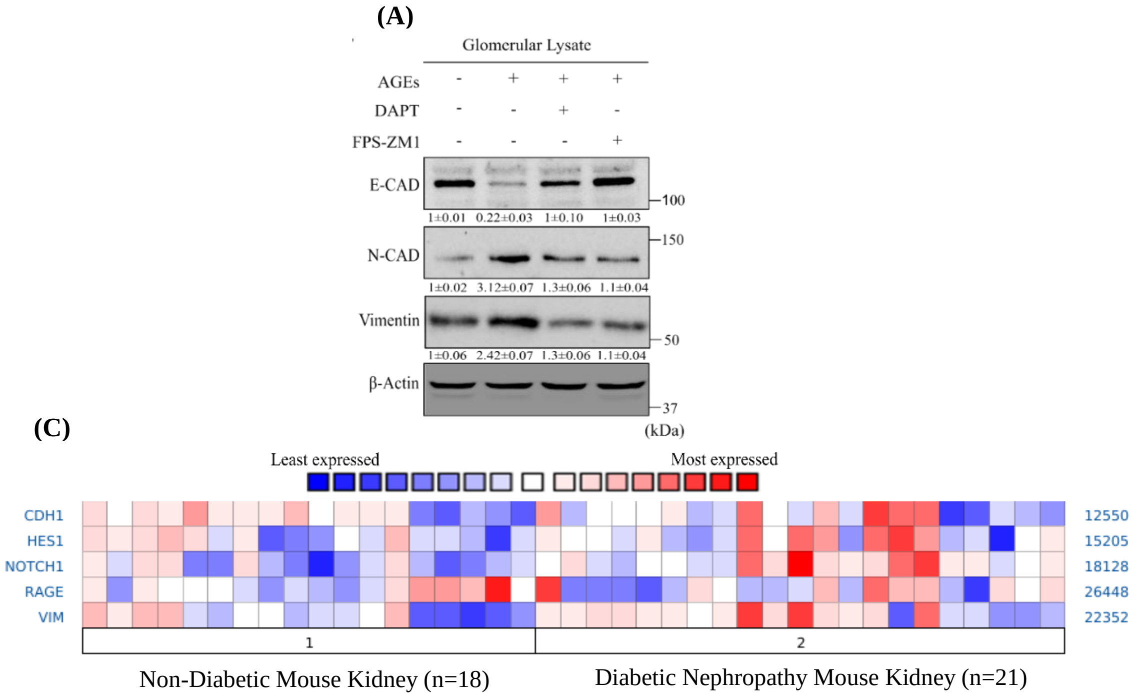

Figure 4b.3: Correlation of AGEs, Notch activation, and EMT. (A) Immunoblotting analysis for E-CAD, N-CAD, vimentin, and β-actin in glomerular lysates from with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 treated mice. The fold change values were presented with the expression of the respective genes after normalizing with β-actin**. (B)** Nephroseq analysis co-expression RAGE, NOTCH1, HES1, CDH1 (N-Cad) and VIM expression levels in non-diabetic (n = 18) vs. DN mouse kidney (n = 21) data set from Hodgin Diabetes Mouse Glomerulus.

4b.1.3 Inhibiting RAGE and Notch1 activation protect AGEs administrated mice from proteinuria.

As we have stated, podocytes are instrumental in regulating glomerular permselectivity and either podocyte injury or loss elicits proteinuria. We measured expression of key slit-diaphragm proteins (podocin and nephrin) decreased in the glomerulus of AGE-treated mice **(Figure 4b.4A-B)**. Since damage to the slit-diaphragm contributes to proteinuria, we measured kidney function and found that the administration of AGEs resulted in reduced GFR and an increased urinary albumin to creatinine ratio (UACR) **(Figure 4b.4C-D)** and proteinuria **(Figure 4b.4E)**. Interestingly treatment of mice with DAPT or FPS-ZM1 rescued the expression of podocin and nephrin and rescued AGE-induced proteinuria **(Figure 4b.4A-E)**. Out data argue that AGEs impair the podocyte function and induce proteinuria through RAGE and Notch1 signaling.

4b.1.4 AGE induced EMT of podocyte elicits glomerulosclerosis *in vivo*.

DN is presented with progressive renal fibrosis [318, 319]. AGEs were shown to induce fibrosis and contribute to the pathology of DN [320]. Therefore, we examined the paraffin-embedded sections of AGE-treated mice for fibrosis. We noticed renal fibrosis in the mice administered with AGEs, as analyzed by PAS and MT staining **(Figure 4b.5A)**. The panel graph indicating the Glomerular damage score was found elevated in AGE-treated mice. It is noteworthy that both DAPT and RAGE inhibitor had prevented AGE-induced fibrosis and glomerular damage **(Figure 4b.5A)**. Next, we measured the expression of fibrotic markers in in mice supplemented with DAPT and FPS-ZM1. Indeed, DAPT and FPS-ZM1 inhibited the expression of AGE-induced fibrotic markers: collagen IV, α-SMA, and fibronectin **(Figure 4b.5B)**.

