STUDIES ON RECEPTOR FOR ADVANCED GLYCATION END-PRODUCTS PATHWAY IN DIABETIC NEPHROPATHY

Thesis submitted for the award of the degree of

Doctor of Philosophy

in

Cell Biology and Molecular Genetics

Under the faculty of Allied Health and Basic Sciences

by

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Under the supervision of

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2022

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ABREVIATIONS

ADAM10 – A Disintegrin and Metalloproteinase Domain-containing protein 10

AGE – Advanced Glycation End-products

ATP – Adeenosine triphosphate

CLR - C-Lectin-like Receptors

CML - N (6) carboxymethyl lysine

DAMP - Damage Associated Molecular Patterns

DIAPH1 – Diaphanous homolog-1

DN – Diabetic Nephropathy

DNA – Deoxyribose Nucleic Acid

ECM – Extracellular Matrix

Egr-1 – Early Growth Response

ERK – Extracellular Signal Regulated Kinase

esRAGE – Endogenously secreted Receptor for Advanced Glycation End-products

GCK - Glucokinase

GFR – Glomerular Filtration Rate

HNF4A – Hepatocyte Nuclear Factor – 4 Alpha

Ig - Immunoglobulin

IL – Interleukin

INS – Insulin

IRAK4 - Inteleukin-1 receptor-associated kinase-4

IRS1 – Insulin receptor Substrate 1

IκB – Inhibitor kappa B

JAK – Janus kinase

JNK – c- Jun N- terminal kinase

MODY - Maturity Onset Diabetes of the Young

MyD88 – Myeloid Differentiation Primary response 88

NF-κB – Nuclear factor kappa light chain enhancer of activated B cells

NLR - Nucleotide binding and oligomerization domain like Receptor

NOD - Nucleotide binding and oligomerization domain

PAMP - Pathogen Associated Molecular Patterns

PI3K – Phospho inositide 3-kinase

PKC – Protein Kinase C

PLC – Phospholipase

PRR - Pathogen Recognition Receptor

RAC1- Ras related C3 botulinum toxin substrate 1

RAGE - Receptor for Advanced Glycation End-products

RIG - Retinoic acid- inducible gene

RLR – RIG-like Receptors

RNA – Ribose Nucleic Acid

sRAGE – Soluble Receptor for Advanced Glycation End-products

STAT – Signal Transducers and Activators Of Transcription

T2DM - Type II diabetes mellitus

TIRAP – Toll/Inteleukin-1 Receptor Domain- Containing Adaptor Protein

TLR – Toll-like Receptors

TNF – Tumour Necrosis Factor

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ABSTRACT

Abstract

Nephropathy is a common complication of diabetes mellitus involving inflammatory damage to the nephrons. Hyperglycaemia is a hallmark of diabetes; and eventually leads to the formation of modified substances called advanced glycation end-products (AGEs). These sugar derivatives are potent activators of inflammation particularly via the receptor for advanced glycation end-products (RAGE) pathway. Inflammatory damage in nephropathy could be due to excessive inflammation particularly by the RAGE pathway and is indicated to be one of the major factors responsible for the development of diabetic nephropathy. Information about the disease mechanism is necessary to halt its progression. This study aimed to evaluate the functional status of the RAGE signalling pathway in diabetic nephropathy.

A three-group observational study involving diabetic nephropathy patients, type II diabetes mellitus patients, and healthy volunteers that included 59 individuals per group was conducted. Gene expression was measured by quantitative reserve transcription polymerase chain reaction and protein levels were measured by enzyme-linked immunosorbent assay.

The first objective of this study was to determine whether diabetic patients are intrinsically prone to produce excessive inflammatory cytokines when exposed to AGEs. To achieve this, blood samples from the study participants were treated with an AGE substance called carboxymethyl lysine (CML). The effect of this treatment was checked by measuring key mediators of the RAGE pathway viz., *NF-κB* gene expression and TNF secretion. The CML-induced fold change in *NF-κB* gene expression was

higher in diabetic nephropathy patients (2.8 times) followed by diabetic patients (2.5 times). The effect was lowest in the healthy volunteers (0.9 times). Similarly, CML-induced fold change in TNF secretion was highest in the diabetic nephropathy patients (1.76 times; p <0.0001; paired t-test) followed by diabetic patients (1.52 times; p <0.0001; paired t-test), and healthy volunteers (1.31 times; p <0.0001; paired t-test). This shows that diabetic patients show an excessive inflammatory response when exposed to CML. Furthermore, the response is highest among diabetic patients with nephropathy.

The next question was whether the excessive inflammatory response to AGEs arises due to poor regulation of the RAGE pathway. The binding of AGEs to membrane-bound RAGE is counterbalanced by the membrane-free form of this receptor called soluble RAGE (sRAGE). The soluble form arises from the membrane-bound receptor due to proteolytic shedding catalysed by the ADAM10 enzyme. This was evaluated by comparing sRAGE production, and *ADAM10* gene expression was compared in the three study groups. Insulin was used to induce sRAGE production and *ADAM10* gene expression in blood cells. The insulin-induced fold change in sRAGE production was lowest in diabetic nephropathy patients (1.2; p <0.0001; paired t test), followed by diabetic patients (1.4; p <0.0001; paired t test). The effect was highest in the healthy volunteers (1.8; p <0.0001; paired t test). This indicates that sRAGE production shows a diminished response to insulin in diabetic patients. Furthermore, insulin treatment resulted in upregulation of *ADAM10* gene expression in healthy volunteers (1.25 times) but not in diabetic nephropathy patients (0.93 times) or

diabetic patients (1.0 times). These results indicate that the major regulators of the RAGE pathway are diminished in diabetic patients.

This study shows that patients with diabetes, particularly those with nephropathy, are hyperresponsive to CML. This hyper-responsiveness may be due to poor regulation of the RAGE pathway. Together, these results show that diabetic patients are intrinsically prone to excessive inflammation when exposed to AGEs. Therefore, modulation of the RAGE pathway may play a prophylactic role in preventing renal complications in diabetic patients. However, the conclusion has to be considered preliminary proof-of-principle and not confirmatory. This is because the RAGE pathway is highly complex and involves several mediators and regulators. Herein, only the key mediators and regulators were checked. A comprehensive analysis is necessary to confirm our hypothesis.

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CHAPTER I
INTRODUCTION

Type II diabetes mellitus (T2DM) is a common metabolic disorder characterised by hyperglycaemia (Rojas-Carranza *et al*., 2018, Kaur *et al*., 2020). It is a major public health burden that affects over 537 million people worldwide and continues to increase at an alarming prevalence of 9% (Saeedi *et al*., 2019, Shah *et al*., 2021, IDF Diabetes Atlas, 2021). India is one of the epicentres of the global diabetes epidemic, with the second-highest diabetes population in the world (~77 million) and a prevalence of 7% (IDF Diabetes Atlas, 2021)

Uncontrolled hyperglycaemia damages the vasculature and can lead to dysfunction of multiple organ systems (Rask-Madsen and King, 2013; Daryabor *et al*., 2020). This vascular damage can further lead to macrovascular complications such as cardiovascular and cerebrovascular disease and microvascular complications such as retinopathy, nephropathy and peripheral neuropathy (Chawla *et al*., 2016). The most common macrovascular complication of T2DM is cardiovascular disease (Viigimaa *et al*., 2020). Among the microvascular complications, nephropathy stands first in prevalence (Barrett *et al*., 2017). Approximately 20-40% of patients with diabetes eventually develop nephropathy (Gheith *et al*., 2015).

Diabetic nephropathy (DN) is characterised by the progressive loss of renal function due to nephron injury. If left untreated, DN may lead to complete loss of renal function, a condition referred to as end-stage renal disease (Romagnani *et al*., 2017, Chen *et al*., 2020).

Excessive inflammation, particularly *via* the Receptor for Advanced Glycation End-products (RAGE) pathway, is indicated to be one of the major factors responsible for the development of DN (Manigrasso *et al*., 2014, Sanajou *et al*., 2018). The RAGE pathway begins with the activation of the cell surface receptor by advanced glycation end-products (AGEs) (Perrone *et al*., 2020). AGEs are metabolic byproducts that arise due to nonenzymatic glycation and subsequent oxidation of proteins and lipids (Fishman *et al*., 2018). The cognate binding of AGEs to RAGE elicits a signal cascade that activates a transcription factor called nuclear factor kappa B (NF-κB), which then leads to the production of proinflammatory cytokines such as tumor necrosis factor (TNF) (Liu *et al*., 2017, Dariya & Nagaraju, 2020).

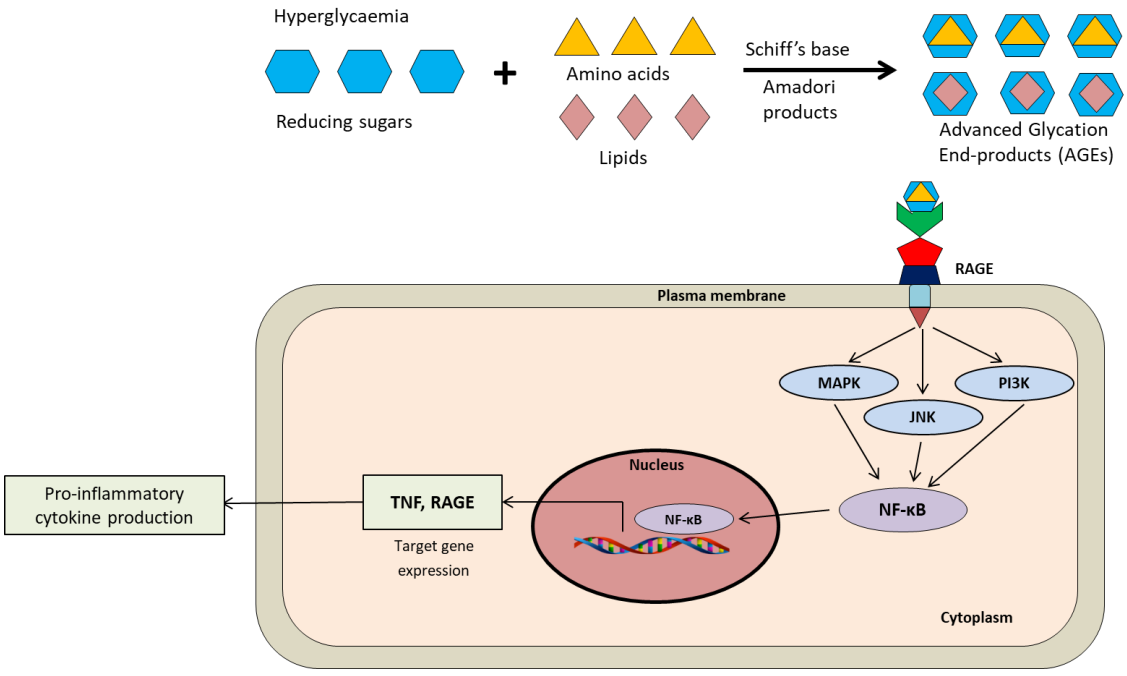

Figure 1: Schematic representation of the RAGE pathway

Current evidence indicates that the RAGE pathway is overactive in DN. Excessive formation of AGEs due to a chronic hyperglycaemic state is considered to be the major reason for this (Parwani & Mandal, 2020). However, the role of other factors, such as the hyperresponsiveness and counterregulation of the RAGE pathway, has not been explored in DN. Therefore, this study aimed to evaluate the functional status of the RAGE signalling pathway in DN.

CHAPTER II

SCOPE, RATIONALE, AND SIGNIFICANCE

SCOPE

Inflammation plays an important role in the pathogenesis of DN (Moreno *et al*., 2018). The overactive RAGE pathway is considered to be responsible for triggering excessive inflammation in DN (Wu *et al*., 2021). Current evidence indicates that the RAGE pathway is overactive in DN due to the excessive formation of AGEs (Sourris *et al*., 2021). The AGE levels are abnormally high in DN due to the underlying hyperglycaemic state. In other words, the RAGE pathway is overactive in DN due to abnormally high levels of RAGE agonists. Elevated AGE levels are an extrinsic factor for the RAGE pathway. In addition to extrinsic factors, intrinsic factors such as sensitivity and regulation may also contribute to overactive RAGE. However, there is limited information in this direction, and hence, this study was undertaken.

RATIONALE

The sensitivity of the RAGE pathway may be relatively higher in DN such that a comparable amount of the agonist can trigger an excessive cellular response. This hyper-responsiveness may involve upregulation of the key transcription factors of the RAGE pathway, such as NF-κB, which in turn results in elevated secretion of proinflammatory cytokines (Hudson and Lippman, 2018). This aspect was evaluated in this study by comparing the AGE-induced expression of *NF-κB* and secretion of proinflammatory cytokines. TNF was selected as the representative cytokine since it is the major cytokine implicated in DN (Shen *et al*., 2020). N(6) carboxymethyl lysine (CML) was used as the

representative AGE since it is the common AGE formed in T2DM patients (Perrone *et al*., 2020). These experiments were carried out using whole blood samples. Blood samples were used as a surrogate because the reavailability of kidney biopsies from study participants was not feasible due to ethical reasons. RAGE is known to be expressed on the surface of blood cells such as monocytes, lymphocytes, and natural killer cells. Furthermore, the aim of this study was to examine the intrinsic factors related to the RAGE pathway. As intrinsic factors are genetically determined, the behaviour of the RAGE pathway is likely to be comparable in renal and blood cells.

The hypothesis of this study was that a fixed amount of CML would induce relatively higher levels of *NF-κB* expression and TNF secretion in the blood samples of DN than in T2DM and healthy volunteers. In other words, samples from DN are expected to show hyperresponsiveness to CML compared to T2DM and healthy volunteers. The research hypothesis is schematically represented in Fig 2.1.

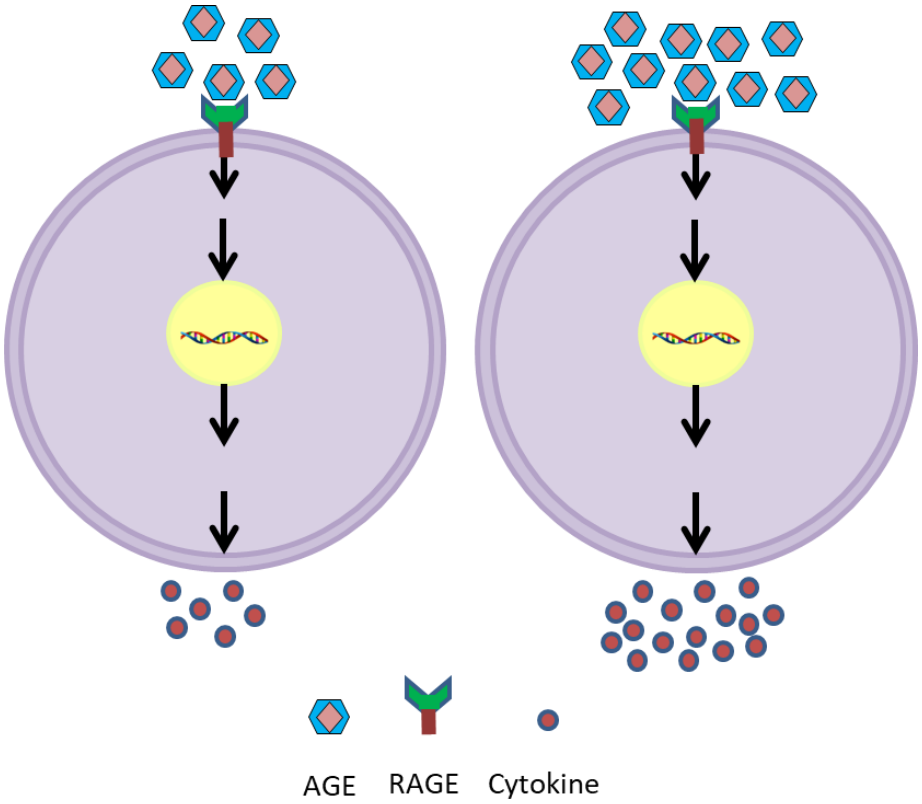

Figure 2.1.A.: Elevated AGE levels result in overactive RAGE pathway

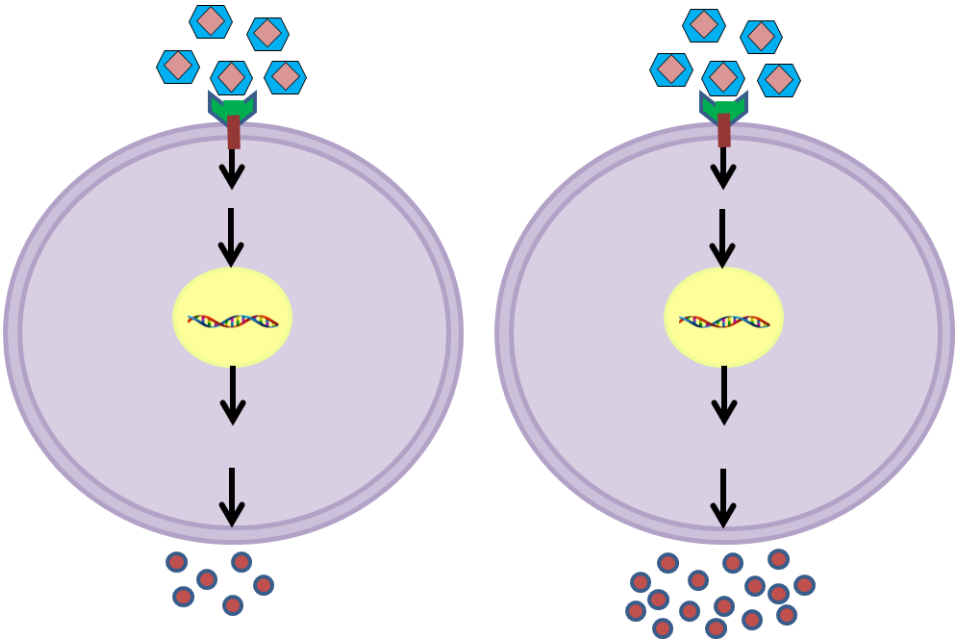

Figure 2.1.B.: Hyper-responsiveness of RAGE to AGE

Activation of the RAGE pathway by AGE agonists is regulated by soluble RAGE (sRAGE) (Erusalimsky, 2021). This form arises due to the proteolytic cleavage of the extracellular domain of membrane-bound RAGE. Proteolysis is catalysed by a membrane-bound protease called **A Disintegrin and Metalloproteinase Domain-containing protein 10 (ADAM10**) (Lee *et al*., 2015). The process of proteolytic release of the ectodomain is referred to as shedding, and the corresponding protease is also referred to as the sheddase (Braley *et al*., 2016). sRAGE retains the capacity to bind AGEs. However, the binding of AGEs to sRAGE does not activate the intracellular signal transduction pathway (Steenbeke *et al*., 2020). Because of this relationship, reduced sRAGE production allows larger amounts of AGEs to bind to RAGE and activate the downstream inflammatory pathway. Thus, the induction of inflammatory signal transduction *via* membrane-bound RAGE is regulated by sRAGE. In other words, sRAGE serves as a decoy to competitively inhibit the binding of AGE ligands to RAGE. This aspect was evaluated in this study by comparing the levels of sRAGE in blood samples collected from DN, T2DM, and healthy volunteers.

The *ADAM10* gene has been shown to be regulated by insulin through the ERK pathway, which activates the transcription factor called upstream factor 1 (Hu *et al*., 2016). Therefore, insulin was used in this study to activate ADAM10 and sRAGE production.

The hypothesis of this study was that insulin treatment of blood samples would result in the upregulation of *ADAM10* expression and elevated sRAGE production in DN compared with T2DM and healthy volunteers. The research hypothesis is schematically represented in Fig 2.2.

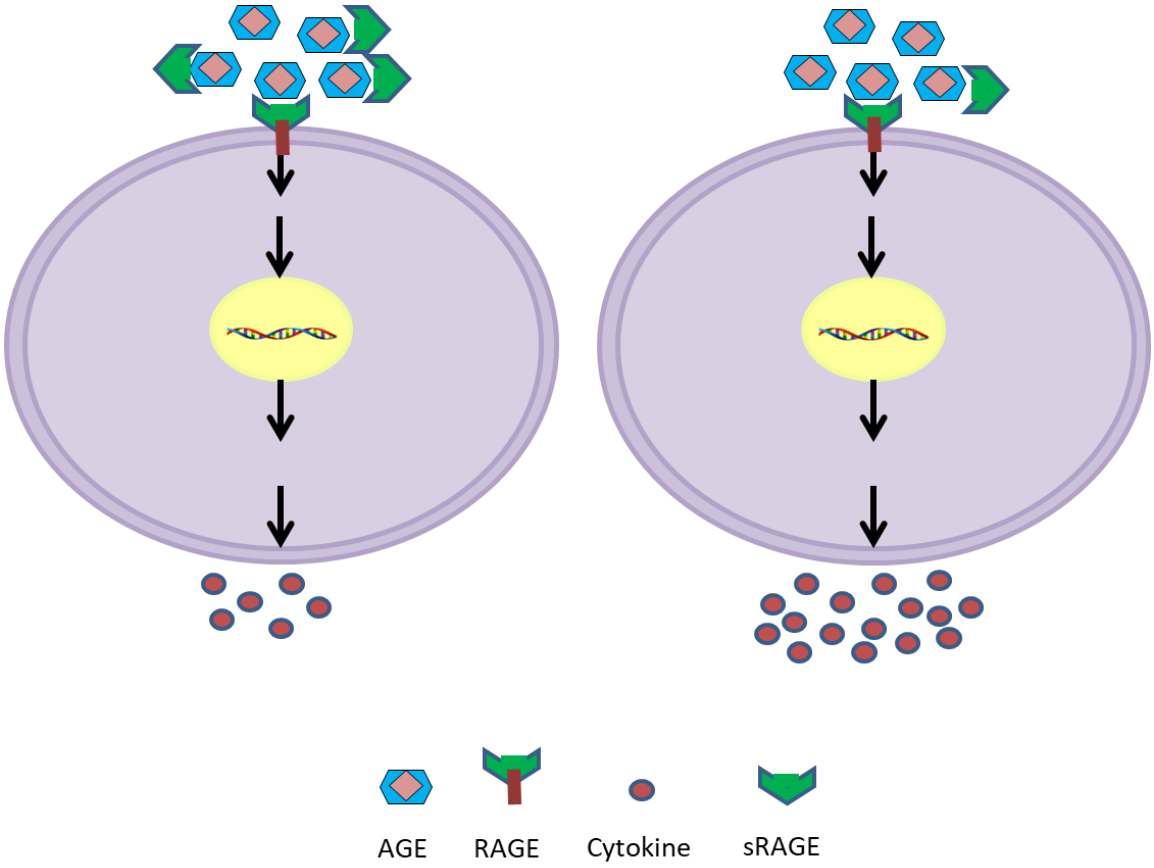

Figure 2.2.: Regulation of RAGE pathway

SIGNIFICANCE

DN is a leading cause of end-stage renal failure. DN contributes to an 80% mortality rate of end-stage renal failure (Thomas *et al.*, 2015). Therefore, the development of prevention and treatment strategies is of vital importance.

The purpose of this study was to evaluate whether the RAGE pathway is abnormally hyperresponsive to AGE substances by evaluating the AGE-induced gene expression of *NF-κB* and TNF production. Additionally, we evaluated whether sRAGE production and *ADAM10* gene expression are affected by insulin stimulation. This would confirm that increased activation of RAGE is due to reduced sRAGE production. The results of this study will also contribute to the elucidation of the molecular mechanism by which RAGE plays a role in the pathogenesis of DN.

If RAGE is found to be hyperresponsive, then anti-RAGE therapies can be developed to counter the development of DN. This study will also establish the role of ADAM10 and insulin in the shedding of RAGE and will contribute to understanding the mechanistic basis for the reduction in sRAGE levels in DN. The knowledge generated in this study will be helpful in developing therapeutic strategies for ameliorating the progression of T2DM into DN

CHAPTER III
AIM AND OBJECTIVES

3.1. Aim

To evaluate the functional status of the RAGE pathway in diabetic nephropathy

3.2. Research questions

a. Is the RAGE pathway hyper-responsive to AGE in DN patients?

b. Is the expression level of ADAM10 reduced in DN patients?

c. Does insulin-induced RAGE shedding decrease in DN patients?