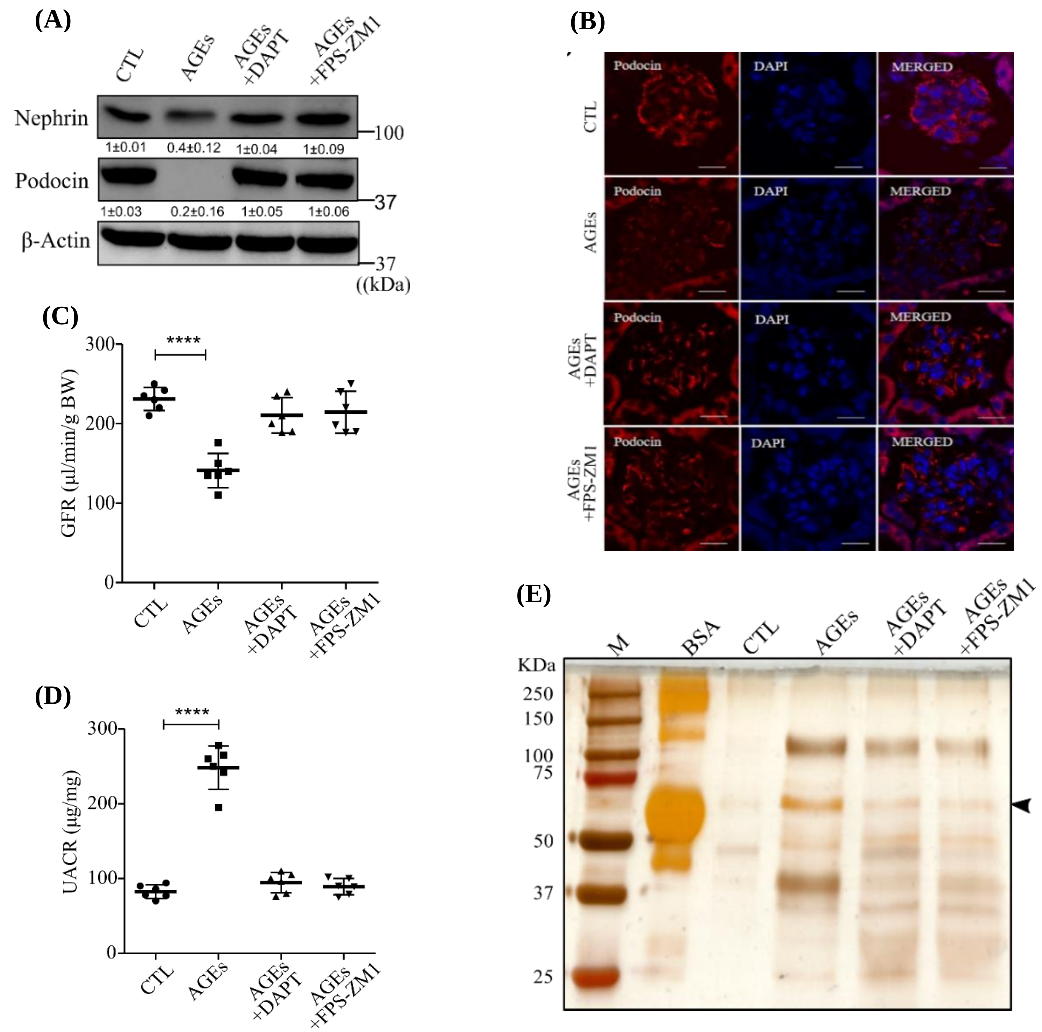

Figure 4b.4: AGEs elicit diminished kidney function. (A) Immunoblotting for podocin and nephrin expression in glomerular lysates and fold change values were presented with the expression of the respective genes after normalizing with β-actin. **(B)** IHC for podocin in the glomerulus from mice treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). **(C)** GFR in mice administered with or without AGEs and administered with DAPT or FPS-ZM1. n=6, ****p<0.0001. **(D)** UACR was estimated in mice treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). ****P<0.0001. **(E)** Urine from animals was subjected to SDSPAGE. BSA was used as a standard. Arrowhead indicates the presence of the albumin in the AGEs treated mice urine sample. M indicates the standard protein marker in kDa.

Glomerular fibrosis is presented with thickening of GBM that may manifest in the podocyte foot process effacement [321]. Since we observed elevated expression of fibrotic markers in AGE-injected mice, we next assessed the morphology of GBM and podocytes in these mice. Although TEM analysis of AGE-administered mice shows thickening of GBM and podocyte foot process effacement, co-treatment with DAPT or FPS-ZM1 abrogated this adverse effect

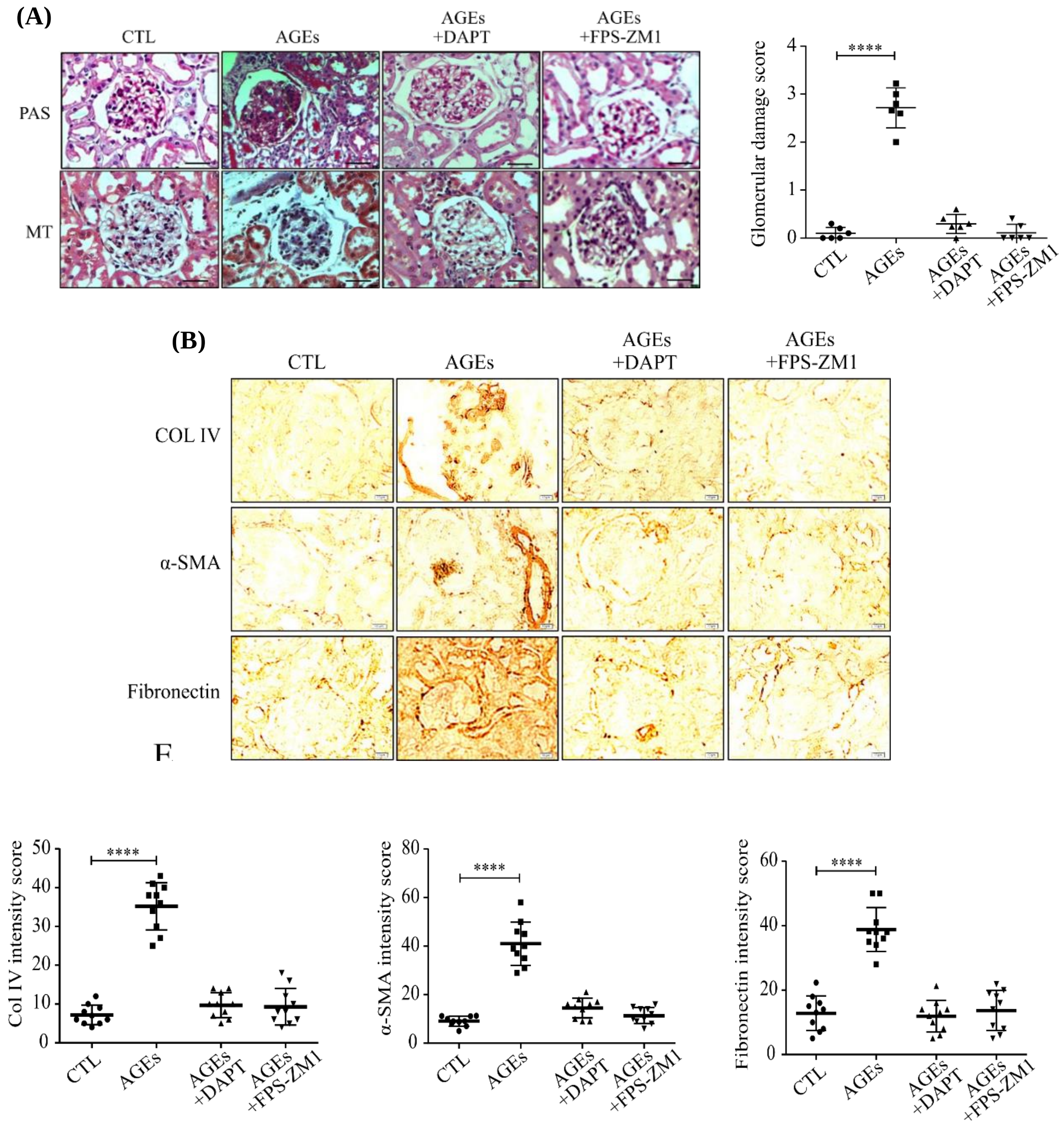

Figure 4b.5: AGEs induce fibrotic markers *in vivo.* (A) Representative images of PAS and MT staining in mice glomeruli from with or without AGEs, AGEs+DAPT, and AGEs+FPS-ZM1 treatment. Magnification ×400. Scale bar=50 µm. The right panel graph representing quantification of glomerular damage. **(B)** IHC for Col IV, α-SMA, and fibronectin in glomerular sections from mice treated with or without AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). Magnification ×400. Scale bar=50 µm. The down panel graph showing the quantified intensity of glomerular expression of Col IV, α-SMA, and fibronectin. ****P<0.0001.

of AGEs on glomerular architecture **(Fig 4b.6A)**. The thickening of GBM might lead to the dehiscence of podocytes [322]. Suztak et al. have reported that the depletion of podocytes occurs at the onset of DN [323]. Next, we assessed whether podocyte depletion occur in AGE-administered mice. Quantification of WT1, a podocyte-specific marker, revealed the presence of detached podocyte in many glomeruli, whereas DAPT and RAGE inhibitor prevented AGE-dependent podocyte depletion **(Fig 4b.6B)**. Together the data confirm that AGE-induced Notch signaling leads to glomerular fibrosis and podocyte depletion.