3.3. Objectives

a. To compare the responsiveness of RAGE to carboxymethyl lysine in patients with diabetic nephropathy, type II diabetic patients and healthy volunteers.

b. To compare the level of ADAM10 expression in diabetic nephropathy, type II diabetic patients and healthy volunteers.

c. To compare the level of insulin-induced mRAGE shedding in diabetic nephropathy, type II diabetic patients and healthy volunteers.

CHAPTER IV

REVIEW OF LITERATURE

Diabetes burden and its types

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia (Yaribeygi *et al*., 2020). It is a major public health burden both in India and across the world (Tandon *et al*., 2018). The prevalence of diabetes in India is approximately 11 to 14% in urban areas and approximately 3 to 8% in rural areas (Mohan *et al.,* 2017). Globally, the prevalence is approximately 7% (IDF Diabetes Atlas, 2021).

Classification of diabetes mellitus

Diabetes mellitus is classified based on the pathogenic process that leads to hyperglycemia (Baynest, 2015). The four categories of diabetes mellitus are designated as follows:

a) Type I Diabetes Mellitus
b) Type II Diabetes Mellitus
c) Gestational Diabetes Mellitus
d) Maturity onset diabetes of the young

Type I diabetes mellitus

Type I diabetes mellitus arises as a result of insulin deficiency (Hamilton *et al*., 2017). It occurs when insulin-producing β-cells are destroyed by the immune system, resulting in a reduction in insulin synthesis (Roep *et al*., 2020). Type I diabetes is associated with genetic factors and the production

of autoantibodies that destroy pancreatic β-cells (Lucier and Weinstock, 2021). Type I diabetes is mainly managed by insulin replacement therapy using recombinant insulin (Sapra and Bhandari, 2021).

Type II diabetes mellitus (T2DM)

T2DM mainly arises due to insulin resistance (Galicia-Garcia *et al*., 2020). Insulin resistance is the impaired response to insulin stimulation by target tissues, mainly liver, muscle, and adipose tissue. Insulin resistance impairs glucose utilisation, resulting in an increase in β cell insulin production and hyperinsulinemia (Freeman and Pennings, 2022). Pancreatic β cells initially compensate for insulin resistance by upregulating the secretion of insulin. Prolonged compensation of insulin by β cells can lead to its failure, in turn affecting insulin production (Boland *et al*., 2017).

Gestational Diabetes Mellitus

Hyperglycemia that develops during pregnancy is known as gestational diabetes mellitus (Mirghani Dirar and Doupis, 2017; Ploughs *et al*., 2018). Gestational diabetes arises due to insulin resistance as a consequence of placental hormonal release. Human placental lactogen is the main hormone related to increased insulin resistance in this condition. Other hormones related to the development of this disease are growth hormone, prolactin, corticotropin-releasing hormone, and progesterone (Napso *et al*., 2018). Placental hormones reduce the sensitivity of insulin receptors, thereby

leading to insulin resistance and hyperglycaemia in pregnancy. The condition is transient in nature and resolves upon completion of the pregnancy (Kampmann *et al.*, 2019).

Maturity onset diabetes of the young (MODY)

MODY is caused by defects in pancreatic islet cell development that impair insulin secretion. This arises due to genetic mutations in genes that influence insulin signalling and glucose metabolism in beta cells. MODY follows an autosomal dominant inheritance pattern. Some of the important genes that contribute to MODY include *GCK, HNF4A, INS,* etc. (Skoczek *et al*., 2021).

Complications of diabetes mellitus

Uncontrolled hyperglycaemia eventually results in damage to several tissues, with the major impact being on the vasculature (Bharti *et al.* 2018). Vascular complications are categorised into two types, mainly microvascular and microvascular complications (Beckman and Creager, 2016).

Macrovascular complications

Macrovascular complications involve damage to larger blood vessels, such as coronary arteries, peripheral arteries, and vessels of the brain (Huang et al., 2017).

Coronary artery disease arises due to atherosclerosis (Yuan *et al*., 2019). Atherosclerosis is characterised by increased lipid deposition in the subendothelial layer of large blood vessels. The continuous deposition of lipids on the inner lining of blood vessels and subsequent calcification leads to plaque formation. This reduces the lumen of the blood vessel, leading to decreased blood supply and resulting in ischemia and organ damage (Yamagishi and Matsui, 2018). Hyperglycemia increases the rate of plaque formation by inhibiting enzymes involved in the dilation of blood vessels, thereby resulting in vascular endothelial dysfunction (Gero, 2018).

Peripheral artery disease is characterised by the formation of atherosclerotic plaque in the vessels that supply blood to the lower extremities. As a consequence, there is reduced flow of oxygenated blood to the limbs, resulting in ischemia. Hyperglycaemic conditions accelerate plaque formation by inducing vascular endothelial dysfunction (Gero, 2018).

Uncontrolled hyperglycaemia is a risk factor for ischaemic stroke. Hyperglycemia exerts direct lipid peroxidation, increasing the formation of atherosclerotic plaque in major blood vessels of the brain, and as a consequence, there is reduced flow of oxygenated blood to the brain, resulting in ischemia (Hill, 2014).

Microvascular complications

Microvascular complications mainly involve damage to capillaries. The common microvascular complications are diabetic retinopathy, diabetic neuropathy and diabetic nephropathy (Pillinger and Kam, 2017; Faselis et al., 2020).

Diabetic Retinopathy

Diabetic retinopathy is a complication that affects the eyes. It is caused by damage to the capillaries that supply the retinal region of the eye. This condition eventually leads to blindness. Hyperglycemia activates various pathways that result in increased vascular permeability and microvascular occlusion. As a consequence, there are intraretinal microvascular abnormalities and neovascularization (Gui *et al*., 2020).

Diabetic neuropathy

Diabetic neuropathy is a neurodegenerative disorder that can affect sensory, autonomic and motor axons. This condition results in loss of sensory function in the lower extremities of the body (Bodman and Varacallo, 2022). Nerve damage arises because of hyperglycaemic-induced damage to the capillaries that supply blood to these nerves (Feldman *et al*., 2019).

Diabetic nephropathy

Diabetic nephropathy (DN) is a microvascular complication affecting the kidneys (Lim, 2014). It is the progressive loss of renal function due to inflammatory damage of the nephrons (Andrade-Oliveira *et al*. 2019). DN is characterised by the development of proteinuria and a decreased glomerular filtration rate (Gheith *et al*. 2015).

Burden of diabetic nephropathy

DN is a common microvascular complication of diabetes. Approximately 25% of patients with T2DM eventually develop nephropathy (Alicic *et al*. 2017). If left untreated, DN can lead to a fatal condition called end-stage renal failure (Umanath and Lewis, 2018).

Stages of diabetic nephropathy

DN progresses through several distinct stages, beginning with a microalbuminuria phase and a terminal phase characterised by clinical proteinuria terminating in end-stage renal failure (Samsu, 2021).

Early symptoms of DN are hyperfiltration and the development of nephromegaly (Tonneijck, *et al.* 2017). Due to presistant hyperglycaemia, the filtration load on the kidney increases, which leads to alterations in kidney function. decrease in glomerular filtration rate (GFR), which is detected by increased creatinine clearance. This leads to renal hypertrophy (Anguiano Gómez *et al.* 2018).

A significant hallmark of DN is the appearance of microalbuminuria (30-300 mg/24 hrs), which is associated with proteinuria, which progresses into macroalbuminuria (>300 mg/24 hrs) (Persson and Rossing, 2017). Patients with constant and persistent proteinuria for an extended period of time eventually progress to the advanced stages of DN (de Boer *et al.* 2020). At this point, there is loss of albumin in the urine, resulting in low serum albumin. Eventually, this leads to the development of edema and a decline in GFR. This leads to end-stage renal failure (Bobkova *et al.*, 2016). The rate at which the loss of renal function occurs varies with each individual, and some enter the end stage 2 to 3 years after the onset and some at 15 years after the onset of diabetes (Alicic *et al.*, 2017). The various stages of DN are discussed in table 1.

Table 1: Stages of diabetic nephropathy

Stage	Characteristic feature	Diabetes duration (yr)	Urinary Albumin Excretion Rate (µg/min)	Glomerular Filtration Rate (ml/min)	Signs
I	Increase in size & activity of the kidney	At diagnosis	Absent	90	Enlarged kidney, higher blood flow and rate of filtration
II	Initial changes in kidney tissue	2-5	<20	60-89	Thickening of basal membrane
III	Onset of nephropathy	5-15	20-200	30-59	Micro-albuminuria, rise in blood pressure
IV	Clinical manifestation of nephropathy	10-25	≥ 200	15 -29	Macro-albuminuria, falling blood flow and filtration rate, high blood pressure
V	Renal insufficiency	15-30	≥ 200	< 15	Rise in serum creatinine, near permanent hypertension

Histological changes

DN is morphologically characterised by thickening of the glomerular basement membrane, a special type of basement membrane that lines the glomerular epithelial cells (podocytes) in the kidney (Marshall, 2016). The glomerular basement membrane is composed of glycoproteins and proteoglycans. The major components of the glomerular basement membrane include collagen, laminin, and glycosaminoglycans, such as heparan sulphate, keratin sulphate, dermatan and chondroitin sulphate, which cover the luminal surface of glomerular endothelial cells (Chew and Lennon, 2018). Proteoglycans include glycosaminoglycan chains and protein cores that are secreted in the extracellular matrix or maintained at the cell surface (Wight, 2018). Glycosaminoglycans are hydrated with negatively charged molecules and have important roles in the barrier; reduced glycosaminoglycans lead to glomerular hyperfiltration and proteinuria in DN (Pourghasem *et al*., 2015).

The structure of the glomerular filtration barrier includes the fenestrated endothelium, glomerular basement membrane, podocyte foot processes and slit diaphragms (Daehn and Duffield, 2021). Reduction or structural changes in one or more elements of this complex can lead to proteinuria (Ebefors *et al*., 2021). Podocytes have an important and core role in the glomerular filtration barrier. Its foot processes interdigitate with each other and combine with the foot processes of neighboring podocytes to

create a physical barrier (Reiser and Altintas, 2016). In DN apoptosis, loss of podocytes has been observed (Wang *et al*., 2019).

In addition, there is also an expansion of the extracellular matrix surrounding the mesangial cells. Mesangium hypertrophy includes mesangial cell hyperplasia and oversecretion of ECM components. Mesangial expansion causes the collapse of the lumen of the capillaries (Ebefors *et al*., 2022). As a result, glomerular volume increases. The increase in IgG and IgM, complement C and fibrin leakage in the glomerulus results in the presence of their sediments in the ECM but also stimulates basement membrane proliferation and inflammation (Pourghasem *et al*., 2015).

Cellular proliferation in the kidney specifically in proximal tubules is also a characteristic histological change in DN (Alicic *et al*., 2017). Numerous growth factors, such as insulin-like growth factor 1, platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor (EGF), contribute to the early proliferation of renal tubules (Shi *et al*., 2018).

Another histological change in the diabetic kidney is the thickening of the basement membrane in the glomerulus and tubules, and the progressive accumulation of extracellular matrix components occurs due to an increase in gene expression and protein synthesis, such as collagen IV, laminin, and

fibronectin (Bülow and Boor, 2019). All these histological changes contribute to the development of glomerulrosclerosis (scaring in the kidney) and are the final and most significant alteration in DN.

The hyperglycaemic state induces biochemical changes in the components of the glomerular basement membrane, such as chemical reduction of laminin, heparin sulphate and other sulphated glycosaminoglycans (Gowd *et al.* 2016). In addition, there is an accumulation of collagen and proteins in the glomerular basement membrane. These changes lead to glomerulosclerosis and basement membrane expansion. The scarring of the tissue and expansion of the membrane can affect the filtration process, and these changes result in the gradual loss of renal function (Chen *et al.* 2020).

Role of inflammation in DN

Inflammation plays an essential role in the development of DN. This conclusion is based on the following observations:

a) Infiltration of inflammatory cells in the kidney
b) Elevated expression of proinflammatory cytokines
c) Elevated expression of growth factors

Infiltration of inflammatory cells into the kidney

Leukocyte infiltration of the kidneys is a common feature in animal models, and its association with disease progression is similar to human diabetic nephropathy (Lim and Tesch, 2012). More than 90% of leukocytes that infiltrate the kidneys are macrophages (Galkina et al., 2006; Kinsey and Okusa, 2012). T cells, macrophages, and dendritic cells are among the most important cell types contributing to the inflammation-mediated acceleration of DN progression (Zheng and Zheng, 2016). Macrophage infiltration hastens the progression of glomerular sclerosis in experimental diabetic kidney models (Guiteras *et al*., 2019, Tian and Chen, 2015). The accumulation of macrophages and T cells in the glomeruli and interstitium of the kidney has been reported (Galkina and Ley, 2006, Lim and Tesch). Chen and coworkers (2022) assessed the various populations of immune cells in both healthy and diabetic kidney biopsies and found that cytotoxic lymphocytes and myeloid dendritic cells were the most common. Further analysis showed that the abundance of almost all immune cell populations in the DN samples was significantly higher than that in the healthy samples.

Elevated expression of proinflammatory cytokines

Chemokines and cytokines are a group of plasma proteins that regulate the inflammatory response.

Chemokines are responsible for attracting immune cells to the site of injury. Fufaa and coworkers (2015) evaluated monocyte chemoattractant protein 1 levels in urine and renal biopsies of DN patients and found that monocyte chemoattractant protein 1 levels were elevated. Further studies by Nadkarni *et al*. (2016) reported that urinary monocyte chemoattractant protein 1 levels are associated with deterioration of renal function. A similar trend was also reported by Satirapoj and coworkers (2018). Monocyte chemoattractant protein 1 is an important chemokine that is responsible for the migration of monocytes and the accumulation of macrophages in the kidneys of DN patients. (Proudfoot, 2015, Lee *et al*., 2017, Sarker *et al*., 2020).

Another important chemokine called colony-stimulating factor 1 has been linked to DN. Lenda *et al*. (2003) observed that colony-stimulating factor 1 levels were higher in renal biopsies in a diabetic mouse model. Colony-stimulating factor 1 is responsible for the survival, proliferation, and differentiation of macrophages, contributing to the sustenance of macrophages in the kidneys.

Several important cytokines, such as TNF, IL-6, and IL-18, have been shown to be elevated in DN (Donate-Correa et al., 2020). The following cytokines and their implications in DN are discussed in detail below:

TNF has been reported to be elevated in both the serum and urine of patients with DN compared with nondiabetic individuals or diabetic subjects without renal disease (Chen *et al*., 2017). Furthermore, Cheng and coworkers (2021) showed that TNF expression is elevated in the renal biopsies of patients with DN. This finding indicates that abnormal gene expression in the kidney may be one of the major sources of TNF in DN.

IL-6 cytokine levels have also been shown to be increased in DN. Serum IL-6 levels are higher in patients with DN than in diabetic patients without renal disease (Taslipinar *et al*., 2011, Magno, *et al*., 2019). Furthermore, Su and coworkers (2017) showed that IL-6 gene expression is elevated in the renal biopsies of DN patients. This finding indicates that abnormal gene expression in the kidney may be one of the major sources of IL-6 in DN.

IL-18 cytokine levels were also found to be increased in the serum and urine of DN patients (Moriwaki *et al*., 2003). Plasma IL-18 levels in patients with DN were elevated compared to those in diabetic patients without renal complications and healthy volunteers (Wong *et al*., 2007). This finding indicates that IL-18 levels are abnormal in DN.

Elevated expression of growth factors

Several growth factors have been implicated in the pathogenesis of DN. Transforming growth factor beta, connective tissue growth factor, and vascular endothelial growth factor are the most common (Shi *et al*., 2018).

Transforming Growth Factor beta gene expression has been shown to be higher in DN patients compared to non-diabetics by Liu and Li, 2019. Transforming Growth Factor beta promotes renal fibrosis by increasing collagen synthesis, suppressing extracellular matrix degradation, promoting collagen cross-linking, and endothelial cell de-differentiation (Zhou *et al*., 2020).

Elevated levels of Connective Tissue Growth Factor in urine and kidney biopsies of diabetic mouse model have been reported by Gerritsen *et al*., (2015). Furthermore, the authors also observed increased Connective Tissue Growth Factor expression in the glomeruli and medullary tubules of the kidneys of diabetic mouse model. A similar trend in renal biopsies of DN patients was observed by Yin and Liu, (2019). Connective Tissue Growth Factor is involved in the regulation of tissue fibrosis.

Expression of Vascular Endothelial Growth Factor was reported to be elevated in glomerular podocytes and tubular cells in diabetic mouse model (Majumder and Advani, 2017). Several studies have reported that the increased levels of Vascular Endothelial Growth Factor in both renal

biopsies and urine in DN patients (Kanesaki *et al*., 2005; Kim *et al*., 2005; Hanefeld *et al*., 2016). Vascular Endothelial Growth Factor is responsible for endothelial cell growth and differentiation, and is critical for vascular permeability and angiogenesis (Zhang *et al*., 2020).

Cellular and molecular basis of inflammation

Inflammation is an innate immune response that resolves tissue damage triggered by a variety of factors, including pathogens and toxic compounds (Chen *et al*., 2017). The inflammatory response is the coordinated activation of signalling pathways that regulate inflammatory mediator levels in resident tissue cells and inflammatory cells (Sugimoto *et al*., 2016). The common mechanism involved in the inflammatory response is summarised below:

a) Recognition of stimuli by cell surface pattern receptors
b) Activation of inflammatory pathways
c) Release of inflammatory markers and chemotaxis
d) Resolution

Recognition of stimuli by cell surface pattern receptors

The inflammatory cascade is initiated by the 'Recognition of infection or damage'. This is typically achieved by the detection of Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Patterns (DAMPs) by the Pattern Recognition Receptors (PRRs) (Zindel and Kubes,

2020). PRRs activate common signalling pathways that culminate in the activation of transcription factors like NF-κB (Liu *et al*., 2017).

Activation of inflammatory pathways

The second step is signal transduction through the transcription factor NF-κB, which is found in all cell types and remains in its inactive state when it is bound to its inhibitor protein, IκB. Upon signal transduction, NF-κB is released from IκB and translocates to the nucleus, where transcription takes place by binding NF-κB to target promoter regions. Prior to transcription, there is translation of proinflammatory cytokines such as IL-1-β, IL-6, and TNF (Yu *et al*., 2020).

Release of inflammatory cytokines and chemotaxis

The release of proinflammatory cytokines affects the cellular processes of other cells. In conjunction with chemokines and various costimulatory molecules, cytokines facilitate the recruitment of effector cells, such as monocytes and neutrophils, to the site of injury or infection. These cells create a cytotoxic environment by further releasing toxic chemicals from their cytoplasmic granules through a process called degranulation (Arango Duque and Descoteaux, 2014). The degranulation process and the rapid release of chemicals require the consumption of both glucose and oxygen, known as a "respiratory burst", and toxic chemicals include reactive oxygen species, reactive nitrogen species, and various other proteinases. These

substances are destructive to both pathogens and hosts (Kany *et al*., 2019). The net effect of these interactions culminates in the cardinal signs of local inflammation, i.e., heat, swelling, redness, pain and loss of function.

Resolution

The final stage of inflammation is resolution, which is a critical step to limit collateral damage to the host. After the first few hours of inflammation, a coordinated programme of resolution is set into motion by tissue resident and recruited macrophages (Watanabe *et al*., 2019). During acute inflammation, these cells produce proinflammatory prostaglandins and leukotrienes but rapidly switch to lipoxins, which block further neutrophil recruitment and instead favor the infiltration of primed macrophages important for wound healing (Sugimoto *et al*.2016).

Mediators of inflammation

Inflammation is a protective response that ensures the removal of harmful stimuli, with a subsequent healing process for the repair of damaged tissue (Sugimoto *et al.*, 2016). The inflammatory process involves an array of proteins that help bring about homeostasis (Liu *et al.*, 2017). Some of the key players include PAMPs, DAMPs, and PRRs (Amarante-Mendes *et al*., 2018).

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Pathogen-associated molecular patterns (PAMPs)

PAMPs are assorted microbial molecules that have different "patterns" or motifs that are recognised by immune cells (Carrillo *et al*., 2017). PAMPs initiate a pathogen-induced inflammatory response (Li and Wu, 2021). PAMPs activate innate immune responses that protect the host from infection through recognition by PRRs (Johswich, 2017). PAMPs are micromolecular conserved patterns within a class of microbes that are derived from microbial components as follows:

a) Cell wall components such as peptidoglycan and lipopolysaccharide
b) Microbial DNA and RNA
c) Flagellin
d) Viral proteins from envelope and capsid
e) Glycolipids and glycoproteins

Damage-associated molecular patterns (DAMPs)

Damage-associated molecular patterns are also known as danger-associated molecular patterns, danger signals or alarmins (Roh and Sohn, 2018). DAMPs are biomolecules that can trigger a noninfectious inflammatory response known as "sterile inflammation" (Chen *et al.*, 2017). DAMPs vary greatly depending on the type of cell and injured tissue (Vénéreau *et al.*, 2015). DAMPs move from a reducing to an oxidising milieu, which results in their denaturation and triggers immune cells to detect the molecular pattern and induce an immune response (Relja and Land, 2020). DAMPs are either nuclear or cytosolic proteins that are released into the extracellular environment as a result of tissue injury (Schaefer, 2014). DAMPs are categorised into:

a) Protein DAMPs: Heat shock proteins, HMGB1 (high mobility box-1), and hyaluronan fragments.
b) Nonprotein DAMPs: uric acid, heparin sulphate and DNA.

PRR and its action

PRRs are the main receptors that play a role in inflammation (Kumar, 2019). These receptors are germline encoded receptors that respond to highly PAMPs and DAMPs (Jang *et al.*, 2015). PRRs are present on innate immune cells, including dendritic cells, monocytes, neutrophils, cytotoxic natural killer cells, macrophages and epithelial cells (Gasteiger *et al.*, 2017).

Classification of PRRs based on localisation

PRRs can be expressed on cell surfaces in the cytoplasm and have specificity for PAMPs and DAMPs from a variety of sources (Li and Wu, 2021).

a) Membrane-bound PRRs: Toll-like receptors (TLRs), C-Lectin-like receptors (CLRs)
b) Cytoplasmic PRRs: Nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), Retinoic acid-inducible gene (RIG)-1-like receptors (RLRs)

Membrane-bound receptors

These receptors are anchored to the plasma membrane of the cell and can detect PAMPs and DAMPs present in the extracellular environment. The major families of PRRs that are included in this group are as follows (Boutrot and Zipfel, 2017):

Toll-like receptors (TLRs)

TLRs are type I membrane proteins characterised by an extracellular domain containing leucine-rich repeats and a cytoplasmic tail that contains a conserved region called the toll/IL-1 receptor domain (TIR) (Ruysschaert and Lonez, 2015). The structure is predominantly expressed in tissues involved in immune functions, such as the spleen and peripheral blood

leukocytes, as well as the lung and gastrointestinal tract (Lakshmi and Jayavardhanan, 2015). TLRs are located on the plasma membrane (TLRs 1, 2, 4, 5, 6, 8, and 10), with the exception of a few that are localised in endosomes (TLRs 3, 7, and 9) (Wang et al., 2021). The main function of each TLR is described in table 2 (Maglione et al., 2015; Fitzgerald et al., 2020).

Table 2: TLRs and their function

TLR	Function
TLR1, 2, 6	Recognition of lipoproteins, lipomanins and lipoteichoic acid from Gram +ve bacteria
TLR3, 10	Identification of viral derived dsRNA
TLR4	Activated by Lipopolysaccharide
TLR5	Detects bacterial flagellin
TLR7,8	Recognition of small synthetic activated molecules
TLR9	Required for response to unmethylated CpG DNA

C-Lectin like receptors (CLRs)

CLRs comprise a large family of receptors that bind to carbohydrates in a calcium-dependent manner (Agier *et al*., 2018). They are mostly expressed on macrophages and dendritic cells, which phagocytoze various

glycoproteins and microbes for pathogen clearance and antigen presentation to T-lymphocytes (Hoving et al., 2014; Bermejo-Jambrina et al., 2018). These receptors are also involved in fungal recognition and clearance (Goyal *et al*., 2018). On the basis of their molecular structure, there are two groups of CLRs:

a) Type I contains carbohydrate-recognition domains (CRDs) and the macrophage mannose receptor
b) Type II contains a single CRD domain and includes dectin 1, dectin 2, macrophage-inducible c-lectin, dendritic cell-specific ICA3-3 grabbing nonintegrin and DCNK lectin group receptor.