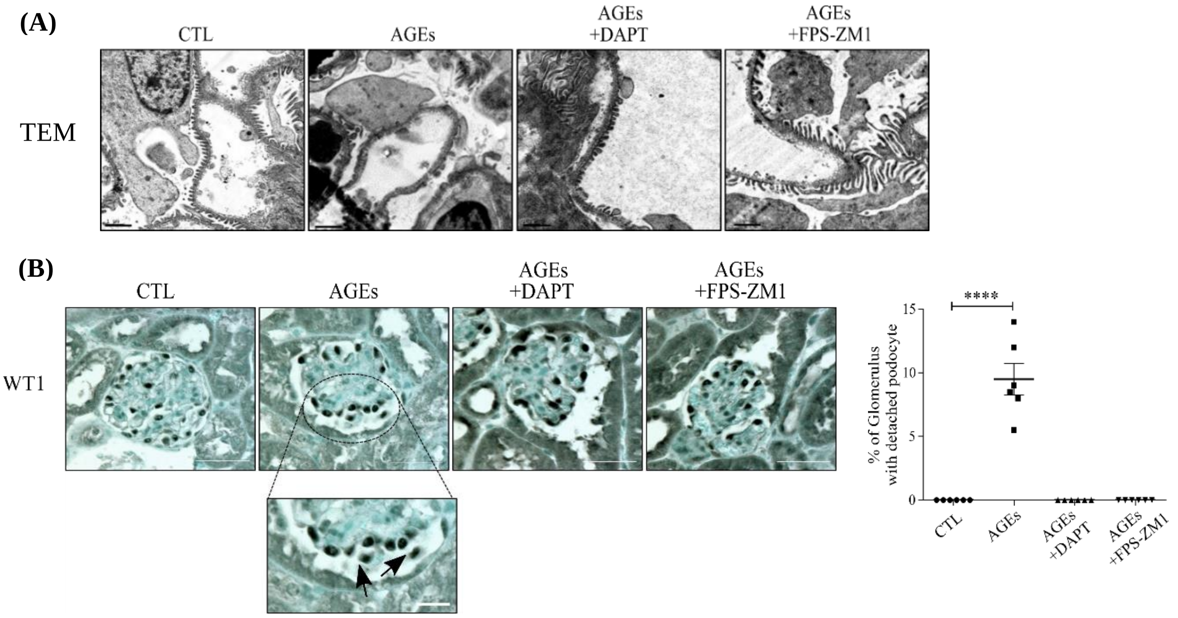

Figure 4b.6: AGEs induce podocyte injury and loss. (A) TEM images showing the damage to podocyte foot processes in glomerulus of mice groups (CTL, AGEs, AGEs+DAPT and AGEs+FPS-ZM1). Scale bar=1 µm. **(B)** Representative images of IHC for WT1 (podocytes) in the glomerulus from mice treated with or without (CTL) AGEs, AGEs+DAPT and AGEs+FPS-ZM1 (n=6). The arrows indicate detached podocytes.

4.2 Discussion

In this present study, we exhibit that AGEs reactivate Notch1 signaling in podocytes that leads to podocyte EMT and increase permeability to albumin in both *in vitro* and *in vivo*. In AGEs administrated mice group, podocyte EMT induces podocyte foot process effacement, and GBM thickening causes glomerulosclerosis. Use of RAGE inhibitor and γ-secretase inhibitor shows that reduction of podocyte EMT in both. The inhibitor administrated mice group protected from podocyte depletion and sclerosis. Importantly, podocyte depletion and proteinuria are common features of DN, whereas blocking of RAGE or γ-secretase successfully abrogated AGE-induced pathophysiological symptoms of DN. The data presented in our study demonstrate that AGEs that accumulate in DN activate Notch signaling in the podocytes elicits in podocyte in EMT and glomerulosclerosis.

Although Notch signaling is crucial during the development of glomerular podocytes, it is not requisite for differentiation of podocytes, beyond the stage of the S-shaped body formation [289]. Notch1 activity was unnoticeable in the healthy adult glomeruli; however, in patients with glomerular diseases, strong upregulation of Notch signaling was observed in podocytes [249]. Suppression of Notch signaling is considered to be a prerequisite for differentiation of renal progenitors into podocyte lineage, whereas persistent Notch activation results in a mitotic catastrophe [289, 324]. Therefore, the significance of re-activation of Notch signaling in adult podocytes remains largely enigmatic. Podocyte-specific upregulation of Notch signaling is associated with progressive glomerulosclerosis, foot process effacement, and severe proteinuria [325]. In our recent study, we demonstrated that in diabetes increased growth hormone (GH)-induced Notch activation resulted in podocyte foot process effacement and glomerular fibrosis, whereas inhibition of γ-secretase improved glomerular function. Similarly, in another study, it was shown that inhibition of Notch signaling in murine models improved proteinuria by reducing podocyte loss [326]. Podocyte-specific deletion of Notch1 has reduced podocyte injury and severity of proteinuria in DN murine models [314].

Interestingly, we noticed activated Notch signaling preferentially in the glomeruli, particularly in the podocytes. Preferential localization of AGEs is observed in selective tissues particularly at the microvasculature, which includes the glomerulus, and the adverse consequences of AGEs were first described in the renal systems [327, 328]. Glomerulus serves as a blood–urine barrier exposed to a large volume of serum AGEs and their reduced clearance in the diabetic milieu [329]. Indeed a progressive decline of kidney function had been demonstrated in subjects with diabetes [244]. Furthermore, elevated levels of glomerular RAGE are typically observed in experimental DN animals [330]. The exclusive podocyte distribution of RAGE and its upregulation in diabetic glomeruli possibly explain AGE-dependent Notch activation selectively in the glomerular region [331]. It is noteworthy that AGEs also stimulate the RAGE expression, and thereby enhanced AGE–RAGE interaction could also provoke adverse renal effect in diabetic conditions. A strong correlation was observed between renal AGEs and incidence of chronic kidney disease, and glomerular AGEs are considered a therapeutic target to combat complications of DN [316]. Several master regulators that activate Notch activity in adult cells include transforming growth factor (TGF) and so on. AGEs stimulate TGF-β1 expression and NF-kB activation [332].

As Notch signaling is not warranted for the podocytes post embryonic development, and aberrant activation of Notch in podocytes is pathological, targeting podocyte Notch signaling may offer protection against diabetic renal complications. Accumulating evidence suggests that either γ-secretase inhibitor or antibodies against Notch ligands or receptors are effective in combating diabetic kidney disease [333]. It is noteworthy that people with DN showed elevated NICD1 expression in podocytes, and blocking the NICD1 expression with γ-secretase inhibitor DAPT prevented the mice from proteinuria. Taken together, we believe that the modulation of

Notch signaling in DN could be a potential target for therapeutic interventions to control the loss of kidney function.

Summary

The kidney play a crucial role in the filtration of blood and the removal of toxic compound in form of urine. AGEs are one of the family of glycotoxins which is excreted through urine by the kidney. The pool of circulatory AGEs is contain both Exo and Endo-AGEs. More Eno-AGEs formation (in diabetes) and consumption of Exo-AGEs increases the circulatory AGEs. This drive the AGES to accumulate at different micro and microvasculature system and create pathological complication [334-338]. Evidences were suggested AGEs accumulate because of both an unbalance between reduced clearance and the increase in production by the kidneys. This involvement in the pathogenesis of DN characterized by proteinuria, podocyte loss, glomerulosclerosis. In this doctorial study, we have investigated the association of AGEs with the podocyte loss in DN patients. Next, we have investigated the mechanism involved in AGEs induced podocte loss. I have found that AGEs induced re-activation of Notch signaling in podocytes and induced cytoskeletal abnormalities. Notch activation is parallel to EMT of podocytes in vitro and AGEs induced glomerulosclerosis, altered podocyte morphology, and proteinuria in vivo.

The salient finding in this study is as follow:

- Poor glycemic control and glycation index in diabetic subjects associate with impaired kidney function.
- Excess AGEs, particularly CML associated with decreased podocin expression, and foot-process effacement of podocytes from type II patients with nephropathy.
- Podocytes in DN patients undergo EMT, which might be responsible for the observed detached phenotype.
- Both our experimental and Nephroseq data suggest that AGE/RAGE activation is associated with glomerular fibrosis.

- AGEs adversely affect kidney function by eliciting podocyte injury and depletion, possibly by promoting podocyte EMT.
- We confirmed the formation of glucose-derived AGEs in D-glucose-BSA preparation.
- Notch signaling is activated in human podocytes exposed to AGEs.
- Podocytes undergo EMT on treatment with AGEs, and Notch activation is required for EMT of AGE-treated podocytes in vitro.
- AGEs activate Notch signaling and induce EMT in glomerular podocytes in vivo.
- AGE-induced Notch signaling leads to glomerular fibrosis and podocyte depletion.
- AGEs impair the podocyte function and induce proteinuria through RAGE and Notch1 signaling.
- Activated Notch signaling in podocytes from diabetic subjects is concomitant with EMT of glomerular podocytes.

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2. Nishad R, Meshram P, Singh AK, et al. *Activation of Notch1 signaling in podocytes by glucose-derived AGEs contributes to proteinuria*. BMJ Open Diabetes Research and Care 2020;8:e001203. doi: 10.1136/bmjdrc-2020-001203

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1. International Conference on *Calcium Signaling Regulatory mechanisms to impact on health and diseases*, RCB, Faridabad (Jan 31st – Feb 1st 2020).
2. 2nd International Online Course on *Flow Cytometry and its Applications in Biomedical Sciences*, University of Hyderabad, Hyderabad (Sep 1st – Sep 4th 2021).
3. International E-Conference on *Recent Research and Innovations in Life Science 2022 - Nutraceuticals in Healthy Ageing*, MMK & SDM Mahila Maha Vidyalaya, Mysuru (Feb 3rd – Feb 4th 2022), Oral Presentation and awarded 3rd prize.
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ORIGINAL RESEARCH
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Advanced-Glycation End-Products Induce Podocyte Injury and Contribute to Proteinuria

Rajkishor Nishad [1]†, Vazeeha Tahaseen [2]†, Rajesh Kavvuri [1], Manga Motrapu [1], Ashish K Singh [1], Kiranmayi Peddi [2]* and Anil K Pasupulati [1]*

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The prevalence of diabetes reaches epidemic proportions. Diabetes is the leading cause of end-stage kidney disease (ESKD) since 30–40% of diabetic patients develop diabetic nephropathy. Albuminuria and glomerular filtration rate used to assess kidney function are considered surrogate outcomes of chronic kidney disease. The search for a biomarker that predicts progression to diabetic kidney disease is intense. We analyzed the association of serum advanced glycation end-products (AGEs) index (AGI) with impaired kidney function in poorly controlled type II diabetic patients. We observed an association between AGI and impaired kidney function in microalbuminuria patients with hyperglycemia. A significant association between AGEs, particularly carboxymethyl lysine (CML), and impaired kidney function were observed. Administration of AGEs to mice showed heavy proteinuria and glomerular abnormalities. Reduced podocyte number in mice administered with AGEs could be attributed to the epithelial-mesenchymal transition of podocytes. Our study suggests CML could be independently related to the podocyte injury and the risk of DN progression to ESKD in patients with microalbuminuria. AGEs in general or CML could be considered a prognostic marker to assess diabetic kidney disease.

Keywords: diabetic kidney disease, advanced glycation end-products, carboxy methyl lysine, podocytes, albuminuria, Introduction, diabetic nephropathy

HIGHLIGHTS

- Advanced glycation end-products (AGEs) index is associated with proteinuria among diabetic subjects.
- Carboxymethyl lysine, a well-characterized AGE, correlated with podocyte injury.
- Administration of AGEs to mice manifested in EMT of podocytes and impaired kidney function.
- Inhibitor against receptor for AGEs prevented podocyte injury and improved proteinuria.