Cytoplasmic PRRs

The PRRs that are localised within the cell in the cytoplasm or enclosed in endosomes are called cytoplasmic PRRs (Hu and Shu, 2018). The major families included here are as follows:

Nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs)

NLRs are conserved cytosolic PRRs. NLRs are mainly involved in recruiting inflammatory caspases for inflammasome assembly (Kim and Shin, 2016). Inflammasomes are cytosolic multiprotein oligomers of the innate immune system responsible for the activation of inflammatory responses. Based on their N-terminal domain, NLRs are categorised into

five families: NLRA, NLRB, NLRC, NLRP, and NLRX (Carriere *et al*., 2021).

RIG-like receptors (RLRs)

RIG-like receptors are key sensors of virus infection, mediating the transcriptional induction of type I interferons and other genes that collectively establish an antiviral host response. Constitute a family of three cytoplasmic PRRs (Abe and Shapira, 2019). They include:

a) Retinoic acid-inducible gene-1-like receptors
b) Melanoma differentiation-associated protein-5
c) Probable ATP-dependent RNA helicase DHX58

RLRs are especially active in the innate immune defence of epithelial, myeloid and central nervous system cells. They are localised in the cytosol of a cell and can detect the presence of viral DNA or RNA (Rehwinkel and Gack, 2020). Upon detection, RLRs activate complex signaling cascades that lead to the production of proinflammatory molecules (Gallagher, 2016).

Role of the RAGE pathway in mediating inflammation in DN

The receptor for advanced glycation end-products (RAGE) is a multiligand receptor present on various cell types that plays a key role in inflammatory processes (Teissier and Boulanger, 2019). RAGE is expressed at low levels in normal tissues and in the vasculature and becomes upregulated at the

sites where its ligands accumulate (Tancharoen *et al*., 2014). RAGE acts as a PRR by recognising tertiary structures, and RAGE has the ability to engage classes of molecules rather than individual ligands, such as advanced glycation end-products (AGEs) (Teissier and Boulanger, 2019; Haque *et al*., 2020). The RAGE pathway has been implicated in DN based on the following observations:

a) Increased AGE deposition

b) Increased RAGE expression

Increased AGE deposition

Studies have shown that renal deposition of AGEs is increased under diabetic conditions. Evidence in this direction has come from human studies, animal studies and cell culture studies. Both animal and human studies have shown that elevated AGE deposition is mostly localised in the glomeruli, renal corpuscles, cortical tubules, Bowman's capsule and tubular basement membrane. Cell culture studies have shown that renal cells are prone to AGE formation when exposed to higher levels of sugars. The various studies that underline the involvement of AGEs in DN pathogenesis are summarised in Table 3.

Table 3: List of studies on renal deposition of AGE in DN

Type of study	Observations	Reference
Human study	• Elevated CML deposition in renal biopsies of DN patients compared to control • CML deposition localised in the glomeruli	Tervaert *et al.*, 2010
Animal study (Mouse model of autophagy and diabetes)	• Increased renal deposition of AGE in diabetic model compared to control • AGE deposition localised in the renal tubular cells	Takahashi *et al.*, 2017
Animal study (Streptozotocin treated mouse model of diabetes)	• Increased renal deposition of AGE in diabetic model compared to control • AGE deposition localised in Bowman's capsule and tubular basement membrane	Hafizur *et al.*, 2017
Animal study (Streptozotocin treated mouse model of diabetes)	• Elevated CML deposition in diabetic model compared to control • CML deposition localized in renal corpuscles, cortical tubules and collecting ducts and loops of Henle • Renal deposition of CML reduced in mice treated with resveratrol	Al-Hussaini *et al.*, 2018

Animal study (Streptozotocin treated mouse model of diabetes)	• Elevated AGE deposition in diabetic model compared to control • AGE deposition localized in the glomeruli • AGE deposition reduced in mice treated with a plant extract (Flavanoid)	Lee *et al*., 2018
Animal study (Streptozotocin treated mouse model of diabetes)	• Increased serum levels of AGE and renal AGE deposition in diabetic model compared to the control • AGE deposition localized in the glomeruli • AGE deposition reduced in mice treated with selected plant extract (*Eucommia ulmoides*)	Do *et al*., 2018
Animal study (Streptozotocin treated mouse model of diabetes)	• Elevated AGE deposition in diabetic model compared to the control • AGE deposition localized in the glomeruli • AGE deposition reduced in mice treated with selected plant extract (*Spatholobus suberectus)*	Do *et al*., 2018
Animal study (Streptozotocin treated mouse model of diabetes)	• Effect of D-ribose on AGE accumulation (D-Ribose potent activator of AGE formation) • Increased AGE deposition in kidneys of diabetic group supplemented with D-Ribose compared to diabetic and control groups.	Hong *et al*., 2018

Cell culture study (Mouse renal glomerular mesangial cell line)	• Effect of D-ribose (D-Ribose potent activator of AGE formation) • Increased AGE formation in D-ribose treated culture compared to glucose treated and control culture	Hong *et al*., 2018

Increased RAGE expression

Studies have shown that renal expression of RAGE is increased under diabetic conditions. Animal studies and cell culture studies have provided evidence in this direction. Animal studies have shown that increased RAGE expression is mostly localised in the glomeruli, renal corpuscles, cortical tubules, Bowman's capsule and tubular basement membrane. The role of RAGE in DN progression is further supported by a knock animal study. Silencing of the *RAGE* gene in a mouse model prevents the progression of diabetes into DN. Cell culture studies have also shown that RAGE expression is increased in renal cells exposed to higher levels of sugars. Interestingly, certain plant extracts are reported to ameliorate sugar-induced increases in RAGE expression. appears to be d In addition, studies have shown the potential role of plant extracts in reducing RAGE expression. The various studies that support the involvement of RAGE in DN pathogenesis are summarised in Table 4.

Table 4: List of studies on RAGE expression in DN

Type of study	Observation	Reference
Animal study (*RAGE* knockout in OVE2 mouse-Diabetic nephropathy model)	• Deletion of *RAGE* prevents progression of diabetic nephropathy compared to diabetic model and control.	Reniger *et al*., 2010
Animal study (Streptozotocin treated mouse model of diabetes)	• RAGE expression elevated in diabetic model compared to control • RAGE expression localised in the renal tubules	Fukami *et al*., 2014
Animal study (Streptozotocin treated mouse model of diabetes)	• Elevated RAGE expression in diabetic model compared to control • RAGE expression localised in the renal cortex	Hou *et al*., 2017
Cell culture (Human renal glomerular endothelial cell line)	• RAGE expression upregulated due to AGE treatment compared to the control.	Hou *et al*., 2017
Animal study (Streptozotocin treated mouse model of diabetes)	• Elevated RAGE expression in diabetic model compared to control • RAGE expression localised in the Bowman's capsule and tubular basement membrane	Hafizur *et al*., 2017

Animal study Streptozotocin treated mouse model of diabetes	• Elevated RAGE expression in diabetic model compared to control • RAGE expression localised in the glomeruli • RAGE expression reduced in mice treated with selected plant extract (*Eucommia ulmoides*)	Do *et al*., 2018
Animal study Streptozotocin treated mouse model of diabetes	• Elevated RAGE expression in diabetic model compared to control • RAGE expression localised in the glomeruli • Reduction on treatment with plant extract (*Spatholobus suberectus)*	Do *et al*., 2018
Animal study Streptozotocin treated mouse model of diabetes	• Effect of D-ribose on RAGE expression (D-Ribose potent activator of AGE formation) • D-ribose supplementation resulted in increased RAGE expression in kidneys of diabetic model compared to the control	Hong *et al*., 2018
Cell culture (Mouse renal glomerular mesangial cell line)	• Effect of D-ribose on RAGE expression (D-Ribose potent activator of AGE formation) • Increased RAGE expression in D-Ribose supplemented culture when compared to glucose and control groups	Hong *et al*., 2018
Cell culture (Renal proximal tubular epithelial cells)	• Increased RAGE expression on AGE treatment compared to control culture.	Jeong *et al*., 2020

Advanced glycation end-products (AGEs)

AGEs comprise a heterogeneous group of compounds that are formed by a nonenzymatic reaction called glycation followed by a spontaneous posttranslational modification in which the carbonyl group of reducing sugars is covalently coupled to proteins, lipids and nucleic acids (Dozio *et al*., 2021).

Formation of AGEs

The Maillard reaction is the first step in the formation of AGEs. It is characterised by reactions between reducing sugars and amines, and these reactions are nonenzymatic in nature. The Maillard reaction results in the formation of Schiff bases that are generated as a result of condensation reactions between the electrophilic carbonyl group of a reducing sugar with free amino groups, essentially lysine or arginine. The subsequent chemical rearrangement leads to the formation of a stable ketoamine called the Amadori product. Schiff bases and Amadori products are reversible reaction products, and they may further react irreversibly with peptides or proteins to form protein cross-links (Szwergold, 2021). Additionally, these compounds may participate in oxidation, dehydration, or polymerization reactions to give rise to numerous other AGEs (Brings et al., 2017). The formation of AGEs is described in Figure 3.

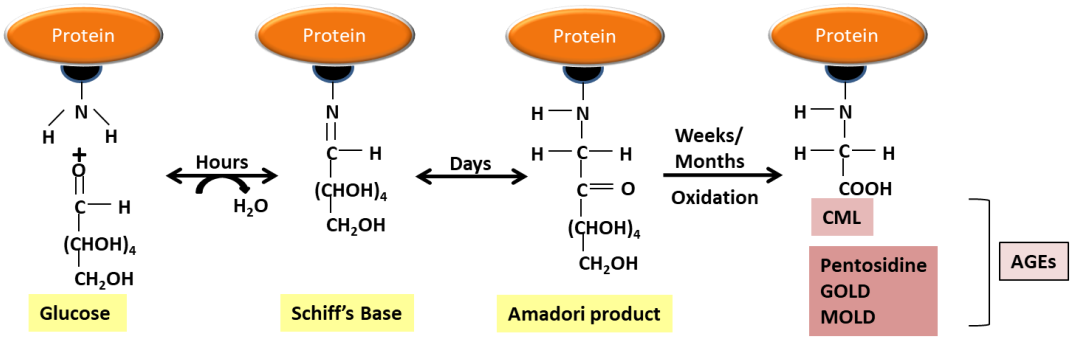

Figure 3: Formation of advanced glycation end products

Classification of AGEs

AGEs are classified into different groups based on their chemical structures and ability to emit fluorescence. They are as follows:

- Fluorescent and cross-linked
- Non-fluorescent and non-crosslinked
- Non-fluorescent protein cross-linking
- Fluorescent non-cross-linked

Fluorescent and cross-linked

These AGEs arise from the cross-linkage of arginine and lysine residues from collagen with ribose but also from hexoses and ascorbic acid. The first isolated and characterised fluorescent cross-linked AGE was pentosidine. Pentosidine is not the only natural fluorescent cross-linked AGE; other fluorescent cross-linked AGEs are pentodilysine, crossline, AGE-XI, vesperlysine A, and vesperlysine C (Henle and Miyata, 2003).

Non-fluorescent and non-crosslinked

These AGEs are formed by the Maillard reaction followed by Amadori product formation and subsequent oxidation. Some of the important nonfluorescent and noncrosslinked AGEs include carboxymethyl-lysine (CML), carboxyethyl-lysine, pyrraline, and imidazolones (Ichihashi *et al*., 2011).

Non-fluorescent protein cross-linking

These AGEs are formed by cross-linking arginine, known as the imidazolium cross-link derived from glyoxal with lysine-arginine (GODIC) and the imidazolium cross-link derived from methylglyoxal with lysine-lysine (MODIC). These compounds are highly reactive molecules and therefore lead to the cross-linking of proteins (Guedes, 2011).

Fluorescent non-cross-linked

These AGEs are imidazolium dilysine cross-links also known as glyoxal-lysine dimer (GOLD) or methylglyoxal-lysine dimer (MOLD) cross-links, which are derived from the reaction between two lysine sidechains and two molecules of glyoxal or methylglyoxal, respectively (Perrone *et al*., 2020).

Sources of AGEs

Based on the source, AGEs are classified into:

a) Exogenous AGEs

b) Endogenous AGEs

Exogenous AGEs

AGEs that arise from a dietary source are classified as exogenous AGEs. Foods that contain high sugar contribute to dietary AGEs. The formation of dietary AGEs can arise by various cooking processes (Takeuchi *et al*., 2017). Factors that influence the formation of this type of AGE are as follows:

a. Temperature (food browning)
b. Water content (dry-heat cooking)
c. P^H

These factors affect chemical reactions that lead to AGE formation. Another source of AGEs that are classified as exogenous are AGEs that arise from cigarette smoke. This smoke contains reactive glycation products that can trigger AGE formation and accumulation (Gkogkolou and Bohm, 2012).

Endogenous AGEs

Endogenous AGEs represent adducts that are produced and slowly accumulate within the body during the normal aging process and under oxidative stress, inflammatory, and hyperglycemic conditions. AGEs can arise from the polyol pathway and lipid peroxidation. This process is independent of the hyperglycemic state. The formation of endogenous AGEs depends mainly on substrate and reactant type/concentration, exposure period, and cell microenvironment (Chaudhuri *et al*., 2018).

AGE clearance

Circulating AGEs are eventually removed from the system by normal physiological processes such as proteolysis. The proteolysis of AGEs results in the formation of peptides and AGE-free adducts (AGE adducts bound to single amino acids) (Gugliucci et al., 2007). The AGE-free adducts are ultimately excreted in the urine after they are released from the proteolysis process. However, the peptides that remain are degraded by the phagocytic action of the epithelial cells of the proximal tubule and consequently degraded by lysosomal action (Fleming *et al*., 2011).

The AGEs that are cross-linked to extracellular matrix proteins are more difficult to clear out because ECM proteins resist proteolysis. These AGEs are also larger and cannot be effectively cleared out and begin to accumulate in the basement membrane of the kidney. A certain amount of clearance of these large AGEs is performed by peripheral macrophages (Byun *et al*., 2017).

Receptor for Advanced Glycation End-products

It is a cell surface receptor belonging to the immunoglobulin superfamily. It comprises three immunoglobulin (Ig) domains, followed by a single transmembrane spanning helix and a short C-terminal cytoplasmic tail. RAGE is made of 550 amino acid residues, a maltotriose, 985 water molecules, and a sulphate ion (Park and Boyington, 2010).

The RAGE polypeptide is folded into three domains. These are extracellular, transmembrane and cytosolic domains. The extracellular region is responsible for the interaction with RAGE ligands. It consists of three immunoglobulin-like regions, one V-type followed by two C-type domains. RAGE contains a single transmembrane-spanning domain that anchors the RAGE polypeptide to the cell membrane. The cytosolic domain is a 43 amino acid polypeptide responsible for signal transduction and essential for intracellular signalling (Ramasamy *et al*., 2011).

Splice variants of *AGER*

Human RAGE is encoded by a gene located on the major histocompatibility complex (MHC) class III region on chromosome 6. RAGE variants are represented by three forms, called N-truncated, dominant negative, and soluble RAGE, that are generated either by natural alternative splicing or by the action of membrane-associated proteases. These variants are similar to the full-length RAGE, also called mRAGE or membrane-bound RAGE, but lack certain domains that are all present in mRAGE (Oliveira *et al*., 2013, Taneja *et al*., 2021). The isoforms of RAGE are represented in Figure 4.

The following are the isoforms of RAGE:

a) N-truncated RAGE lacks the V-domain in the extracellular region and therefore cannot interact with ligands.

b) Dominant-negative RAGE lacks a cytosolic domain, which results in no intracellular signal transduction, although it can bind to ligands.

c) Endogenously secreted RAGE lacks transmembrane and cytosolic domains and arises as a result of alternative splicing.

d) Soluble RAGE lacks the transmembrane domain and has the ability to circulate out of the cell and act as decoys by preventing ligands from binding to full-length RAGE.

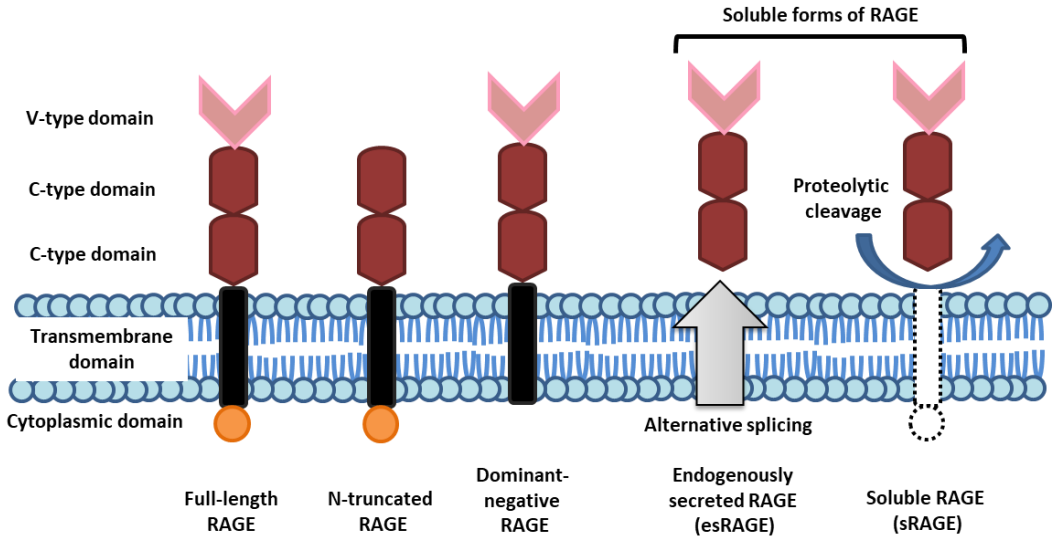

Figure 4: Isoforms of RAGE

RAGE expression

The expression of RAGE depends on the organ, developmental stage, and cellular condition. A relatively low level of RAGE is expressed in all tissues, with the exception of lung tissue, which exhibits high RAGE levels (Neeper *et al*,1992). Under severe pathophysiological settings such as diabetes, chronic inflammation, or neurodegenerative disorders, the expression of RAGE is increased in different tissues, such as vasculature,

hematopoietic cells, and the central nervous system. (Yan *et al*, 2009. Bierhaus *et al*, 2006).

RAGE expression is observed in many cell types, including epithelial cells, such as alveolar cells of the lung, enterocytes, mucus-secreting cells and Paneth cells of the colon and rectum, proximal tubular cells and collecting duct cells of the kidney, hepatocytes and cholangiocytes of the liver, ductal cells of the pancreas, basal glandular cells and urothelial cells of the prostate, keratinocytes in the skin, and Sertoli cells of the testis.

RAGE expression is observed in blood and immune cells such as monocytes, Kupffer cells, T cells, dendritic cells, NK cells and B cells. Endothelial cells in the eye, heart, liver, skin, placenta, testis and prostate are also rich in RAGE expression. RAGE expression is also seen in smooth muscle, neurons and microglia. RAGE is also expressed in mesenchymal cells such as fibroblasts, Ito cells of the liver and peritubular cells of the testis (Proteinatlas.org).

RAGE signalling pathway

The binding of AGEs to their receptor RAGE activates a range of signaling pathways. Homodimerization of RAGE has been identified to be essential for RAGE signalling. The AGE-RAGE interaction results in activation of protein kinase C and tyrosine phosphorylation of the JAK-STAT, PI3K-Akt, MAPK and calcium signaling pathways (Ramasamy *et al*., 2011).

AGEs have been shown to induce the formation of the following complexes:

a) RAGE - DIAPH1 - RAC1/CDC42
b) RAGE - Src/IRS1/PLC - PKC-α – ERK1/2
c) RAGE - TIRAP/MyD88/IRAK4 - PI3K - AKT
d) RAGE – Src/JAK - STAT
e) RAGE – PKCβII-Egr-1- JNK

All these signaling cascades result in the translocation and activation of the transcription factors NF-κB and AP-1 followed by the subsequent upregulation of NF-κB- and AP-1-dependent genes, leading to the expression of proinflammatory cytokines and the upregulation of RAGE expression and causing a positive feedback loop leading to chronic inflammation (Kim *et al*., 2019). The major component of AGE/RAGE signaling is oxidative stress-induced pathways. AGEs induce oxidative stress through the activation of NADPH oxidases. Increased intracellular oxidative stress leads to stimulation of PKC and ERK1/2, resulting in the translocation and activation of NF-κB and subsequent upregulation of NF-κB-dependent genes, which ultimately produces deleterious effects on cells, such as overexpression of proinflammatory cytokines and upregulation of RAGE expression (Burr and Stewart, 2021). Activation of the RAGE pathway results in various cellular processes, as discussed in Figure 5.

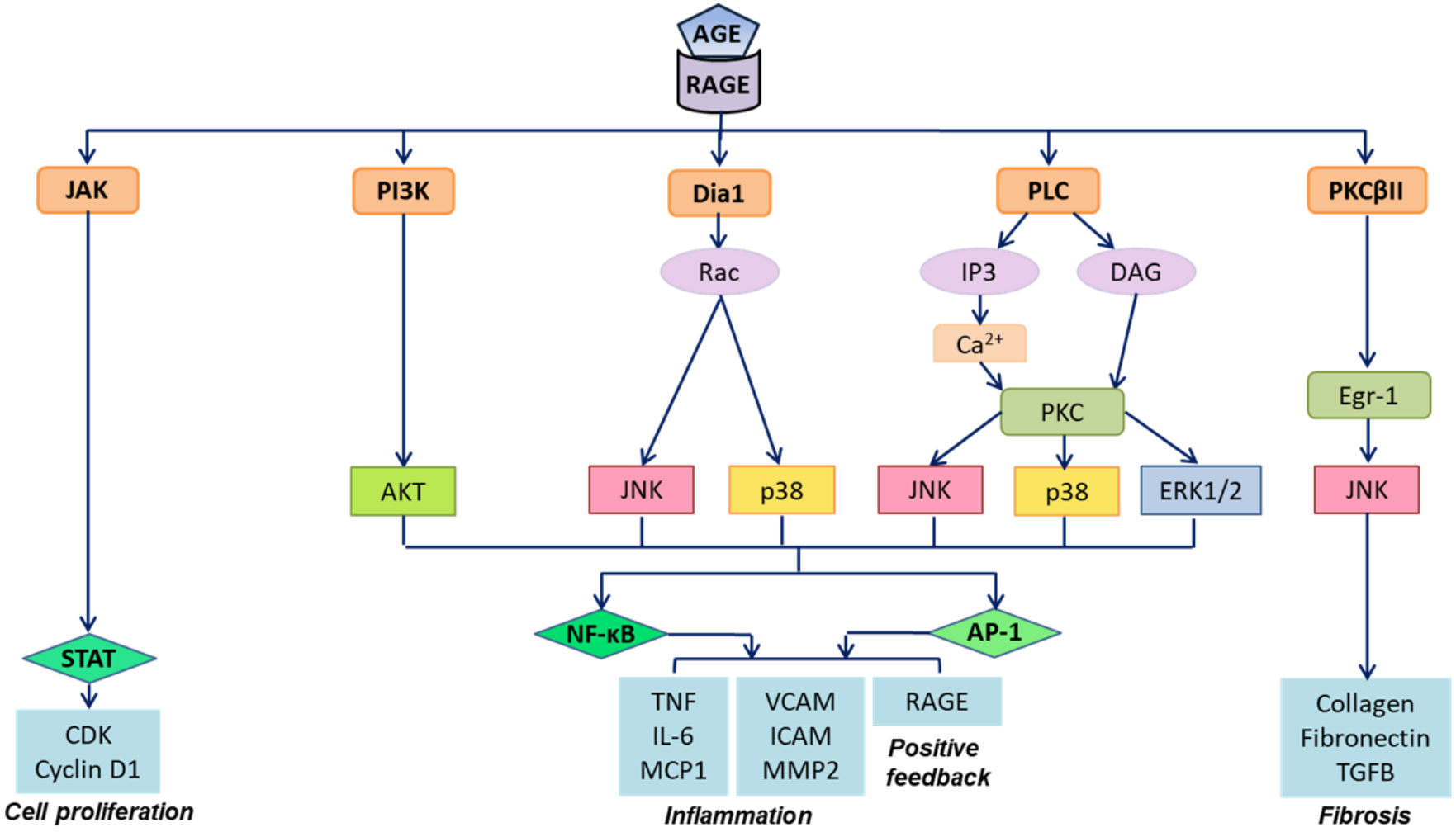

Figure 5: RAGE signaling pathway

Regulation of the RAGE pathway

RAGE pathway activation results in a positive feed-forward loop, wherein inflammatory cytokines activate NF-κB. NF-κB in turn triggers RAGE expression and subsequent NF-κB activation. This can lead to chronic inflammation and result in damage to surrounding tissue. Therefore, regulation of the RAGE pathway is an extremely important aspect to prevent the progression of chronic inflammatory diseases (Xu *et al*., 2018).