INTRODUCTION

Diabetes has long been a growing epidemic, and Asia accounts for 60% of the world's diabetic population (1, 2). The increased prevalence of diabetes led to a surge in macro and microvascular complications such as visual impairment, coronary heart disease, stroke, neuropathy, and diabetic nephropathy (DN). DN is a chronic disease that accounts for 44% of new end-stage kidney disease

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Activation of Notch1 signaling in podocytes by glucose-derived AGEs contributes to proteinuria

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ABSTRACT

Introduction Advanced glycation end-products (AGEs) are implicated in the pathogenesis of diabetic nephropathy (DN). Previous studies have shown that AGEs contribute to glomerulosclerosis and proteinuria. Podocytes, terminally differentiated epithelial cells of the glomerulus and the critical component of the glomerular filtration barrier, express the receptor for AGEs (RAGE). Podocytes are susceptible to severe injury during DN. In this study, we investigated the mechanism by which AGEs contribute to podocyte injury.

Research design and methods Glucose-derived AGEs were prepared in vitro. Reactivation of Notch signaling was examined in AGE-treated human podocytes (in vitro) and glomeruli from AGE-injected mice (in vivo) by quantitative reverse transcription-PCR, western blot analysis, ELISA and immunohistochemical staining. Further, the effects of AGEs on epithelial to mesenchymal transition (EMT) of podocytes and expression of fibrotic markers were evaluated.

Results Using human podocytes and a mouse model, we demonstrated that AGEs activate Notch1 signaling in podocytes and provoke EMT. Inhibition of RAGE and Notch1 by FPS-ZM1 (N-Benzyl-4-chloro-N-cyclohexylbenzamide) and DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butylester), respectively, abrogates AGE-induced Notch activation and EMT. Inhibition of RAGE and Notch1 prevents AGE-induced glomerular fibrosis, thickening of the glomerular basement membrane, foot process effacement, and proteinuria. Furthermore, kidney biopsy sections from people with DN revealed the accumulation of AGEs in the glomerulus with elevated RAGE expression and activated Notch signaling.

Conclusion The data suggest that AGEs activate Notch signaling in the glomerular podocytes. Pharmacological inhibition of Notch signaling by DAPT ameliorates AGE-induced podocytopathy and fibrosis. Our observations suggest that AGE-induced Notch reactivation in mature podocytes could be a novel mechanism in glomerular disease and thus could represent a novel therapeutic target.

Significance of this study

What is already known about this subject?

- Diabetes is presented with elevated advanced glycation end-products (AGEs) in serum and tissues including the kidney.

What are the new findings?

- AGEs induce Notch activation in glomerular podocytes.
- Notch activation resulted in epithelial to mesenchymal transition of podocytes.
- Administration of AGEs resulted in glomerulosclerosis and proteinuria.
- Inhibition of receptor for AGEs or Notch activation abrogates AGE-induced proteinuria.

How might these results change the focus of research or clinical practice?

- Inhibitors of γ-secretase, a key enzyme that triggers Notch activation, could ameliorate AGE-induced Notch activation to prevent proteinuria in diabetic conditions.

INTRODUCTION

Glomerular podocytes are terminally differentiated visceral cells and provide epithelial coverage to the glomerular capillaries. Podocytes, owing to their unique structure and localization, regulate glomerular permselectivity, contribute to the glomerular basement membrane (GBM), and counteract intracapillary hydrostatic pressure. Therefore, podocytes are considered instrumental in regulating the normal function of the glomerulus and are indispensable for the ultrafiltration of blood and the formation of primary urine. Platelet-derived growth factor and vascular endothelial growth factor derived from podocytes are required for the maintenance of parietal epithelial cells and endothelial cells, respectively.[1 2] Podocyte injury and loss are the early cellular changes in glomerular diseases that are clinically evidenced by proteinuria and renal failure due to glomerulosclerosis.[3] The number of podocytes was found to decline in diabetic nephropathy (DN).[4] Since intact podocytes were identified from the urine of patients with proteinuria, it was proposed that podocytes could detach from underlying GBM.[5] The transition of podocytes from epithelial to highly motile

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Cell Death & Disease

ARTICLE Open Access

Growth hormone induces mitotic catastrophe of glomerular podocytes and contributes to proteinuria

Rajkishor Nishad[1], Dhanunjay Mukhi[1], Ashish Kumar Singh[1], Manga Motrapu[1], Kumaraswami Chintala[1], Prasad Tammineni[2] and Anil K. Pasupulati[1]

Abstract

Glomerular podocytes are integral members of the glomerular filtration barrier in the kidney and are crucial for glomerular permselectivity. These highly differentiated cells are vulnerable to an array of noxious stimuli that prevail in several glomerular diseases. Elevated circulating growth hormone (GH) levels are associated with podocyte injury and proteinuria in diabetes. However, the precise mechanism(s) by which excess GH elicits podocytopathy remains to be elucidated. Previous studies have shown that podocytes express GH receptor (GHR) and induce Notch signaling when exposed to GH. In the present study, we demonstrated that GH induces TGF-β1 signaling and provokes cell cycle reentry of otherwise quiescent podocytes. Though differentiated podocytes reenter the cell cycle in response to GH and TGF-β1, they cannot accomplish cytokinesis, despite karyokinesis. Owing to this aberrant cell cycle event, GH- or TGF-β1-treated cells remain binucleated and undergo mitotic catastrophe. Importantly, inhibition of JAK2, TGFBR1 (TGF-β receptor 1), or Notch prevented cell cycle reentry of podocytes and protected them from mitotic catastrophe associated with cell death. Inhibition of Notch activation prevents GH-dependent podocyte injury and proteinuria. Similarly, attenuation of GHR expression abated Notch activation in podocytes. Kidney biopsy sections from patients with diabetic nephropathy (DN) show activation of Notch signaling and binucleated podocytes. These data indicate that excess GH induced TGF-β1-dependent Notch1 signaling contributes to the mitotic catastrophe of podocytes. This study highlights the role of aberrant GH signaling in podocytopathy and the potential application of TGF-β1 or Notch inhibitors, as a therapeutic agent for DN.

Introduction

Glomerular complications are the predominant cause of end-stage kidney disease, and clinical conditions, such as diabetes and hypertension are associated with glomerular dysfunction and proteinuria. Glomerular podocytes are highly differentiated specialized visceral cells that account for ~30% of glomerular cells. These cells provide epithelial coverage to the capillaries and together with glomerular basement membrane (GBM) and perforated endothelial cells, constitute a glomerular filtration barrier. The unique cytoplasmic extensions of podocytes are known as foot processes, which attach to the GBM and interdigitate with neighboring foot processes to form the slit diaphragm (SD). The sophisticated architecture of SD contributes to the glomerular permselectivity. The process of progressive podocyte damage characterized by podocyte hypertrophy, detachment of podocytes, and, finally, irreversible loss of podocytes has been observed in human and experimental models of nephropathy and glomerular diseases[1]. Injury and depletion of podocytes, leading to podocyte insufficiency and capillary collapse, have been implicated in glomerulosclerosis and resulting chronic kidney disease.

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REVIEW

Detrimental effects of hypoxia on glomerular podocytes

Ashish K. Singh[1] · **Lakshmi P. Kolligundla**[1] · **Justus Francis**[1] · **Anil K. Pasupulati**[1]

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Abstract

Hypoxia-inducible factor1 (HIF1) plays a pivotal role in ensuring cells adapt to low-oxygen conditions. Depletion of oxygen, a co-substrate during hydroxylation of prolyl (P402 and P564) residues of HIF1α, evades HIF1α ubiquitination and enables its dimerization with HIF1β to mediate global transcriptional response to hypoxia. Though HIF1 is largely considered eliciting a protective role during physiological or pathological hypoxia or ischemia, elevated HIF1 during chronic hypoxia contributes to glomerular diseases' pathology and proteinuria. The glomerulus is responsible for renal permselectivity and excretion of ultra-filtrated urine. Podocytes are the glomerulus' major cell types and are instrumental for glomerular filtration, permselectivity, and glomerular basement membrane maintenance. Podocyte injury is expected to impair the efficiency of glomerular filtration and manifestation of glomerulosclerosis and proteinuria. Accumulated evidence suggests that podocytes are susceptible to various insults during chronic hypoxia, including podocyte EMT, slit-diaphragm dysfunction, foot process effacement, and cytoskeletal derangement due to accumulation of HIF1. This review discusses how hypoxia/HIF1 signaling regulates various features and function of podocytes during exposure to chronic hypoxia or inducing HIF1 by various chemical modulators.

Keywords Hypoxia · HIF · Podocyte · EMT · Slit-diaphragm · Proteinuria

Abbreviations

CKD	Chronic kidney disease
EMT	Epithelial-to-mesenchymal transition
EPO	Erythropoietin
ESRD	End-stage renal disease
GBM	Glomerular basement membrane
GFB	Glomerular filtration barrier
HIF	Hypoxia-inducible factor
MET	Mesenchymal to epithelial transdifferentiation
PHD	Prolyl hydroxylase
PKD	Polycystic kidney disease
SD	Slit-diaphragm

Key Points • Podocytes, which regulate glomerular permselectivity, are susceptible to hypoxic injury.

• Accumulation of HIF1α results in epithelial-mesenchymal transition of podocytes.

• HIF1α suppresses podocyte slit-diaphragm proteins and thus impairs their permselectivity.

• Podocyte compromises their shape due to cytoskeletal deformities induced by HIF1α.

Ashish K. Singh and Lakshmi P. Kolligundla contributed equally to this work.

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Introduction

Kidneys regulate several functions, including erythropoiesis, electrolyte, water, and acid–base balance, and are thus indispensable in ensuring the body's homeostasis. Human kidneys elicit these functions with the collective effort of ~ 2.0 million nephrons [27]. Nephron, the functional unit of the kidney, consists of two regions: glomerulus and tubule. The glomerulus is responsible for filtering water and small molecules from the blood to form primary urine. Whereas the tubular system ensures selective reabsorption and secretion, thus fine-tune the final composition of urine. Under normal conditions, kidneys help excrete ultra-filtrated urine with a tightly regulated composition [1].