The RAGE pathway is mainly regulated by the proteolytic cleavage of full-length RAGE by a metalloproteinase called ADAM10. The shedding of RAGE from the cell surface gives rise to its soluble isoform sRAGE. sRAGE competes for RAGE ligands and thereby acts as a decoy receptor (Braley et al., 2016). The metalloproteinase ADAM10 cleaves protein substrates close to the extracellular membrane leaflet. This "ectodomain shedding" regulates the turnover of a number of transmembrane proteins involved in cell adhesion and receptor signalling (Degani *et al*., 2020). It can initiate intramembrane proteolysis followed by nuclear transport and signaling of the cytoplasmic domain. sRAGE in the plasma arises due to proteolytic cleavage of mRAGE. ADAM10 is involved in the shedding of type I membrane receptors such as mRAGE. Therefore, plasma levels of sRAGE can be influenced by the expression status of ADAM10 (Reynaert *et al*., 2016).

A heterogeneous population of soluble isoforms of RAGE comprising splice variants of RAGE and isoforms that arise due to ectodomain shedding.

esRAGE is the common variant detected. sRAGE mainly acts as a decoy receptor both locally and systemically. Targeting mechanisms that increase ectodomain shedding of RAGE can ameliorate the effects of the RAGE pathway, and in turn, the increased levels of sRAGE will affect the management of AGE-RAGE interactions without the activation of pathways that can cause subsequent inflammatory derangement and tissue damage (Yamamoto and Yamamoto., 2012).

Role of RAGE in diseases

RAGE binds to the β-sheet fibrillar structures in the amyloid components characteristic of Alzheimer's disease. RAGE can also bind to proinflammatory cytokine-like mediators of the S100/calgranulin family, and amphoterin, also called HMGB1, is a nuclear protein that is released upon necrosis and can exert proinflammatory activity extracellularly. In diabetic vessels, RAGE ligands such as advanced glycation end-products (AGEs) can also initiate a signaling cascade and result in the release of proinflammatory cytokines. Binding of different ligands to RAGE does not accelerate their clearance or degradation but rather begins a sustained period of cellular activation mediated by receptor-dependent signalling (Watanabe and Son, 2021).

Binding of the different ligands, such as AGEs, S100/calgranulin, and HMGB1, to RAGE on different cells, such as endothelial cells, neuronal cells, smooth muscle cells or inflammatory cells, such as monocytes, may activate a range of signalling pathways, including ERK1/2 (p44/p42) MAP kinases, p38

and SAPK/JNK MAP kinases, rho GTPases, phosphoinositol-3 kinase and the JAK/STAT pathway, as well as downstream activation of NF-κB. The interactions of RAGE with its ligands trigger the generation of ROS, at least in part via the activation of NADPH oxidase. RAGE is also involved in mediating inflammatory cell recruitment through the expression of proinflammatory cytokines and chemokines via NF-κB activation. Taken together, these features of RAGE allow the receptor to propagate cellular dysfunctions in a number of pathophysiological situations, such as atherosclerosis and microvascular complications of diabetes, such as neuropathy, retinopathy and nephropathy (Yan *et al*., 20119).

Lacunae in knowledge

The involvement of inflammation in the progression of diabetes into nephropathy is well established. However, the molecular mechanisms that lead to aberrations in inflammatory pathways, particularly the RAGE pathway, are not clearly known. AGE substances are known to be elevated in DN; however, the hyperresponsiveness of the RAGE pathway to AGEs has yet to be explored.

The regulation of the RAGE pathway is controlled by the counterpart of RAGE, i.e., sRAGE, which arises due to proteolytic cleavage of mRAGE. ADAM10 is a type I membrane protease known to be involved in the shedding of type I membrane receptors such as RAGE. Therefore, plasma levels of sRAGE can be influenced by the expression status of ADAM10.

Insulin-induced RAGE shedding has also been linked to RAGE pathway regulation; however, it is not known whether insulin affects RAGE shedding via ADAM10 expression. Insulin resistance is an important pathophysiological feature in T2DM. Presently, there is no information as to whether insulin insensitivity also affects ADAM10 activation.

A review of the literature shows that the RAGE pathway has an imminent role in the pathophysiology of DN. Additionally, the regulation of the RAGE pathway has yet to be explored. However, the paucity of information on the functional status of the RAGE pathway in DN has yet to be evaluated; therefore, this study was undertaken.

CHAPTER V

MATERIAL AND METHODS

5.1. Study design

This study was carried out as a three-group comparative study. The first group comprised patients with DN, the second group comprised patients with T2DM, and the third group comprised healthy volunteers. The study design is given in Figure 5.1. Patients meeting the inclusion and exclusion criteria were recruited after obtaining informed consent. Clinical and demographic details were collected from the patient's medical records.

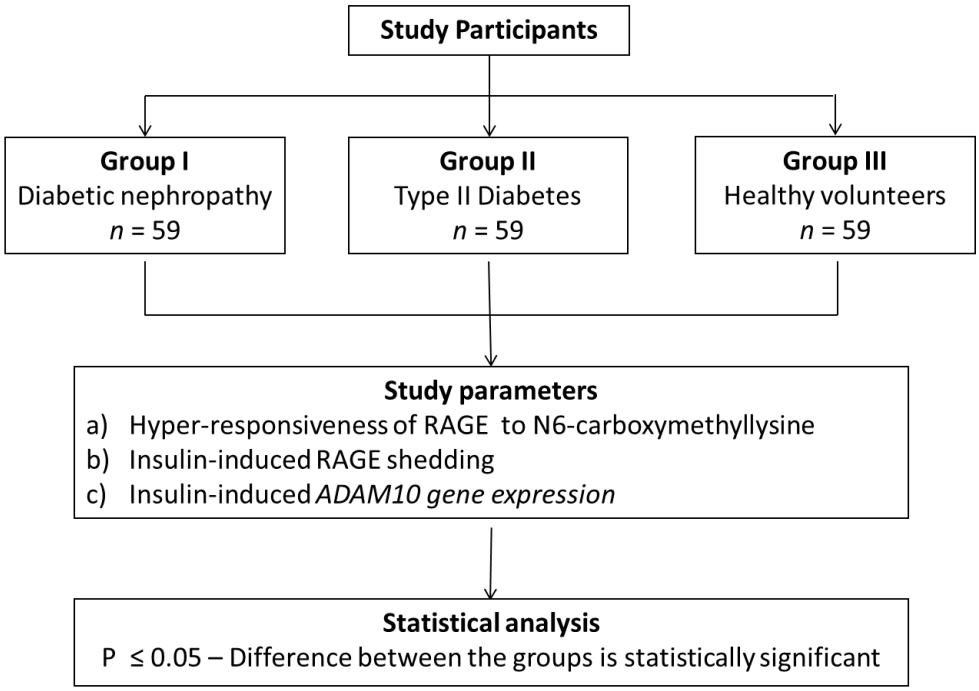

Figure 5.1: Schematic representation of the study design

5.2. Study participants

Patients with DN and T2DM were recruited from the Department of General Medicine, R.L. Jalappa Hospital Research Centre, the recruiting hospital of the Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Age- and

gender-matched healthy volunteers were recruited from the local population. The period of the study was from 2019 to 2021.

5.3. Sample size calculation

The sample size was calculated based on the levels of NF-κB in diabetic patients and healthy volunteers (daViega *et al.* 2019). The sample size was calculated using an open-source web-based tool viz., OpenEpi Version 3.01. The number of participants required for the study to achieve a power of 90% was found to be 59.

5.4. Criteria for patient selection

Patients/participants were included in the three study groups by considering the inclusion and exclusion criteria listed in Table 5.4:

Table 5.4.: Patient selection criteria

Criteria	**Group I** **Diabetic Nephropathy**	**Group II** **Type II Diabetes**	**Group III** **Healthy Volunteers**
Inclusion	a) Persons of both genders of age 45-65 years b) Stage 4 & 5 Diabetic Nephropathy	a) Persons of both genders of age 45-65years b) Hypertension c) Euglycemic	a) Age and Gender matched individuals b) Normoglycemic c) Normal renal function
Exclusion	a) Stage 1-3 Diabetic nephropathy b) Chronic comorbidities	a) Microvascular complications b) Renal complications	a) History of chronic diseases

5.4.1. Diagnostic criteria

Patients were diagnosed with T2DM based on the diagnostic criteria of ICMR (Varghese and Jialal, 2021, ICMR guidelines for management of type II diabetes, 2018):

a) Fasting plasma glucose higher than normal (FPG ≥ 126 mg/dl)
b) Glycated hemoglobin higher than normal (HBA1c > 6%)
c) Volunteers with FPG ≤ 110 mg/dl were included in the control group.

Patients were diagnosed with nephropathy if the following criteria were observed:

a) Creatinine levels higher than normal (Normal range: 0.6 to 1.2 mg/dL)
b) eGFR value lower than normal (Normal: 125 ml/min 180 L/day and 2 ml/sec)
c) Blood urea nitrogen was higher than the normal range (normal range 8-24 mg/dL).

5.5. Ethical issues

The study was initiated after obtaining permission from the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India (SDUMC/KLR/IEC/218/2018-19). Informed consent was obtained from the participants in writing before recruitment.

5.6. Blood culture setup

Fresh blood samples (3 ml) were collected in a sterile EDTA vacutainer and used for the cell culture experiment within an hour. Cultures were set up by

combining 500 µL of whole blood with 495 µL of Rosewell Park Memorial Institute 1640 medium (supplemented with 10% fetal bovine serum and 1% antibiotic). The cultures were incubated at 37°C for 24 hours in a 5% CO_2 atmosphere. Three cultures were set up for each sample. The first culture was treated with N(6)-carboxymethyl lysine (Cat #14580; Sigma Aldrich, USA) to a final concentration of 10 µM. The second culture was treated with insulin (Cat #12585014; Thermo Scientific, USA) to a final concentration of 25 mIU/mL, and the third culture was treated with phosphate buffer saline (vehicle control).

5.7. Preparation of RNA and cDNA transcripts

Following incubation, the blood cultures were centrifuged at 3000 rpm. The supernatant representing the conditioned media was stored at -80°C in aliquots. The cell pellet was used for isolating total RNA by the TRIzol method (Cat #15596018 Thermo Scientific, USA). The protocol for RNA isolation by the TRIzol method was as follows: first, the blood pellet was washed twice with 8 mL of erythrolysis buffer. Once a white pellet was obtained, 1 mL of TRIzol solution was added, gently mixed, and then transferred into a sterile 1.5 mL Eppendorf tube, followed by centrifugation at 3000 rpm for 10 mins. This step was performed to homogenise the pellet in TRIzol. Following this step, 200 µL of chloroform per 1 mL of TRIzol was added and gently mixed by inverting the tube. It was then incubated at room temperature for 2 to 3 min and then centrifuged for 15 min at 12000 rpm at 4°C. After this step, there was a formation of three phases. The upper layer was transferred into a fresh Eppendorf tube, and 500 µL of absolute isopropanol was added and incubated at

room temperature for 10 to 15 mins. The tube was then centrifuged for 10 min at 12000 rpm at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 mL of 75% ethanol and then spun for 5 min at 10000 rpm at 40°C. The supernatant was then discarded, and the pellet was allowed to dry at room temperature. Once the pellet was completely dried, it was resuspended in 50 µL of RNase free water. The isolated total RNA was used to prepare cDNA (Cat #1708891 iscript cDNA synthesis kit; BioRad, USA). cDNA samples were stored at -20°C until further analysis. The cDNA synthesis thermal cycling program was as follows: the initial step was 25°C for 5 mins, followed by 60°C for 20 mins, followed by 95°C for 3 mins for the inactivation of the reverse transcriptase enzyme and a final step of 40°C for infinity.

5.8. Gene expression analysis

The comparative threshold cycle (Ct) method was used to quantify the relative gene expression of the target genes *NF-κB* and *ADAM10* after normalisation to the housekeeping gene *GAPDH*. The primer sequences used for gene expression analysis are described in Table 5.8.1. The mRNA levels were measured by quantitative polymerase chain reaction (qPCR) using the SYBR green method (Cat # 1725271 SsoAdvanced Universal SYBR Green; BioRad, USA). The thermal cycling conditions are described in Table 5.8.2. The fold change in gene expression was determined by calculating $2^{-\Delta\Delta CT}$, where:

ΔCT = Ct (target gene) – Ct (housekeeping gene)

$\Delta\Delta CT = \Delta Ct$ (treated) – ΔCt (untreated)

The ΔCt values were used for statistical comparison between treated and untreated samples within each study group. The log $2^{-\Delta\Delta CT}$ values were used for statistical comparison between study groups.

Table 5.8.1.: Primer sequences used for gene expression analysis:

Gene	Primer sequence	Amplicon size (bp)
GAPDH	FP: GAT CAT CAG CAA TGC CTC CT RP: GAC TGT GGT CAT GAG TCC TTC	110
NF-κB	FP: TAC CGA CAG ACA ACC TCA CC RP: CAG CTT GTC TCG GGT TTC TG	150
ADAM10	FP: ACT GCT GAT GAG AAG GAC CC RP: CCA GAC CAA GTA CGC CAT CA	125

Table 5.8.2.: Thermal cycling conditions for gene expression analysis:

Step	Temperature (^{0}C)			Duration
	GAPDH	*NF-κB*	*ADAM10*	
Initial denaturation	95	95	95	3 min
Denaturation	95	95	95	10 sec
Annealing and extension	55	62.4	59	30 sec
Cycle repetitions	39	39	39	

5.9. Determination of sRAGE and TNF levels:

The conditioned media was used to assess the levels of sRAGE and TNF by enzyme-linked immunosorbent assay. The levels were assessed on induction with insulin or CML and compared to the uninduced conditioned media. Commercially available kits were used to estimate sRAGE (#SEA645Hu, Cloud-Clone Corp., USA) and TNF (#SEA133Hu, Cloud-Clone Corp., USA) following the manufacturer's instructions.

5.10. Statistical analysis

Statistical analysis and graphical representation of the data were carried out by using GraphPad Software, Version 5.0 (La Jolla, California, USA). The data were checked for normality using the Shapiro–Wilk test. The mean and standard deviation were calculated if the data were normally distributed. If the data were not normally distributed, the median and interquartile range were calculated. Parametric tests were used to compare data showing a normal distribution, and nonparametric tests were used for data not following a normal distribution. A P value ≤ 0.05 was considered statistically significant.

CHAPTER VI

RESULTS

6.1. CML-induced *NF-κB* gene expression is increased in diabetic nephropathy:

CML-induced *NF-κB* gene expression was evaluated in patients with diabetic nephropathy, patients with T2DM, and healthy volunteers (n=59 per group). ΔCt was calculated for the normalised gene expression of *NF-κB* and is represented in Figure 6.1.1. CML treatment resulted in higher normalised expression (ΔCt) of the *NF-κB* gene in diabetic nephropathy (p <0.0001; Wilcoxon signed-rank test) but not in T2DM (p <0.0001; Wilcoxon signed-rank test) and healthy volunteers (p = 0.08; Wilcoxon signed-rank test).

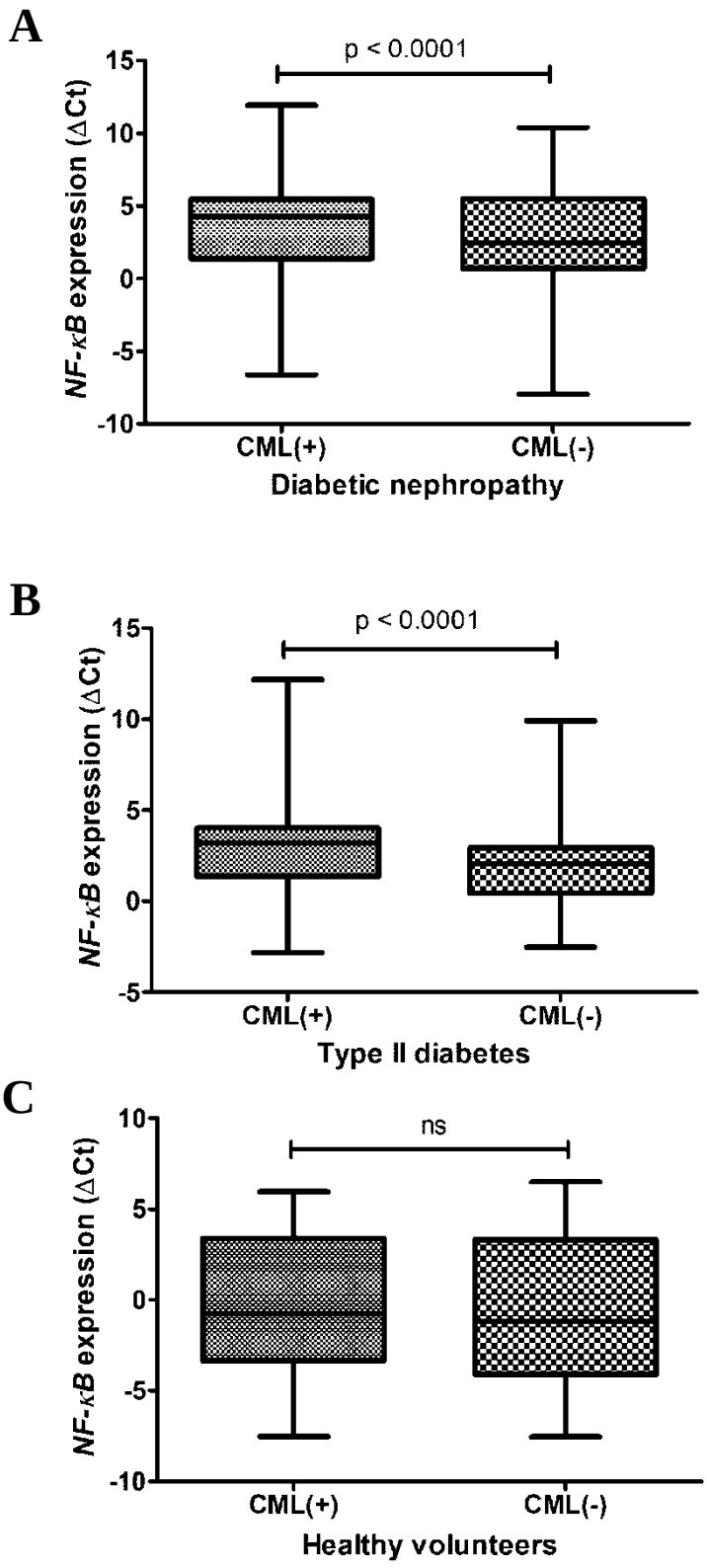

Figure 6.1.1.: Effect of CML on *NF-κB* gene expression in the study groups. **A**. Normalized gene expression in Diabetic nephropathy group. **B**. Normalized gene expression in T2DM group. **C**. Normalized gene expression in Healthy volunteer group.

The CML-induced fold change ($2^{-\Delta\Delta Ct}$) in *NF-κB* expression was 2.8x in diabetic nephropathy, 2.5x in T2DM, and 0.9x in healthy volunteers. The fold change (log $2^{-\Delta\Delta Ct}$) of the *NF-κB* gene was compared between the three study groups. The log $2^{-\Delta\Delta Ct}$ was significantly different among the three groups (p <0.0001; ANOVA). The results are depicted in Figure 6.1.2. The log $2^{-\Delta\Delta Ct}$ was significantly different when compared between the groups in the following combinations: DN vs. T2DM (p <0.001; unpaired t test), DN vs. healthy volunteers (p <0.001; unpaired t test), and T2DM vs. healthy volunteers (p <0.05; unpaired t test). The results are depicted in Figure 6.1.3. These results indicate that CML treatment upregulates the expression of the *NF-κB* gene in diabetic nephropathy and T2DM but not in healthy volunteers.

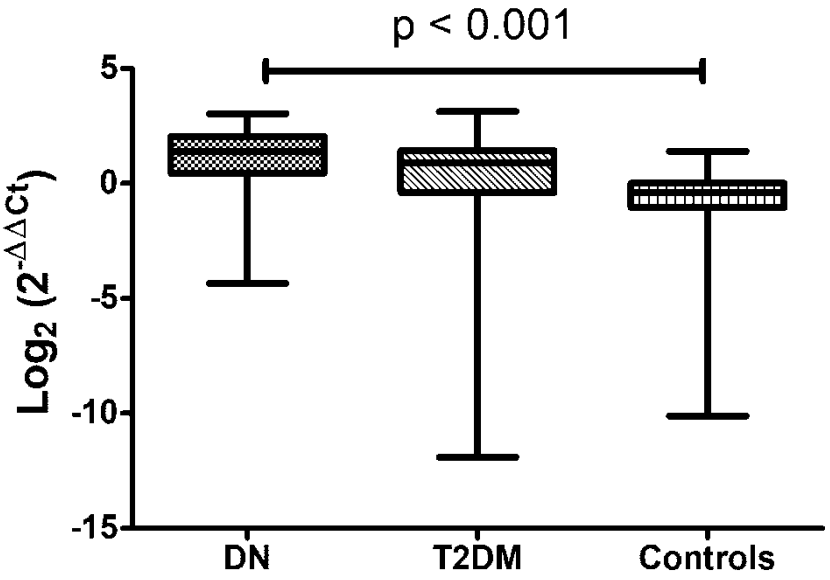

Figure 6.1.2.: Comparison of CML-induced changes in *NF-κB* gene expression between the study groups. Fold change of gene expression between the three study groups (n= 59 per group).

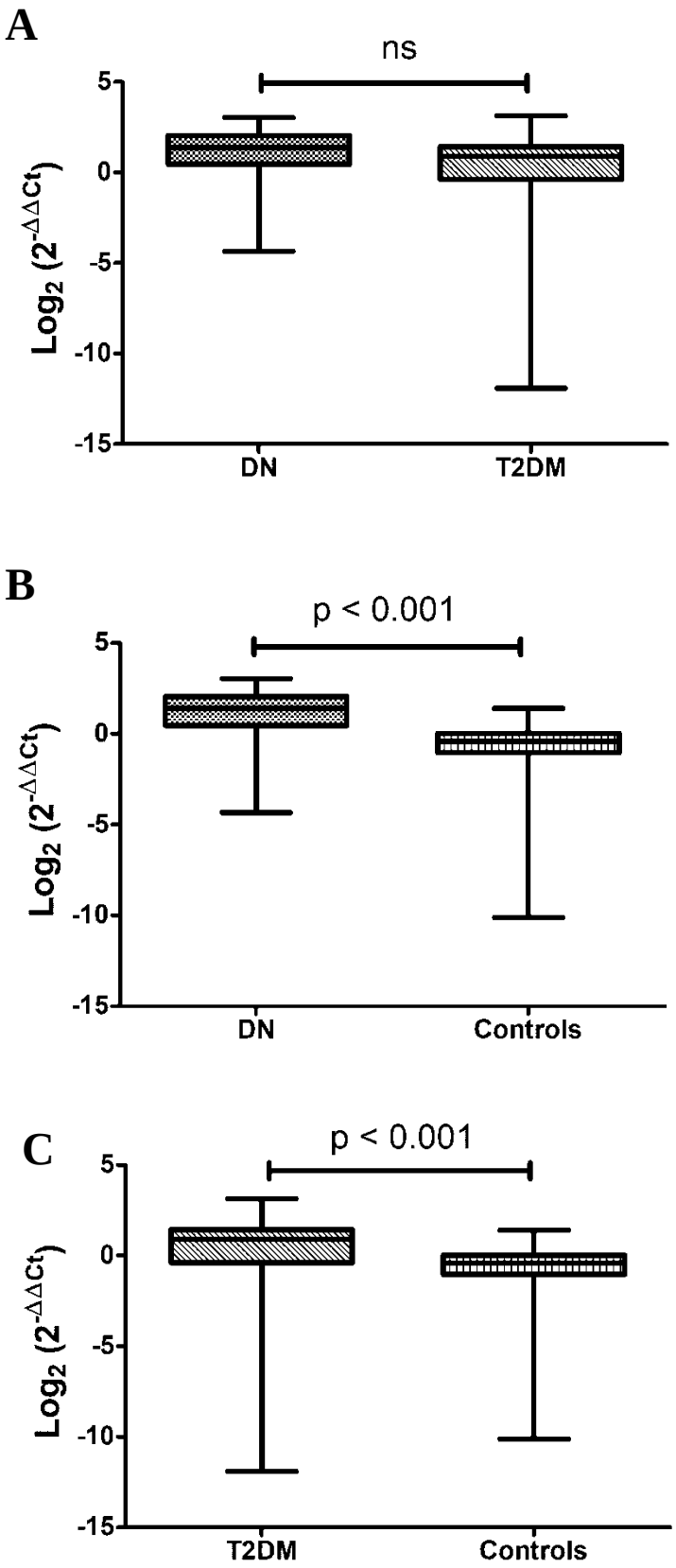

Figure 6.1.3.: Comparison of CML-induced changes in *NF-κB* gene expression between the study groups. **A**. DN and T2DM groups, **B**. DN and healthy volunteer groups, **C**. T2DM and healthy volunteer groups (*n*= 59 per group).

6.2. Effect of clinico-biochemical parameters on CML-induced *NF-κB* gene expression:

CML-induced *NF-κB* expression was compared with two major clinical parameters viz., measures of blood sugar control and measures of renal function. A statistically significant positive correlation (but of weak magnitude) was observed with the measures of blood sugar control, such as fasting blood sugar (r = 0.358, p <0.0001; Spearman's correlation) and glycated haemoglobin (r = 0.369, p <0.0001; Spearman's correlation). This indicates that the magnitude of *NF-κB* expression is related to the magnitude of glycemic control. Similarly, the relationship between responsiveness to CML and renal function was assessed. A statistically significant positive correlation (but of weak magnitude) was observed with the measures of renal function, such as blood urea nitrogen (r = 0.245, p <0.001; Spearman's correlation) and serum creatinine (r = 0.223, p <0.001; Spearman's correlation). Additionally, a statistically significant negative correlation (but of weak magnitude) was observed with the estimated glomerular filtration rate (r = -0.201, p <0.001; Spearman's correlation). The results of the correlation analysis are depicted in Table 6.2. The correlation graphs are plotted in Figures 6.2.1- 6.2.5.

Table 6.2: Correlation of clinico-biochemical parameters with CML-induced *NF-κB* gene expression of the study participants

Parameter	*NF-κB* Fold change	
	Spearman's Correlation (r)	***p* value**
Fasting blood sugar	0.358	< 0.0001
Glycated Haemoglobin	0.369	< 0.0001
Serum Creatinine	0.223	< 0.001
Estimated glomerular filtration rate	-0.201	< 0.001
Blood Urea Nitrogen	0.245	< 0.001

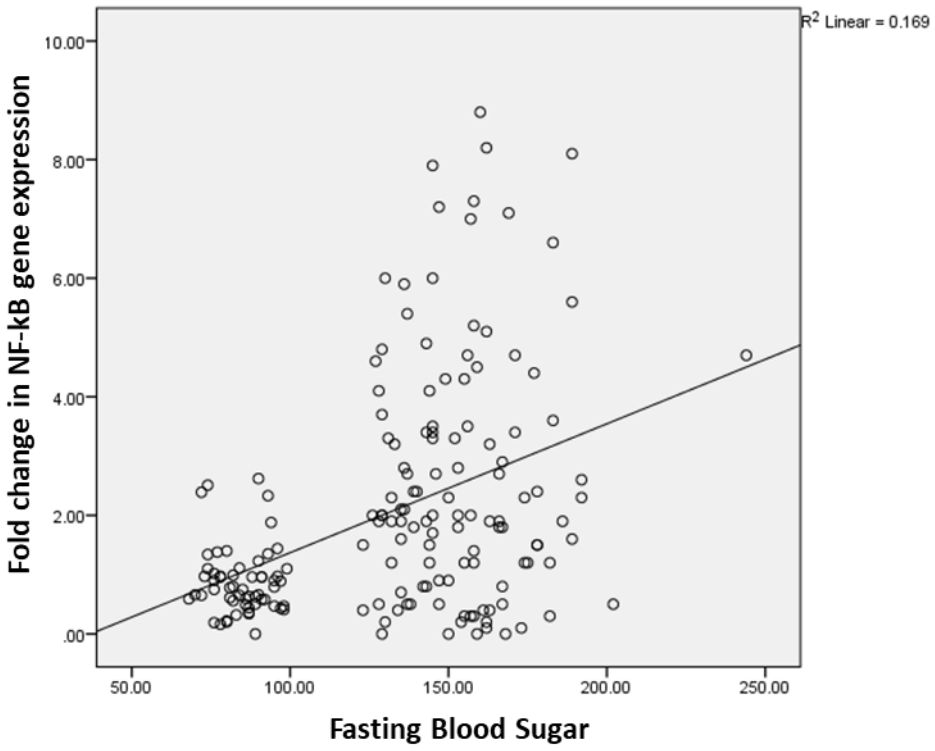

Figure 6.2.1: Correlation between fasting blood sugar levels and CML-induced changes in *NF-κB* gene expression among the three study groups

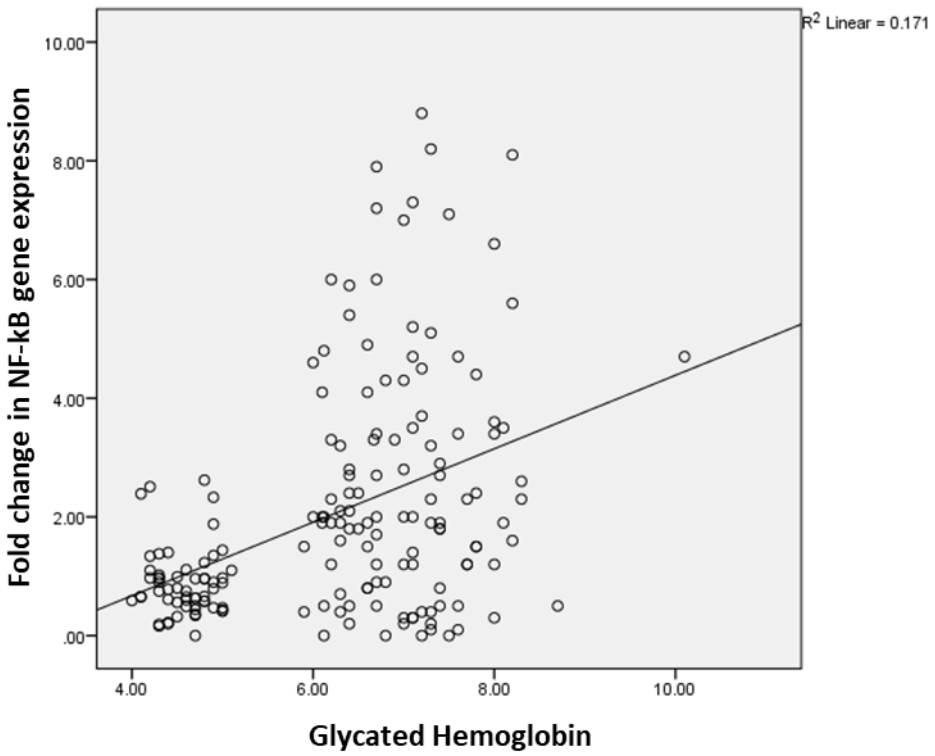

Figure 6.2.2: Correlation between glycated haemoglobin levels and CML-induced changes in *NF-κB* gene expression among the three study groups

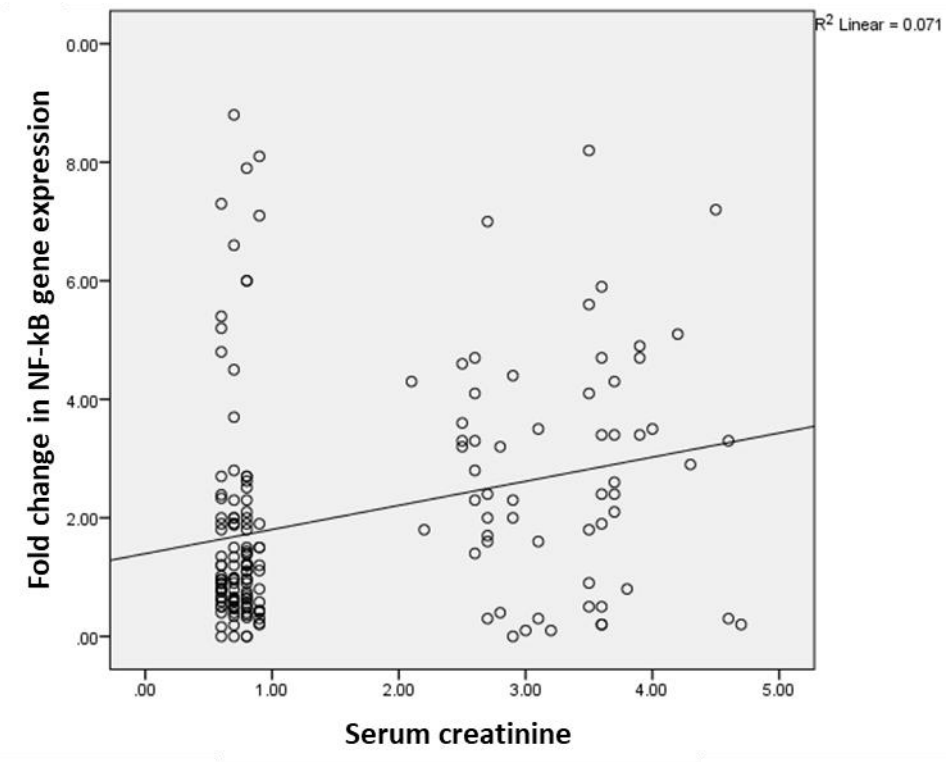

Figure 6.2.3: Correlation between serum creatinine levels and CML-induced changes in *NF-κB* gene expression among the three study groups

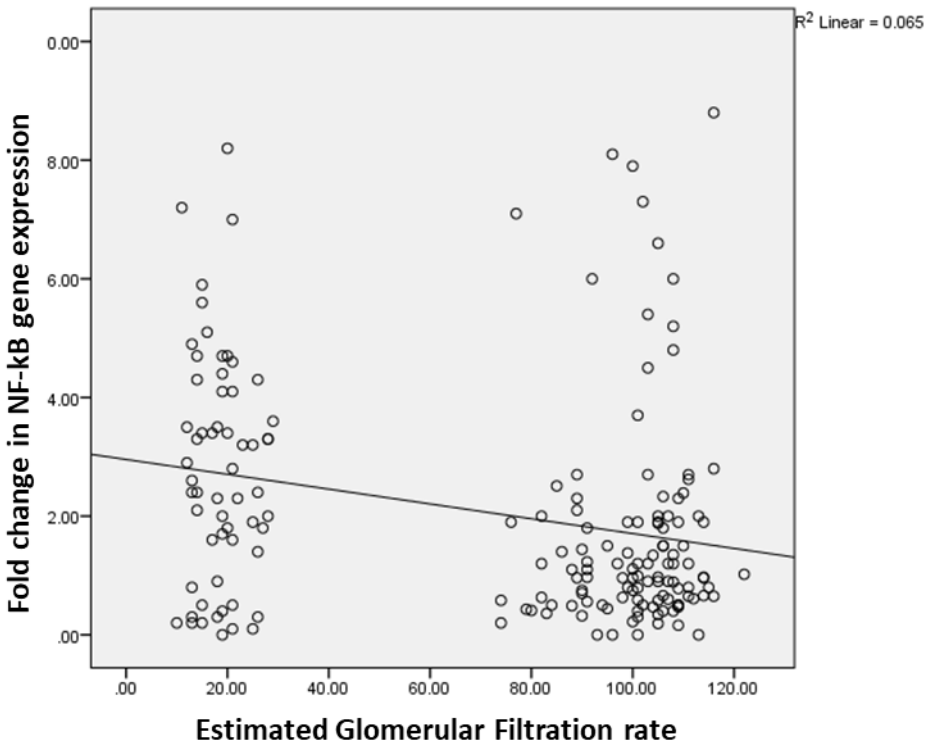

Figure 6.2.4: Correlation between estimated glomerular filtration rate and CML-induced change in *NF-κB* gene expression among the three study groups

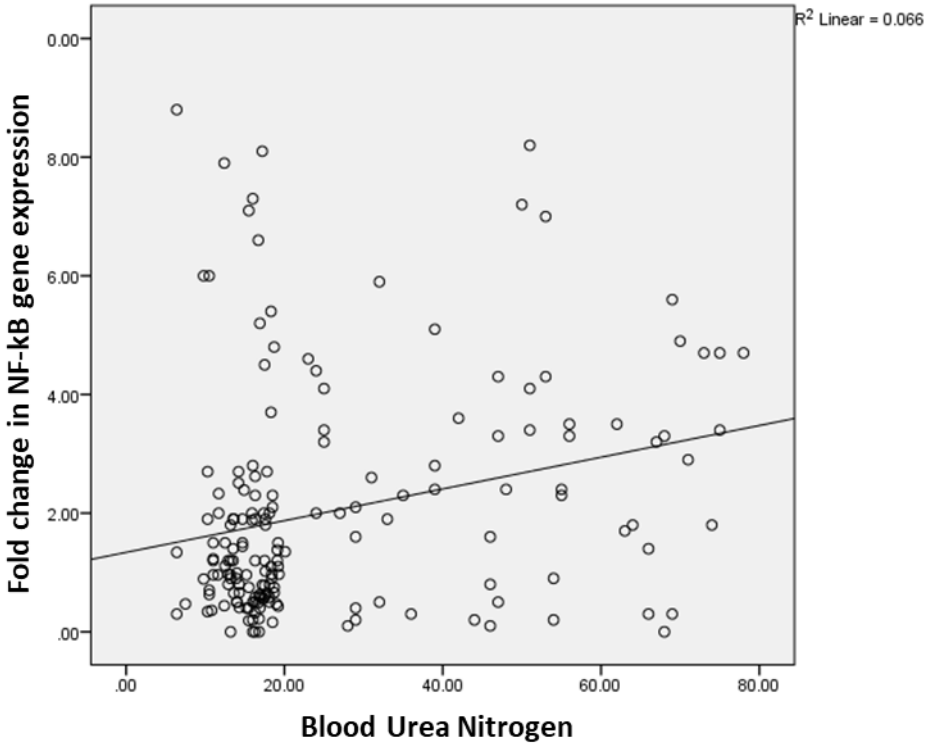

Figure 6.2.5: Correlation between blood urea nitrogen and CML-induced changes in *NF-κB* gene expression among the three study groups

6.3. CML-induced TNF production is elevated in diabetic nephropathy:

CML-induced TNF production was evaluated in patients with diabetic nephropathy, patients with T2DM, and healthy volunteers (n=59 per group). The effect of CML treatment on TNF production in the individual study groups was compared. CML treatment resulted in elevated TNF production in all three study groups. The highest increase in CML-induced TNF production was observed in the diabetic nephropathy group. The average fold increase was 1.76 ± 0.32 (p < 0.0001) in diabetic nephropathy, 1.47 ± 0.16 (p < 0.0001) in T2DM, and 1.30 ± 0.31 (p < 0.0001) in the healthy volunteers. The results are shown in Figure 6.3.1. The fold change in CML-induced TNF production was also compared between the three study groups.

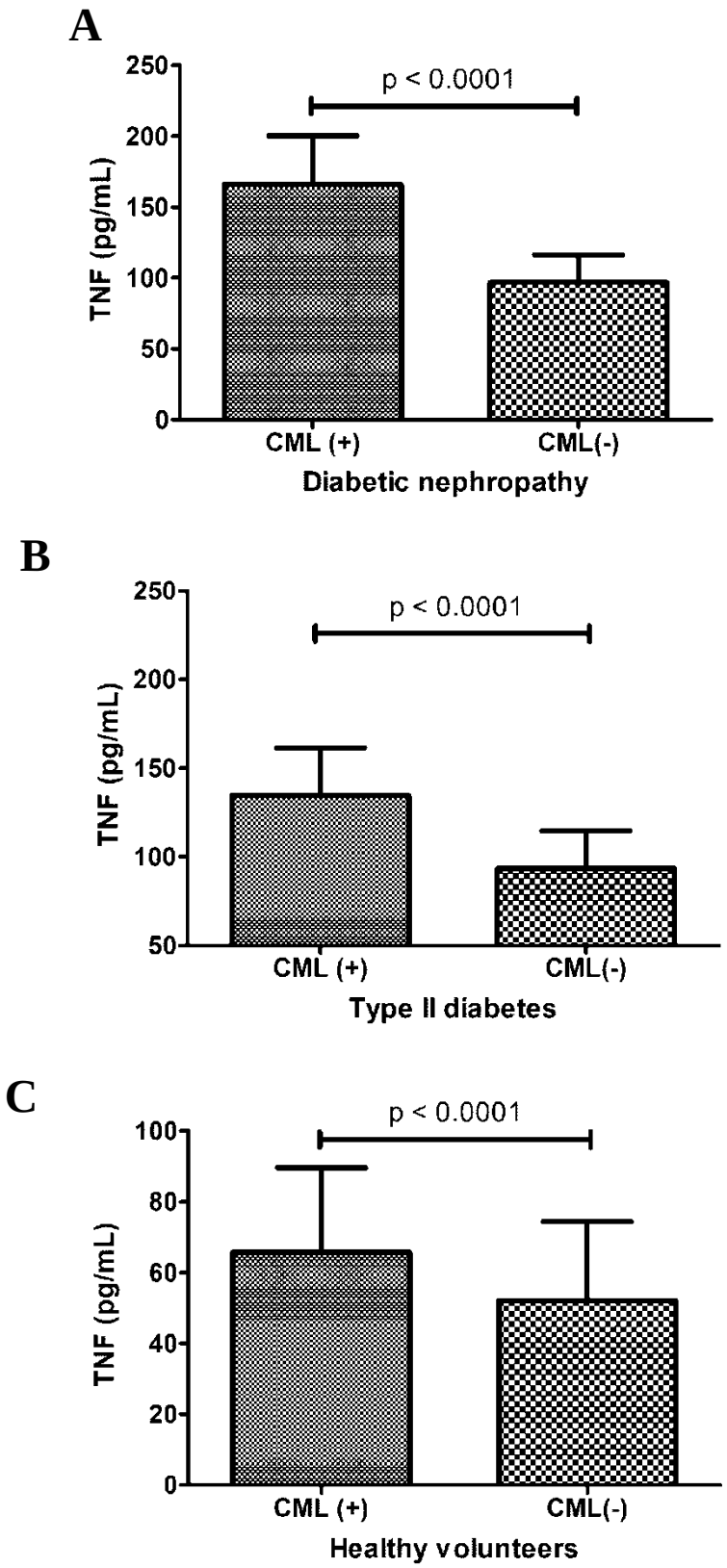

Figure 6.3.1: Effect of CML on TNF production in the study groups.

A. TNF levels in the conditioned media in the diabetic nephropathy group, **B.** TNF levels in the treated and untreated conditioned media in the T2DM group, **C**. TNF levels in the treated and untreated conditioned media in the healthy volunteers group (*n*= 59 per group).

A significant difference was observed in the CML-induced fold change between the three study groups ($p < 0.0001$; ANOVA). The results are shown in Figure 6.3.2. The CML-induced fold change was significantly different between the groups in the following combinations: DN vs. T2DM ($p < 0.0001$; unpaired t test), DN vs. healthy volunteers ($p < 0.0001$; unpaired t test), and T2DM vs. healthy volunteers ($p < 0.05$; unpaired t test). The results are depicted in Figure 6.3.3. These results indicate that CML treatment enhances TNF production in diabetic nephropathy and T2DM compared to healthy volunteers.

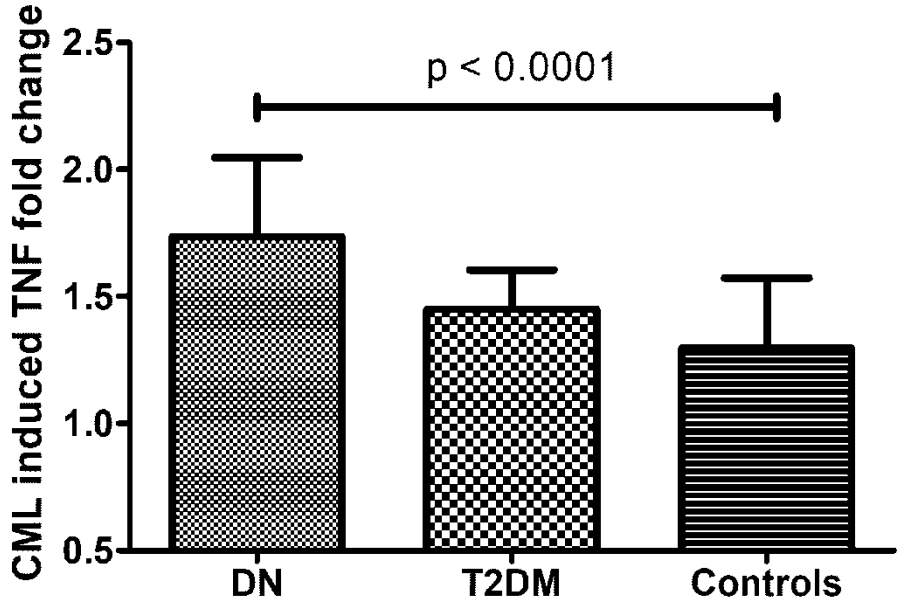

Figure 6.3.2: Comparison of CML-induced TNF fold change among the study groups (n= 59 per group).