Urinary excretion of albumin at varying degrees is a well-known predictor of renal outcome [2]. Microalbuminuria (30 to 300 mg/24 h) followed by macroalbuminuria (≥ 300 mg/24 h) over months to years indicate progressive renal function loss. Proteinuria for an extended period is

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ORIGINAL ARTICLE

Metformin prevents hypoxia-induced podocyte injury by regulating the ZEB2/TG2 axis

Lakshmi P. Kolligundla | **Rajesh Kavvuri** | **Ashish K. Singh** | **Dhanunjay Mukhi** | **Anil K. Pasupulati**

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Abstract

Aim: Podocytes, a vital component of the glomerular filtration barrier, are vulnerable to various noxious stimuli, including Hypoxic. HIF1α that transduces hypoxic adaptations induces Transglutaminase 2 (TG2), which catalyses cross-linking of extracellular matrix proteins. In this study, we investigated the mechanism of regulation of TG2 by HIF1α.

Methods: HIF1α was induced in podocytes by treating with FG4592 (Roxadustat) or hypoxia (1% oxygen) and in mice by treating with FG4592. Gene expression and protein analysis of ZEB2, TRPC6 and TG2 were performed in both experimental models. Histological and kidney function analyses were performed in mice.

Results: Data mining revealed co-expression of HIF1α, ZEB2, TRPC6 and TG2 in the chronic kidney diseases (CKD)-validated dataset. We observed elevated expression of ZEB2, TRPC6 and TG2 in FG4592-treated podocytes. Ectopic expression of ZEB2 resulted in high TRPC6 expression, elevated intracellular calcium levels and increased TG2 activity. Blocking the TRPC6 channel or inhibiting its expression partially attenuated FG4592-induced TG2 activity, whereas suppression of ZEB2 expression significantly abolished TG2 activity. Furthermore, we noticed the induction of the ZEB2/TRPC6/TG2 axis in podocytes in mice administered with FG-4592. Metformin ameliorated the HIF1α-induced podocyte injury and proteinuria in mice administered with FG-4592.

Conclusion: This study demonstrates that HIF1α stimulates both TG2 expression and activity via ZEB2/TRPC6 axis, whereas abrogation of HIF1α by metformin prevented hypoxia-induced glomerular injury. Metformin could be explored to treat proteinuric diseases such as CKD, sleep apnea and renal Ischemia–reperfusion-injury, where hypoxia is considered a risk factor.

KEYWORDS
HIF1α, metformin, podocytes, proteinuria, transglutaminase 2, ZEB2

Summary at a Glance

Accumulating HIF1α in podocytes resulted in elevated expression of ZEB2 and its target TRPC6. Elevated ZBE2 induces Transglutaminase (TG2) expression, whereas TRPC6 induces calcium influx and contributes to enhanced TG2 activity. Metformin ameliorated HIF1α induced ZEB2/TG2 axis and prevented hypoxia-induced podocyte injury and proteinuria.

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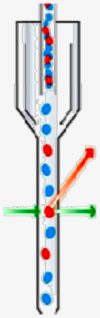

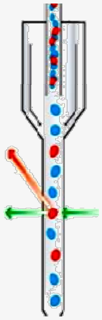

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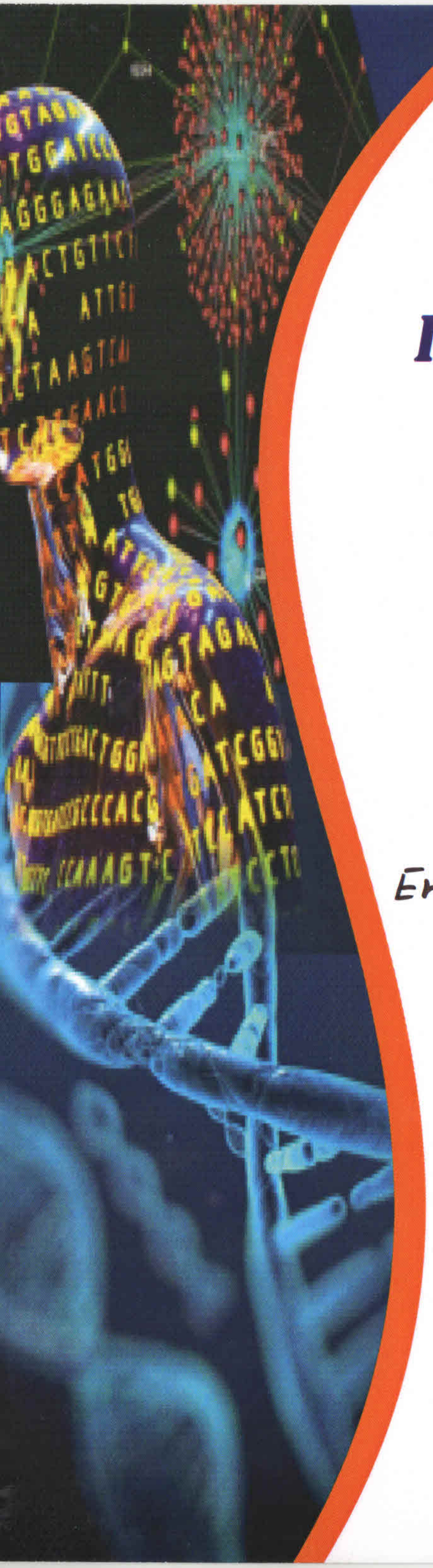

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Advanced glycation end-products induce podocyte injury and contribute to diabetic nephropathy

by Ashish Kumar Singh

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