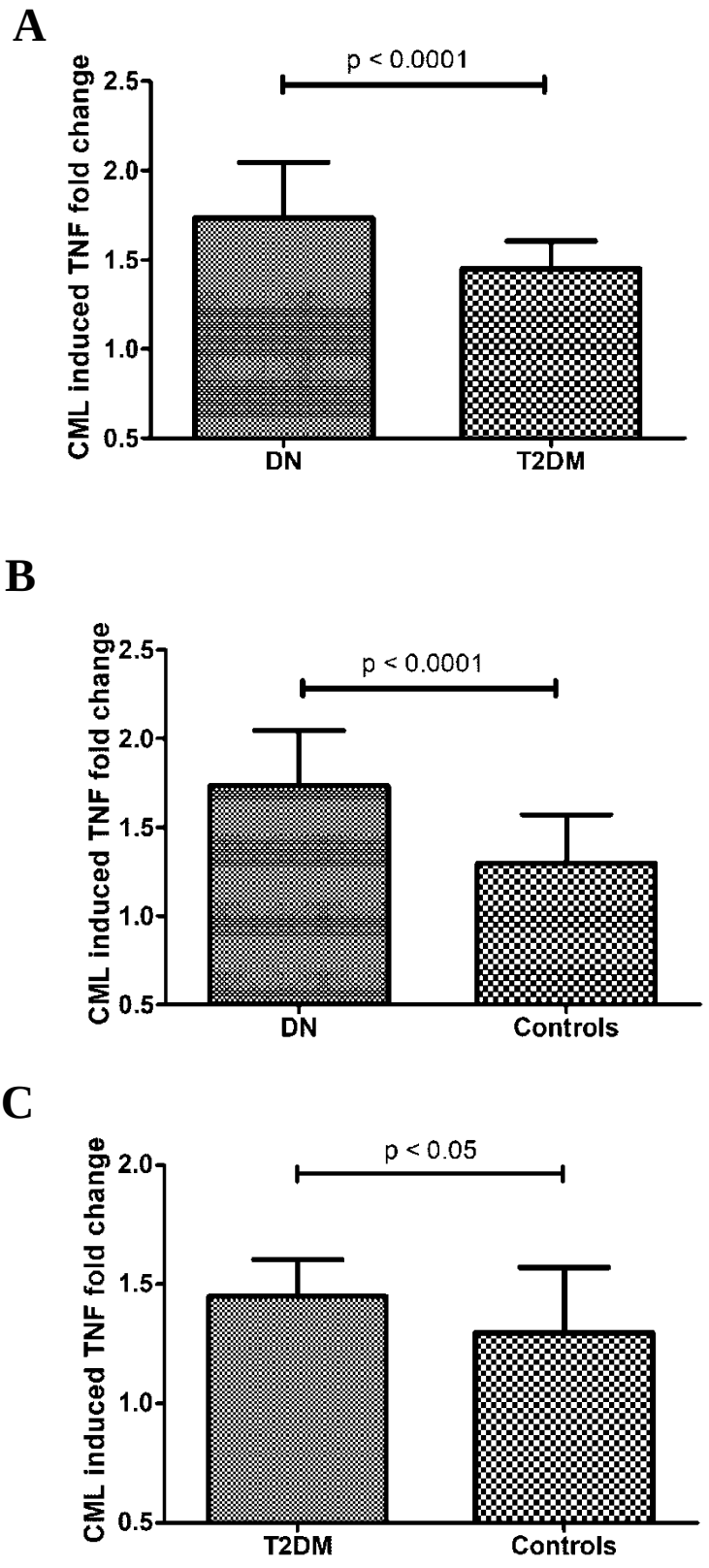

Figure 6.3.3: Comparison of CML-induced TNF fold change between the groups. **A:** CML-induced TNF fold change between the DN and T2DM groups, **B:** CML-induced TNF fold change between the DN and healthy volunteer groups, **C**. CML-induced TNF fold change between the T2DM and healthy volunteer groups (*n*= 59 per group).

6.4. Effect of clinico-biochemical parameters on CML-induced TNF secretion:

CML-induced TNF secretion was also compared with two major clinical parameters viz., measures of blood sugar control and measures of renal function. A statistically significant positive correlation (but of weak magnitude) was observed with the measures of blood sugar control, such as fasting blood sugar and ($r = 0.292$, $p < 0.0001$; Spearman's correlation) glycated haemoglobin ($r = 0.293$, $p < 0.0001$; Spearman's correlation). This indicates that the magnitude of TNF secretion is related to the magnitude of glycemic control. Similarly, the relationship between responsiveness to CML and renal function was assessed. A statistically significant positive correlation (but of weak magnitude) was observed with the measures of renal function, such as blood urea nitrogen ($r = 0.356$, $p < 0.001$; Spearman's correlation) and serum creatinine ($r = 0.380$, $p < 0.0001$; Spearman's correlation). Additionally, a statistically significant negative correlation (but of weak magnitude) was observed with the estimated glomerular filtration rate ($r = -0.4$, $p < 0.01$; Spearman's correlation). The results of the correlation analysis are depicted in Table 6.4. The correlation graphs are plotted in Figures 6.4.1-6.4.5.

Table 6.4.: Correlation of clinico-biochemical parameters with CML-induced TNF secretion of the study participants

Parameter	TNF Fold change	
	Spearman's Correlation (r)	***p* value**
Fasting blood sugar	0.292	< 0.0001
Glycated Haemoglobin	0.293	< 0.0001
Serum Creatinine	0.380	< 0.0001
Estimated glomerular filtration rate	-0.4	< 0.01
Blood Urea Nitrogen	0.356	< 0.001

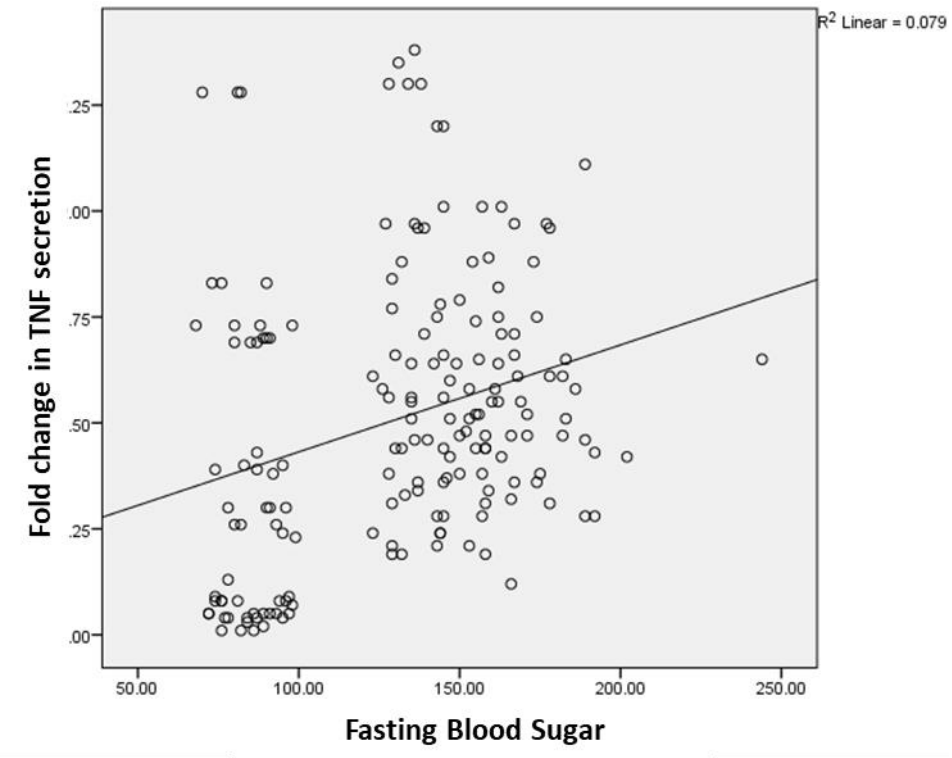

Figure 6.4.1: Correlation between fasting blood sugar levels and CML-induced changes in TNF secretion

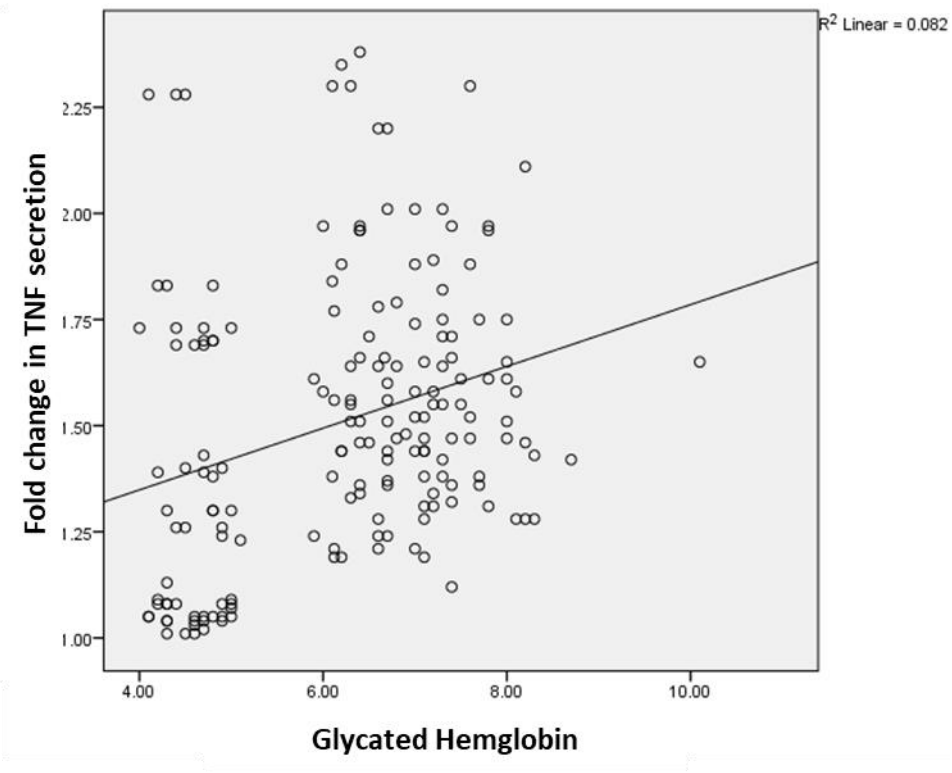

Figure 6.4.2: Correlation between glycated hemoglobin levels and CML-induced changes in TNF secretion

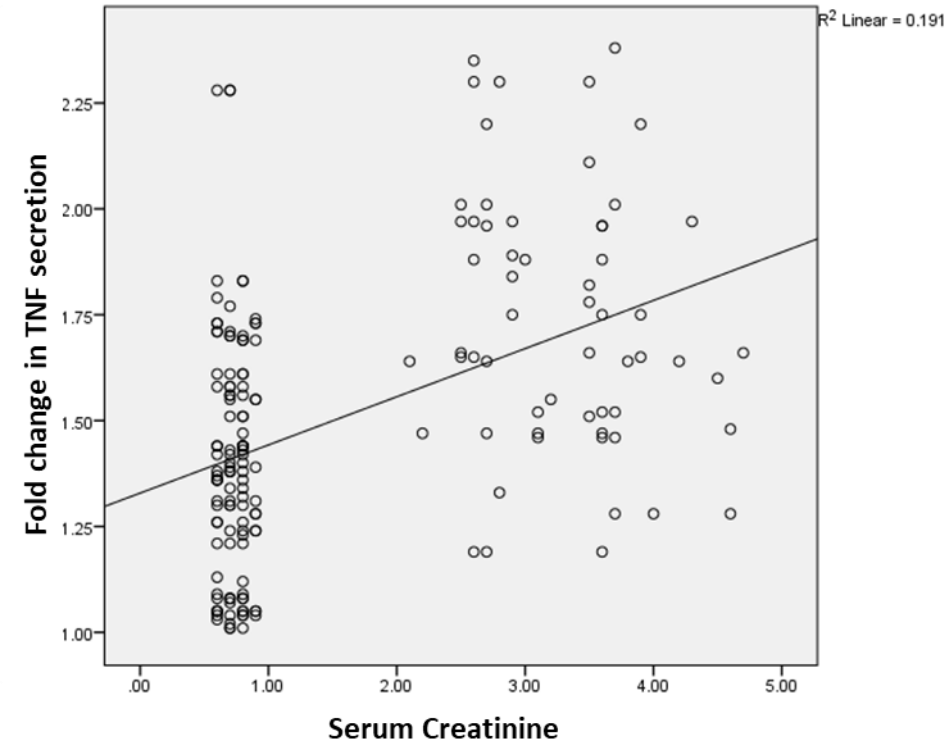

Figure 6.4.3: Correlation between serum creatinine levels and CML-induced changes in TNF secretion

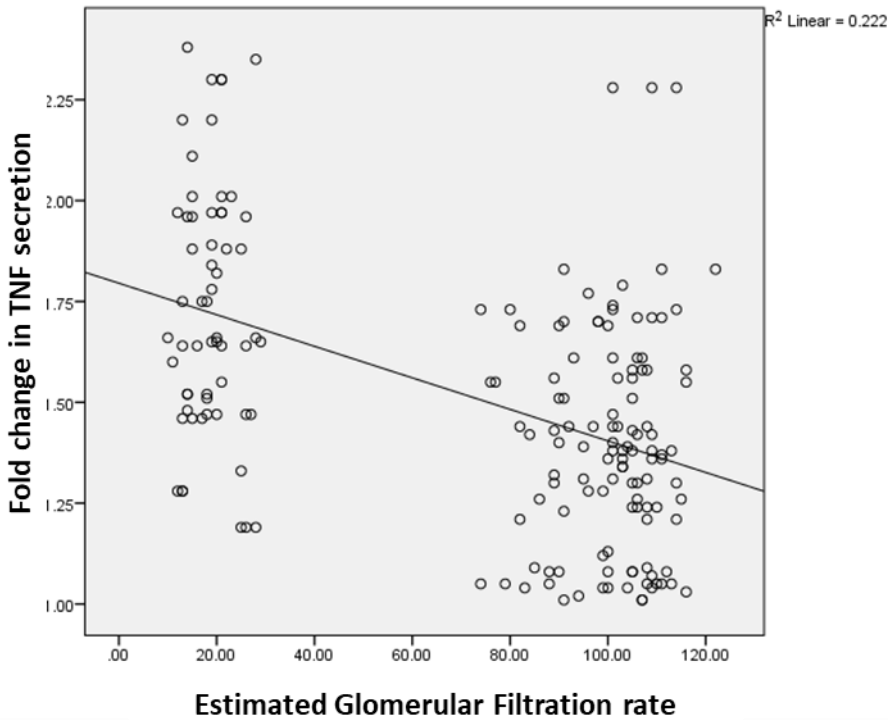

Figure 6.4.4: Correlation between estimated glomerular filtration rate and CML-induced change in TNF secretion

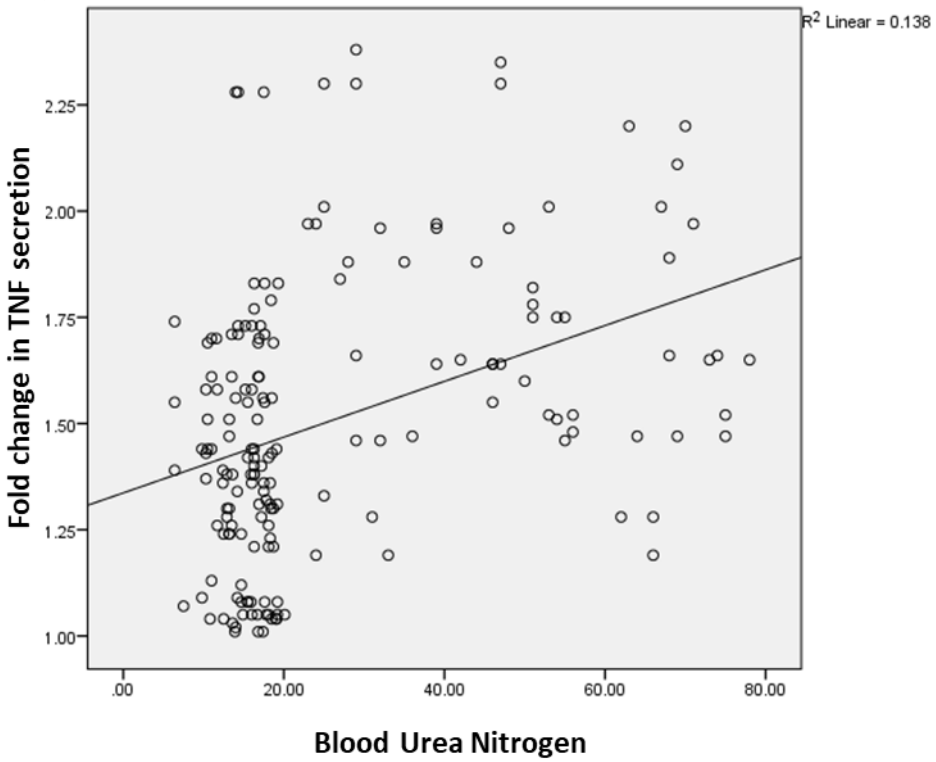

Figure 6.4.5: Correlation between blood urea nitrogen and CML-induced changes in TNF secretion

6.5. CML-induced changes in *NF-κB* gene expression and TNF secretion are correlated:

The CML-induced fold change in the gene expression of *NF-κB* was correlated with the fold change in TNF production among the three study groups. The correlation between *NF-κB* gene expression and TNF production was weakly positive (r = 0.219, p = 0.041, Pearson's correlation). The results are presented in Figure 6.5. These results indicate that as the level of NF-kB increases, TNF production also increases.

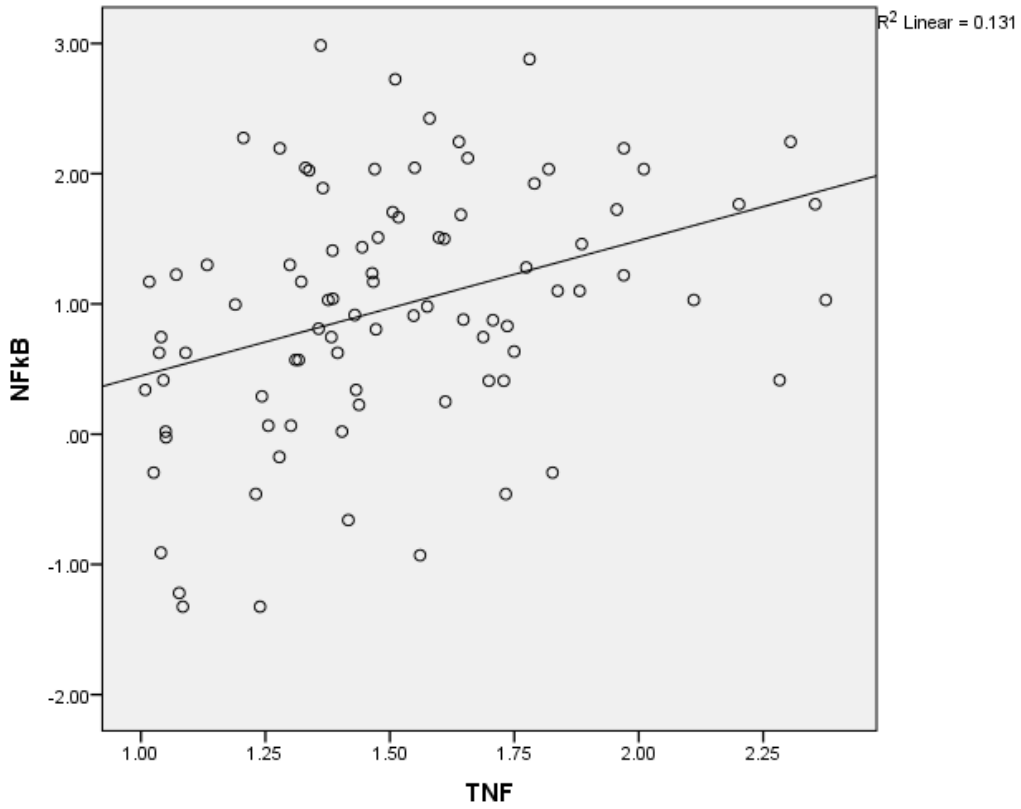

Figure 6.5: Correlation between CML-induced changes in *NF-κB* gene expression and TNF secretion

Insulin-induced sRAGE production is decreased in diabetic nephropathy:

Insulin-induced sRAGE production was evaluated in patients with diabetic nephropathy, patients with T2DM, and healthy volunteers (n=59 per group). The effect of insulin treatment on sRAGE production was compared in each study group. Insulin treatment resulted in higher levels of sRAGE in all three groups. The average fold increase was 1.4 ± 0.10 ($p < 0.0001$, paired t test) in diabetic nephropathy, 1.2 ± 0.11 ($p < 0.0001$, paired t test) in T2DM and 1.8 ± 0.19 ($p < 0.0001$, paired t test) in healthy volunteers. The highest fold change was observed in the case of healthy volunteers when compared to diabetic nephropathy and T2DM. The results are shown in Figure 6.6.1.

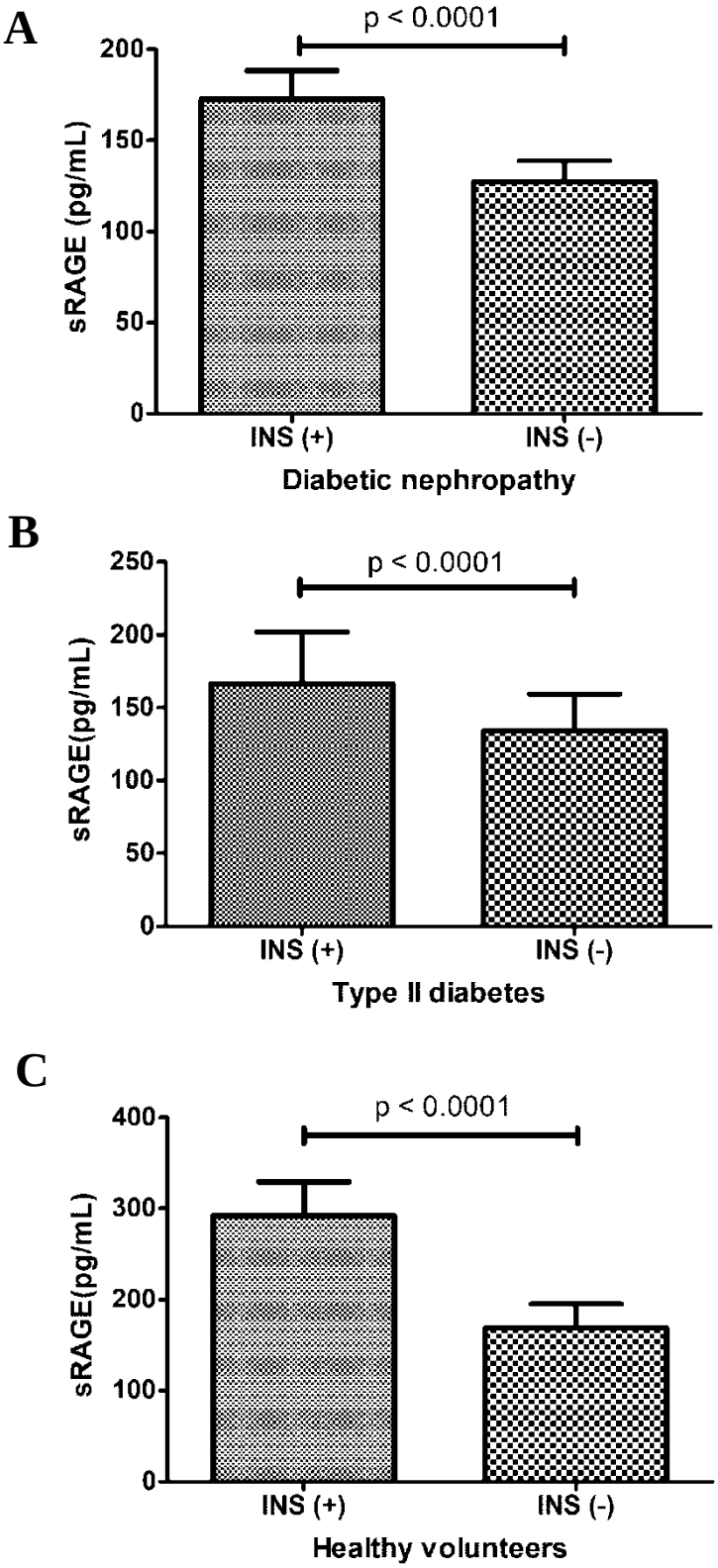

Figure 6.6.1: Effect of insulin on sRAGE production in the study groups. sRAGE levels in the treated and untreated conditioned media in the **A.** Diabetic nephropathy group, **B.** T2DM group, **C.** Healthy volunteer group (*n*= 59 per group).

Insulin-induced sRAGE production was compared between the three study groups. There was a significant difference in the fold change among the three groups (p <0.001; ANOVA). The results are shown in Figure 6.6.2. The insulin-induced fold change was significantly different between the groups in the following combinations: DN vs. T2DM (p <0.0001; unpaired t test), DN vs. healthy volunteers (p <0.0001; unpaired t test), and T2DM vs. healthy volunteers (p <0.0001; unpaired t test). The results are depicted in Figure 6.6.3. These results indicate that insulin treatment leads to significantly higher sRAGE production in healthy volunteers.

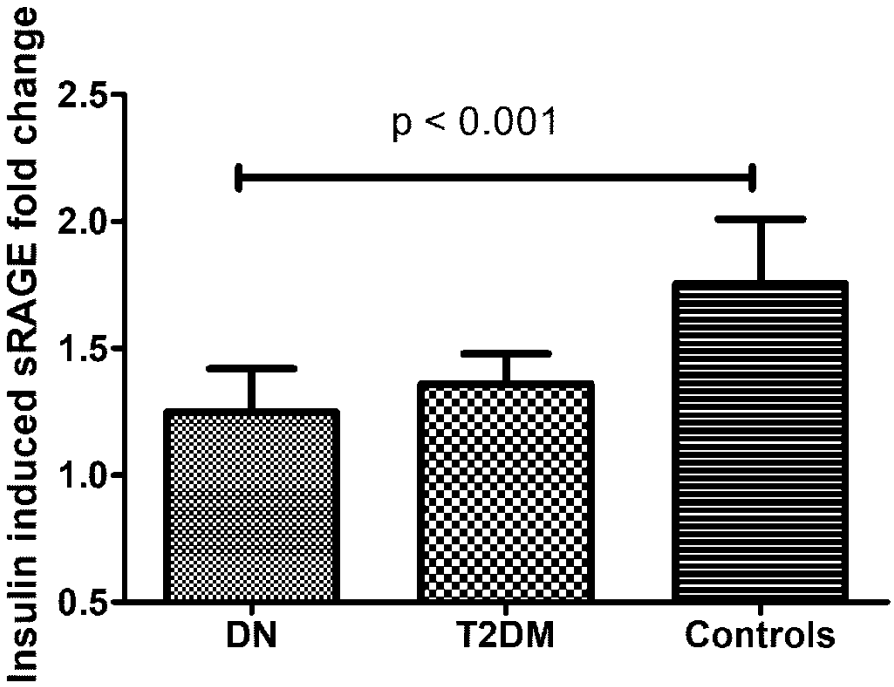

Figure 6.6.2: Comparison of insulin-induced sRAGE fold change between the study groups (n= 59 per group).

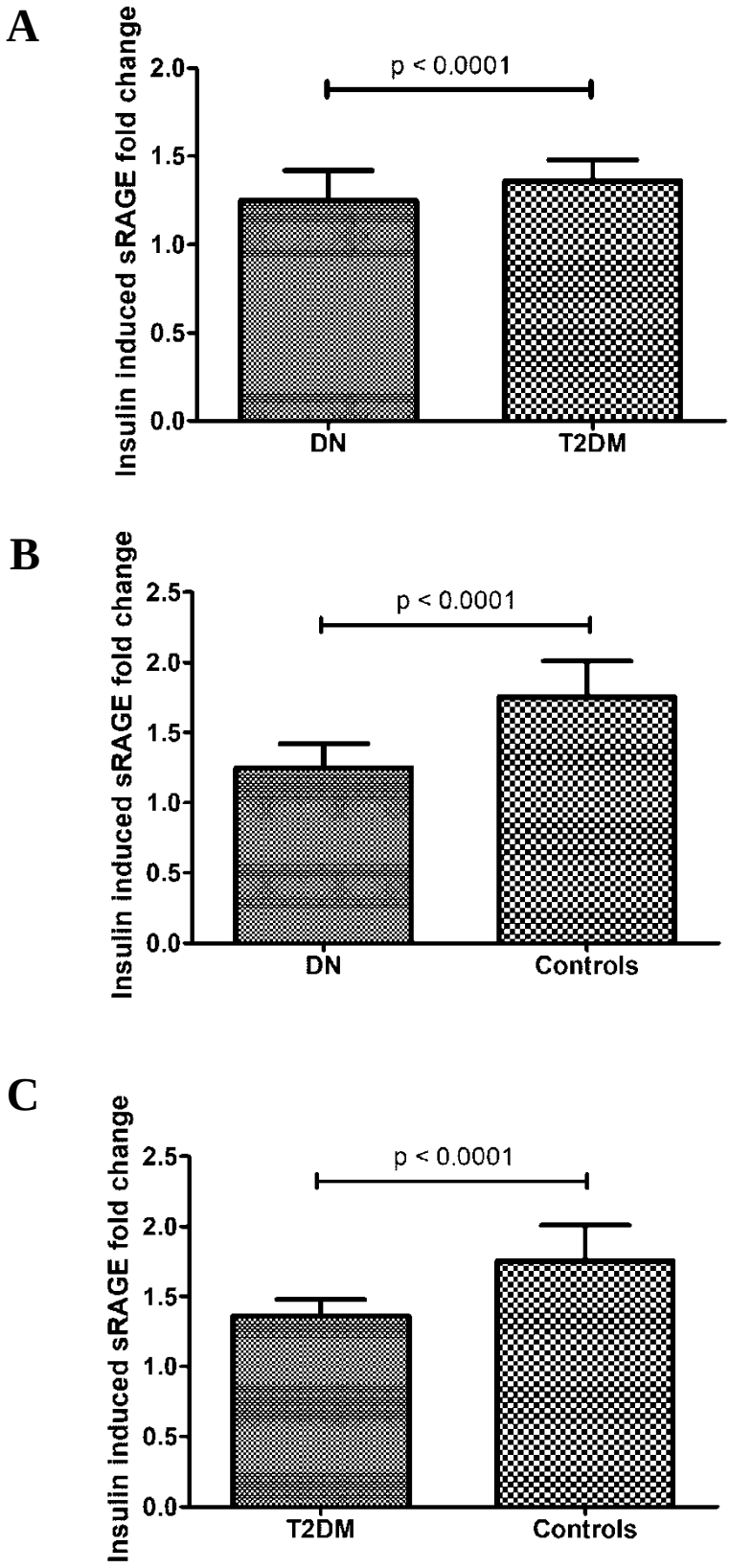

Figure 6.6.3: Comparison of insulin-induced sRAGE fold change between the groups. A. DN and T2DM, B. DN and healthy volunteers, C. T2DM and healthy volunteer groups.

6.7. Effect of clinico-biochemical parameters on insulin-induced sRAGE production:

Insulin-induced sRAGE production was also compared with two major clinical parameters viz., measures of blood sugar control and measures of renal function. A statistically significant negative correlation (but of weak magnitude) was observed with the measures of blood sugar control, such as fasting blood sugar ($r = -0.583$, $p < 0.001$; Spearman's correlation) and glycated haemoglobin ($r = -0.581$, $p < 0.001$; Spearman's correlation). Capacity for sRAGE production is related to capacity for glycemic control. Similarly, the relationship between renal function and sRAGE production was assessed. A statistically significant negative correlation (but of weak magnitude) was observed with the measures of renal function, such as blood urea nitrogen ($r = -0.151$, $p < 0.05$; Spearman's correlation) and serum creatinine ($r = -0.151$, $p < 0.05$; Spearman's correlation). This implies that decreased sRAGE levels may contribute to the deterioration in renal function. The results of the correlation analysis are depicted in Table 6.7. The correlation graphs are plotted in Figures 6.7.1 - 6.7.5

Table 6.7.: Correlation of clinico-biochemical parameters with insulin-induced sRAGE production of the study participants

Parameter	sRAGE Fold change	
	Spearman's Correlation (r)	***p* value**
Fasting blood sugar	**-0.583**	<0.001
Glycated Haemoglobin	**-0.581**	<0.001
Serum Creatinine	**-0.155**	<0.05
Estimated glomerular filtration rate	0.130	0.087
Blood Urea Nitrogen	**-0.151**	<0.05

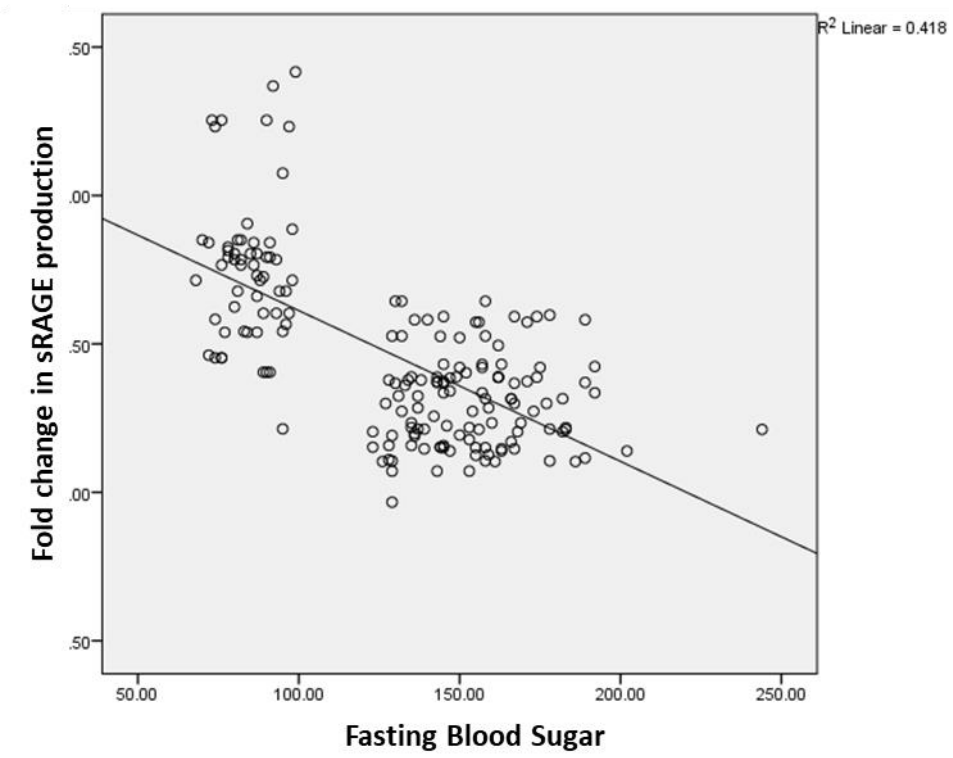

Figure 6.7.1: Correlation between fasting blood sugar levels and insulin-induced changes in sRAGE production

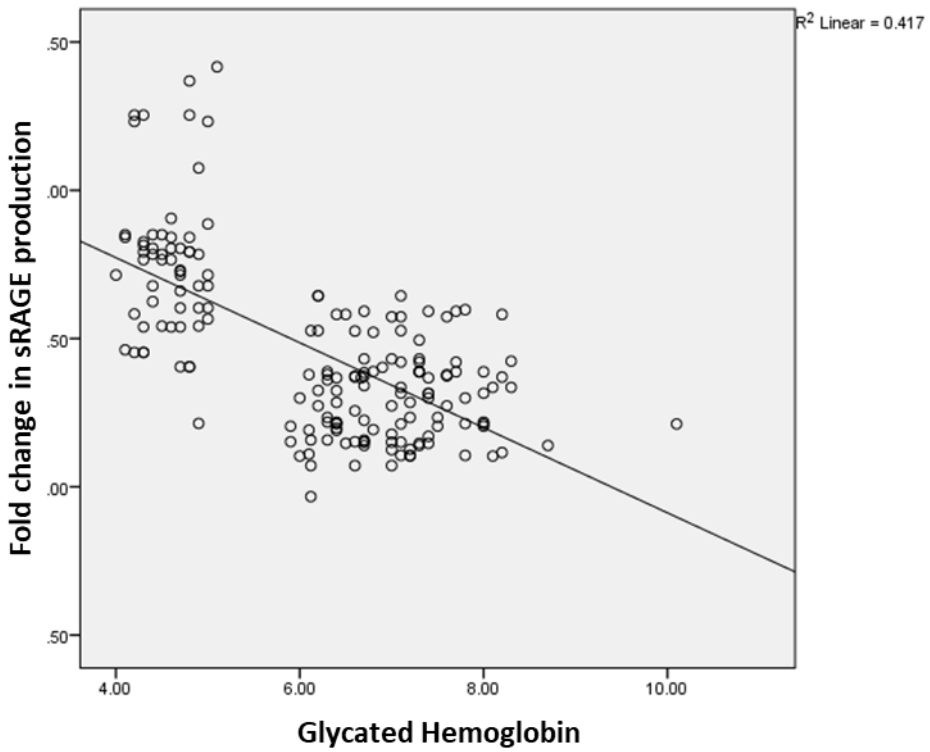

Figure 6.7.1: Correlation between glycated hemoglobin levels and insulin-induced change in sRAGE production

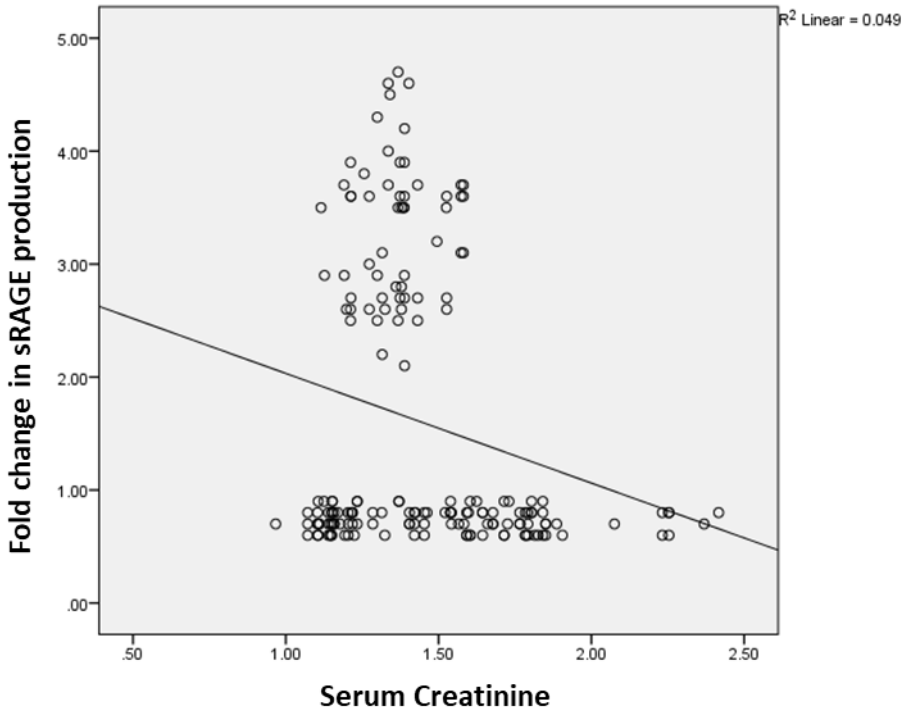

Figure 6.7.1: Correlation between serum creatinine levels and insulin-induced changes in sRAGE production

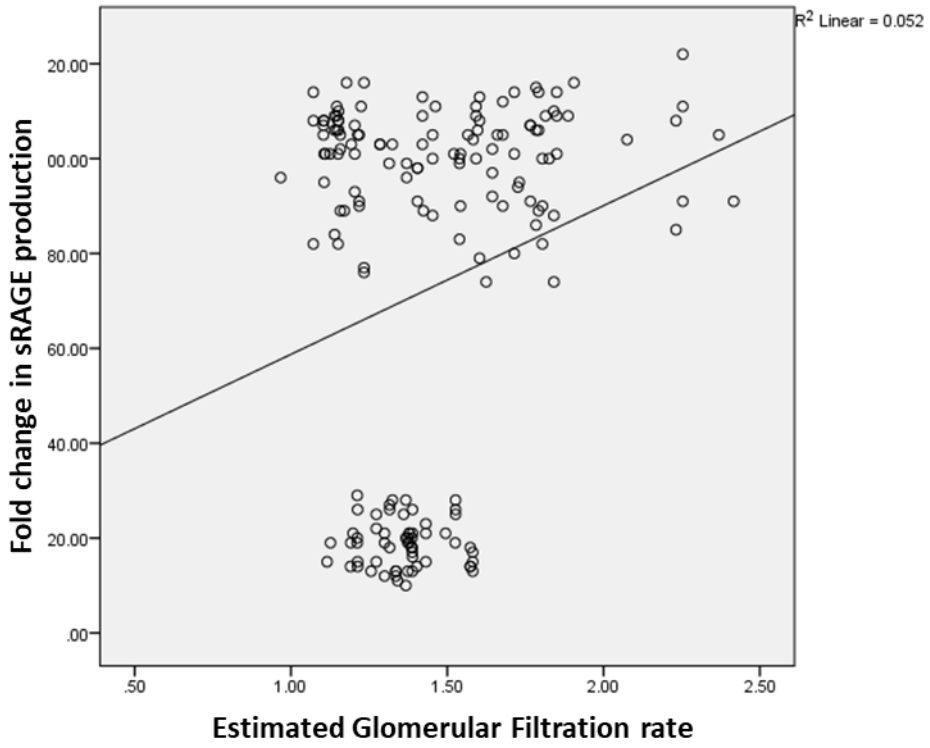

Figure 6.7.1: Correlation between estimated glomerular filtration rate and insulin-induced change in sRAGE production

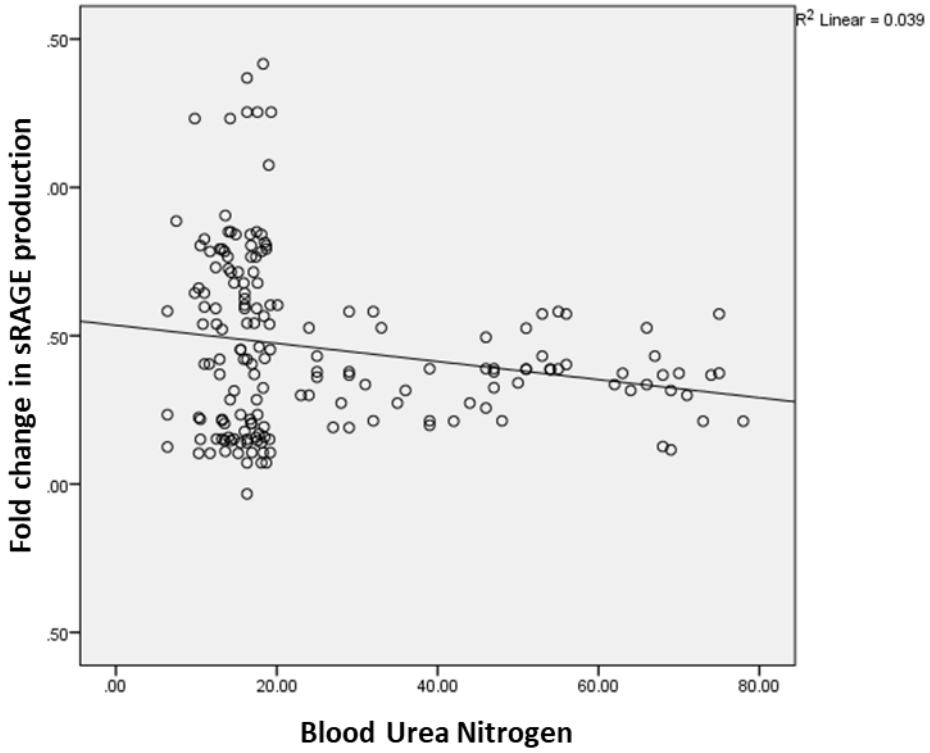

Figure 6.7.1: Correlation between blood urea nitrogen levels and insulin-induced changes in sRAGE production

6.8. Insulin-induced *ADAM10* gene expression is upregulated in healthy volunteers:

CML-induced *ADAM10* gene expression was evaluated in patients with diabetic nephropathy, patients with T2DM, and healthy volunteers (n=59 per group). ΔCt was calculated for the normalised gene expression of *ADAM10* and is represented in Figure 6.8. Insulin treatment resulted in higher normalised expression (ΔCt) of the *ADAM10* gene in healthy volunteers (p <0.001; paired t test), whereas there was no significant change in diabetic nephropathy (p = 0.159; paired t test) and T2DM (p = 0.143; paired t test).

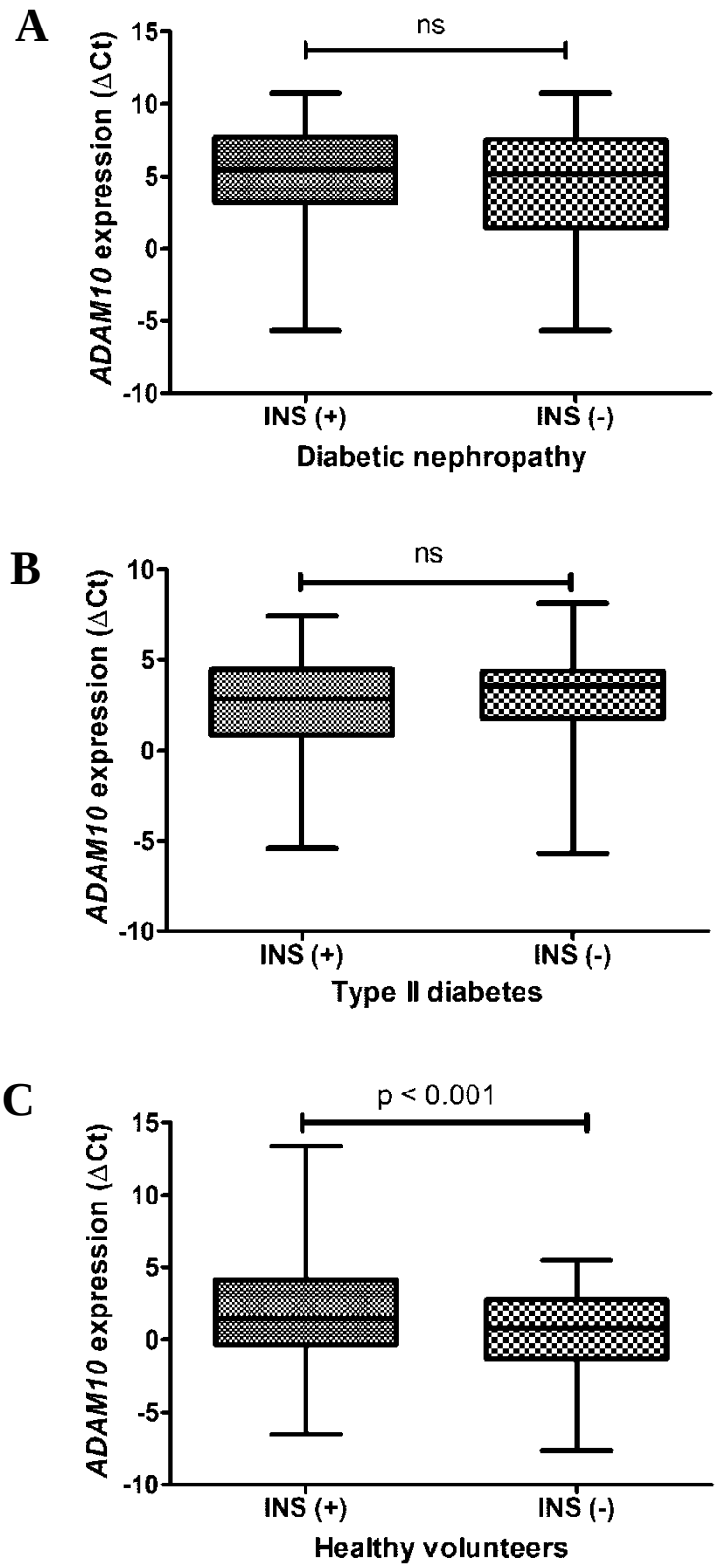

Figure 6.8: Effect of insulin on *ADAM10* gene expression in the study groups. Normalised expression A. Diabetic nephropathy group, B. T2DM group, C. Healthy volunteer group

The insulin-induced fold change ($2^{-\Delta\Delta Ct}$) in *ADAM10* gene expression was 0.93x in DN, 1x in T2DM, and 1.27x in healthy volunteers. The fold change ($\log 2^{-\Delta\Delta Ct}$) of the *ADAM10* gene was compared between the three study groups. The $\log 2^{-\Delta\Delta Ct}$ was significantly different among the three groups ($p < 0.001$; ANOVA). The results are presented in Figure 6.8.1. The insulin-induced fold change was significantly different between the groups in the following combinations: DN vs. T2DM ($p < 0.001$; unpaired t test), DN vs. healthy volunteers ($p < 0.0001$; unpaired t test), and T2DM vs. healthy volunteers ($p < 0.001$; unpaired t test). The results are depicted in Figure 6.8.2. These results indicate that insulin treatment leads to significant upregulation of *ADAM10* gene expression in healthy volunteers but not in diabetic nephropathy and T2DM.

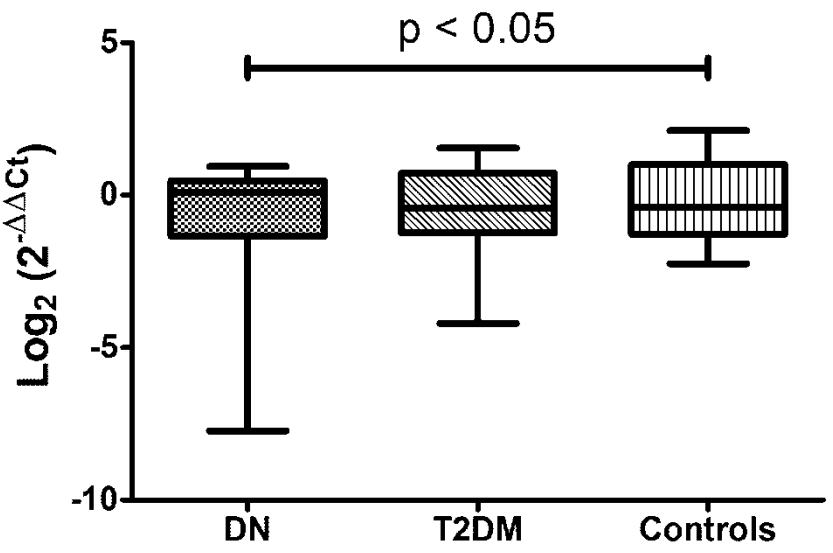

Figure 6.8.1: Comparison of insulin-induced changes in *ADAM10* gene expression between the study groups. Fold change in *ADAM10* gene expression between the three study groups (n= 59 per group).

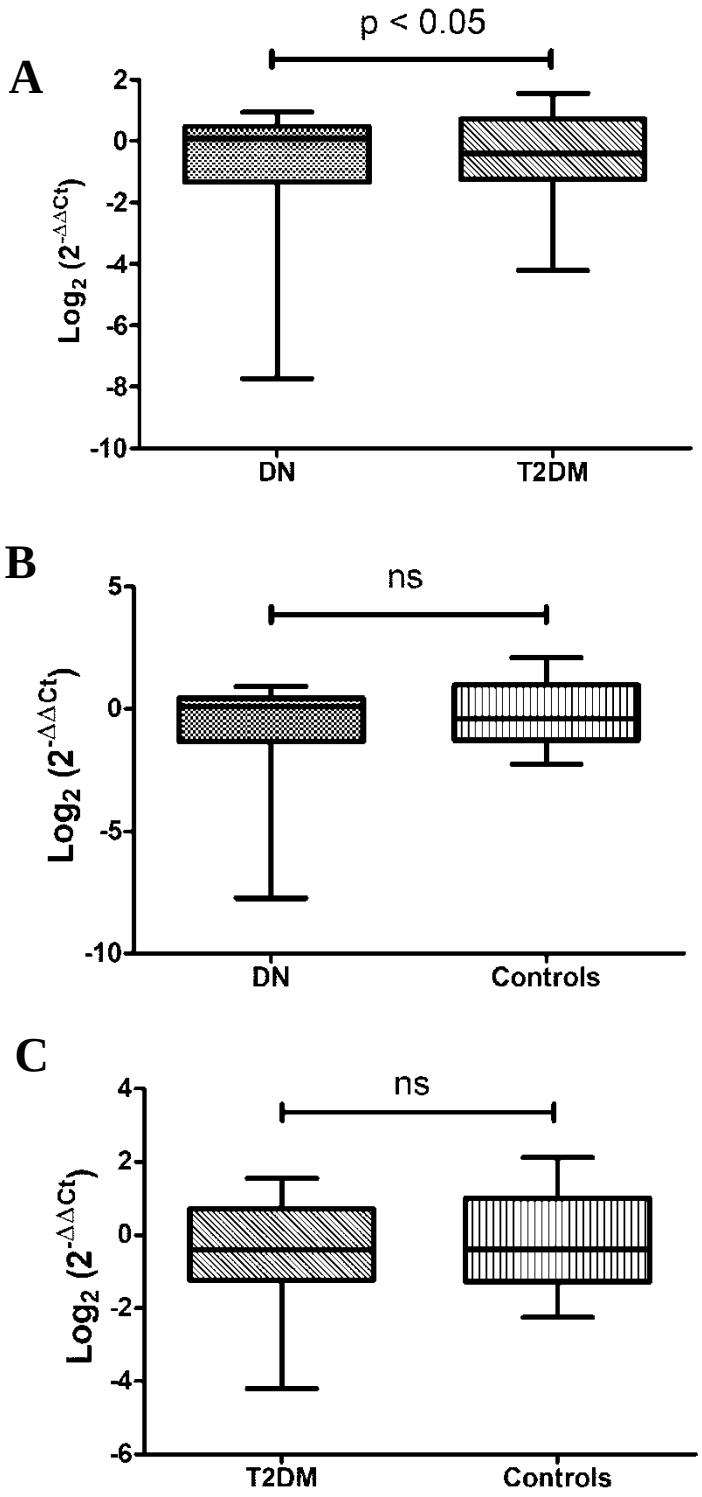

Figure 6.8.2: Comparison of insulin-induced fold change in *ADAM10* gene expression between the study groups. A. DN and T2DM groups, B. DN and healthy volunteers, C. T2DM and healthy volunteers (*n*= 59 per group).

6.9. Effect of biochemical parameters on insulin-induced *ADAM10* gene expression:

Insulin-induced *ADAM10* expression was also compared with two major clinical parameters viz., measures of blood sugar control and measures of renal function (n = 177). A statistically significant negative correlation (but of weak magnitude) was observed with the measures of blood sugar control, such as fasting blood sugar (r = -0.461, $p < 0.0001$; Spearman's correlation) and glycated haemoglobin (r = -0.473, $p < 0.0001$; Spearman's correlation). This finding indicates that *ADAM10* expression is inversely associated with glycemic control. Similarly, the relationship between renal function and sRAGE production was assessed. A statistically significant negative correlation (but of weak magnitude) was observed with the marker of renal function serum creatinine (r = -0.243, $p < 0.001$; Spearman's correlation) and blood urea nitrogen (r = -0.299, $p < 0.0001$; Spearman's correlation). A statistically significant positive correlation (but of weak magnitude) was observed with the estimated glomerular filtration rate (r = 0.201, $p < 0.001$; Spearman's correlation). The results of the correlation analysis are depicted in Table 6.9. The correlation graphs are plotted in Figures 6.9.1-6.9.5.

Table 6.9: Correlation of clinico-biochemical parameters with insulin-induced *ADAM10* gene expression of the study participants

Parameter	*ADAM10* Fold change	
	Spearman's Correlation (r)	*p* value
FBS	-0.461	< 0.0001
HbA1c	-0.473	< 0.0001
S. Creatinine	-0.243	< 0.001
eGFR	0.201	< 0.001
BUN	-0.299	< 0.0001

*p value < 0.05, correlation is statistically significant

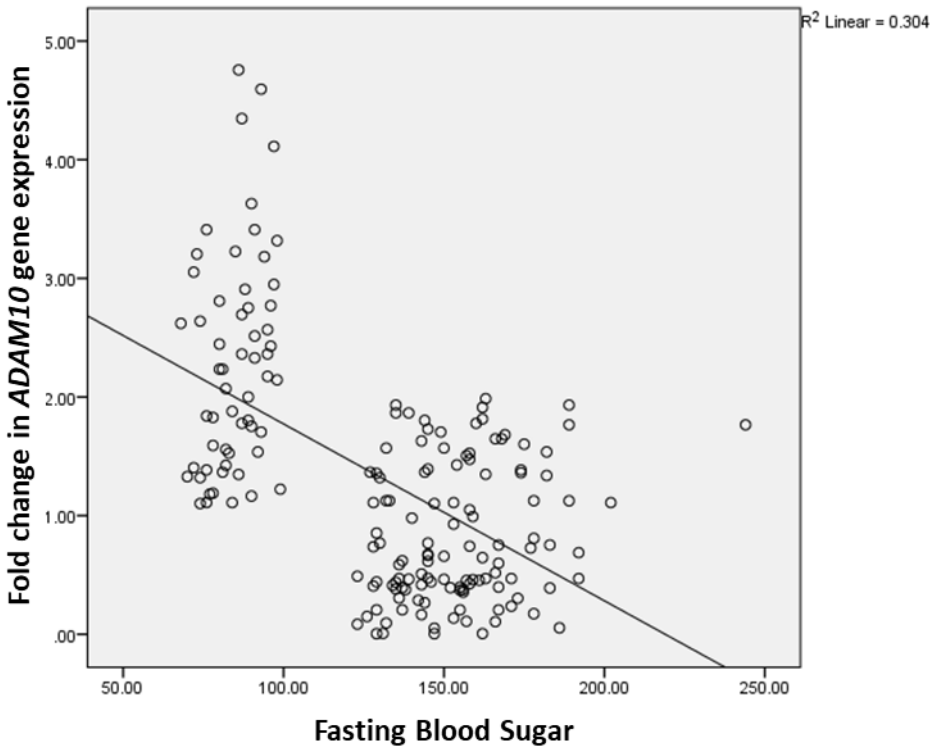

Figure 6.9.1: Correlation between fasting blood sugar levels and insulin-induced fold change in *ADAM10* gene expression

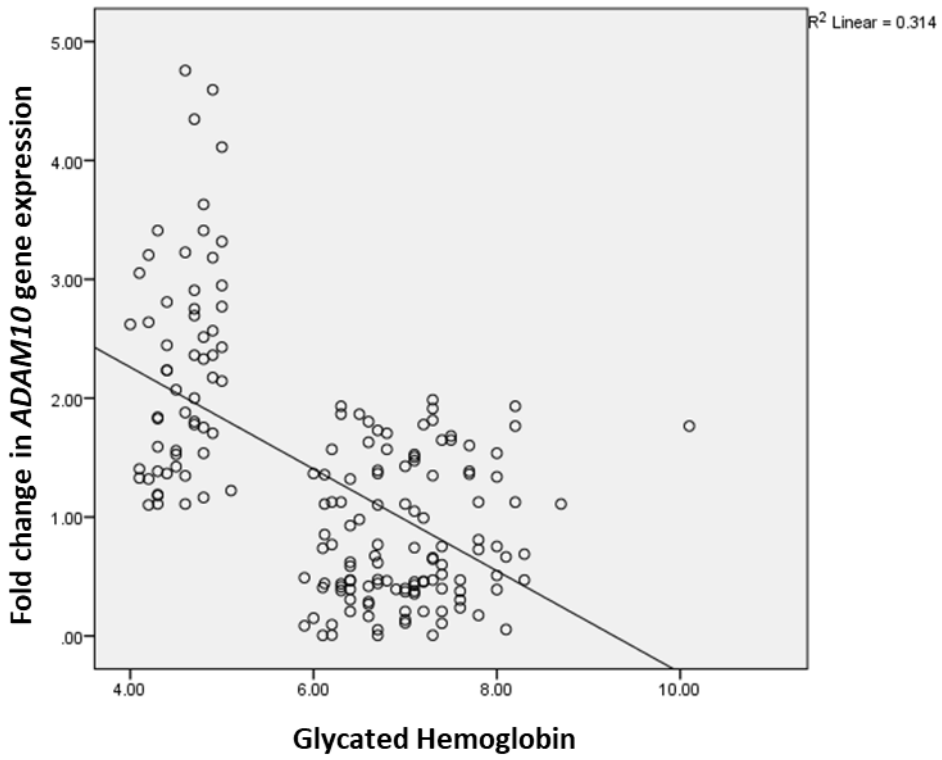

Figure 6.9.2: Correlation between glycated hemoglobin levels and insulin-induced fold change in *ADAM10* gene expression

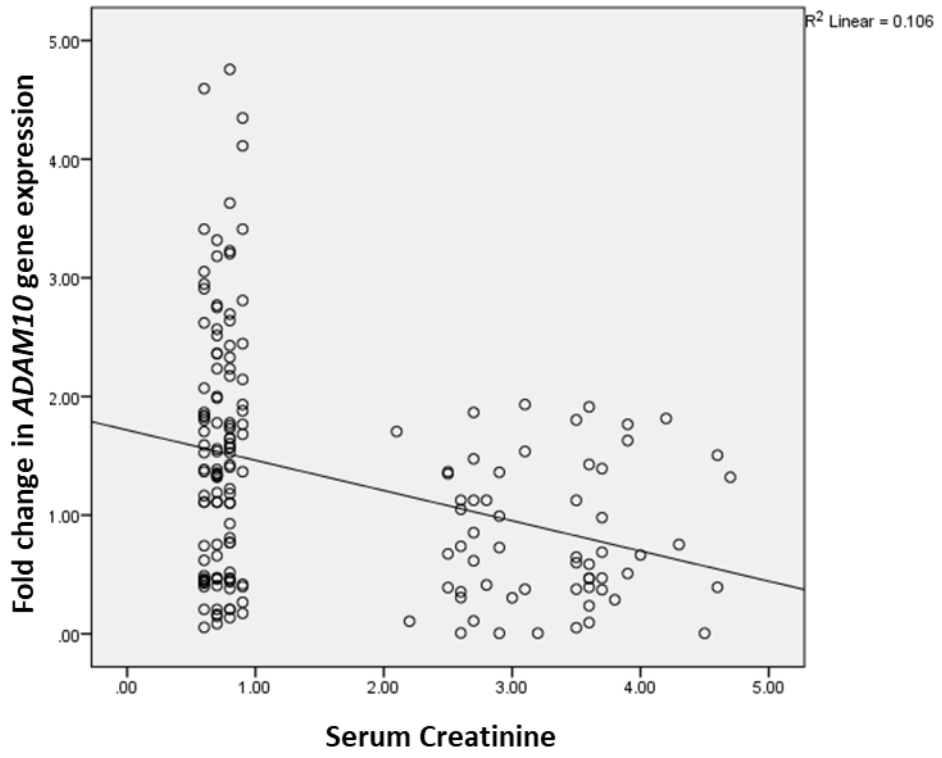

Figure 6.9.3: Correlation between serum creatinine levels and insulin-induced fold change in *ADAM10* gene expression

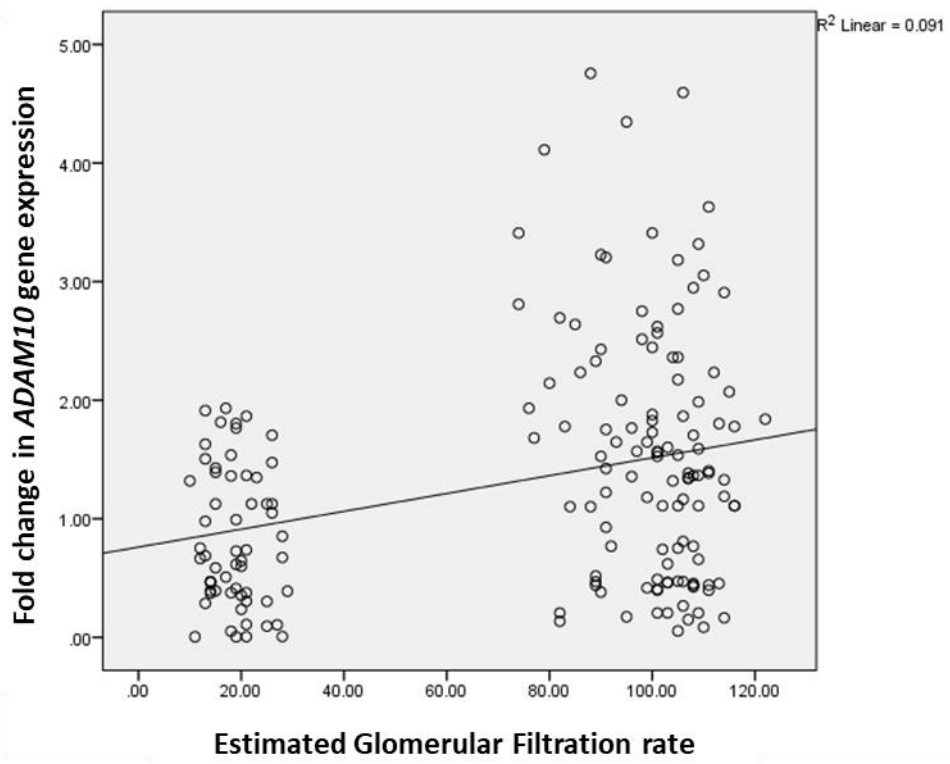

Figure 6.9.4: Correlation between estimated glomerular filtration rate and insulin-induced fold change in *ADAM10* gene expression

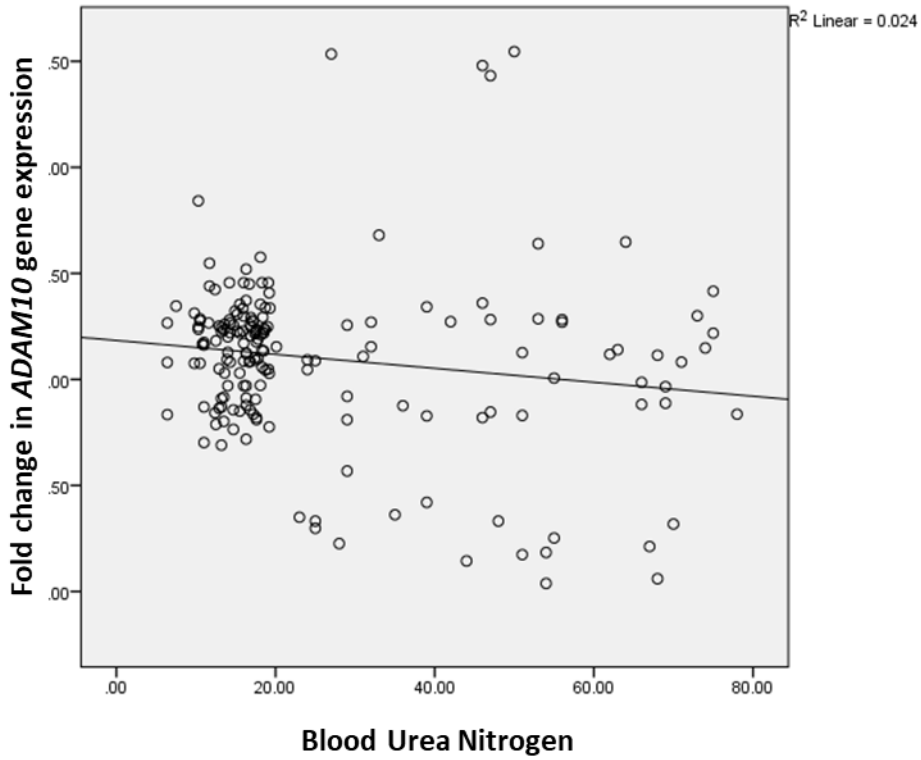

Figure 6.9.5: Correlation between blood urea nitrogen levels and insulin-induced fold change in *ADAM10* gene expression

6.10. Insulin-induced changes in *ADAM10* gene expression and sRAGE production are correlated:

Insulin-induced fold change in the gene expression of ADAM10 was correlated with the fold change in sRAGE production among the three study groups. The correlation between *ADAM10* gene expression and sRAGE production was found to be moderately positive (r = 0.529; p = 1.37 $X10^{-7}$; Pearson correlation). The results are presented in Figure 6.10. These results indicate that as the level of ADAM10 increases, sRAGE production increases.

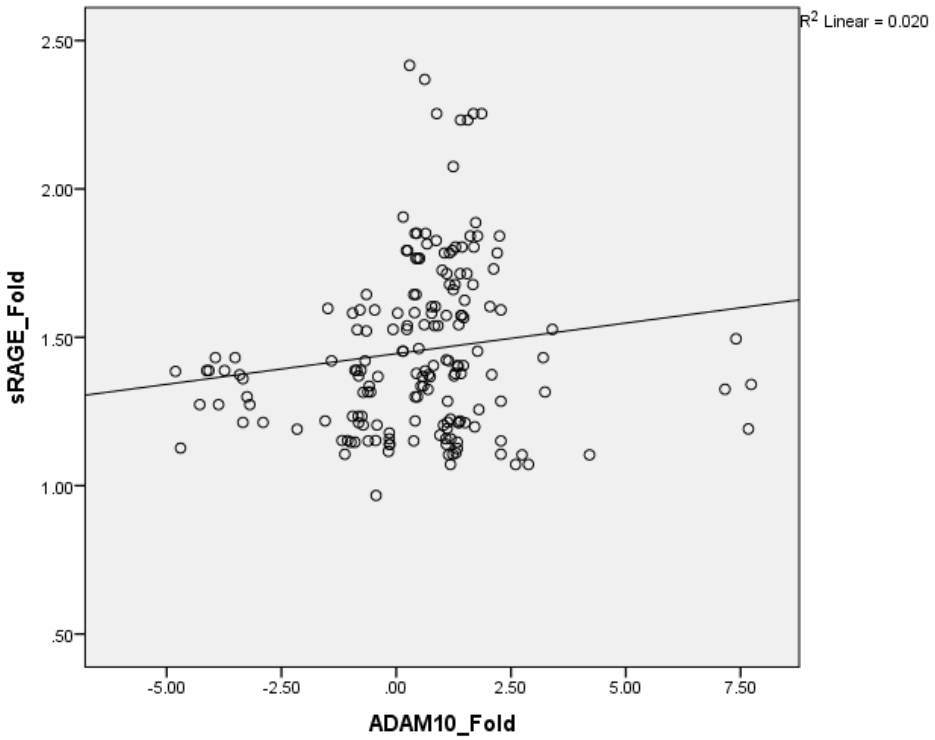

Figure 6.10: Correlation between insulin-induced changes in *ADAM10* gene expression and sRAGE production.

CHAPTER VII
DISCUSSION

The aim of this study was to evaluate the functional status of the RAGE pathway in DN. The main findings of this study are as follows:

a) CML-induced *NF-κB* gene expression and TNF secretion were higher in DN and T2DM patients than in healthy volunteers
b) Insulin-induced sRAGE production was higher in healthy volunteers than in DN and T2DM patients
c) Insulin-induced *ADAM10* gene expression was upregulated in healthy volunteers compared to DN and T2DM patients

The inference and the significance of each finding are discussed individually below.

Inference 1: DN and T2DM patients are hyper-responsive to CML

CML treatment of blood samples showed higher *NF-κB* gene expression and TNF secretion in DN and T2DM patients than in healthy volunteers. CML is an agonist of RAGE (Rabbani and Thornalley, 2018). The binding of CML to RAGE activates the downstream signaling cascade that results in the upregulation of the *NF-κB* gene, and as a consequence, there is an increase in the synthesis of NF-κB protein. This protein is a transcription factor that translocates from the cytoplasm to the nucleus and regulates inflammatory gene expression by binding to the promoter region of cytokine genes such as TNF. The higher *NF-κB* gene expression and TNF secretion observed in this study indicate that DN and T2DM patients are hyper-responsive to CML.

CML-induced *NF-κB* expression and TNF secretion were also compared to measures of glycemic control (fasting blood sugar, glycated hemoglobin) and renal function (serum creatinine, estimated glomerular filtration rate, and blood urea nitrogen).

CML-induced *NF-κB* gene expression showed a positive correlation (weak magnitude) with the measures of glycemic control (fasting blood sugar and glycated hemoglobin) and renal function (serum creatinine and blood urea nitrogen). Additionally, a negative correlation was observed with respect to estimated glomerular filtration rate and CML-induced *NF-κB* gene expression. CML-induced TNF secretion showed a positive correlation with glycemic control and renal function. However, the effect size of the correlation was weak for both measures. This relationship indicates that the magnitude of CML-induced TNF secretion is weakly related to the magnitude of glycemic control and renal function.

To the best of our knowledge, this is the first study to compare the effect of CML on the RAGE pathway in DN. Previous studies have focused on the analysis of *NF-κB* expression and serum TNF levels in DN patients. Upregulation of the *NF-κB* gene in DN has been reported in various samples, such as PBMCs, renal biopsy, and urine (da Veiga *et al*., 2019, Mezzano *et al*., 2004). In addition, the expression of the *NF-κB* gene has been demonstrated to be proportional to the stages of DN (Yi *et al*., 2014). Furthermore, serum levels of TNF have been shown to be relatively higher in DN and T2DM patients (Chen *et al*., 2017). This study shows that CML may be responsible for the

elevated NF-κB and TNF levels observed in previous reports. The obtained results prove that the RAGE pathway is hyper-responsive to CML because of the upregulation of *NF-κB* gene expression and the increase in TNF secretion upon CML stimulation.

Inference 2: sRAGE production is impaired in DN

In this study, insulin treatment of blood samples resulted in increased sRAGE production in healthy volunteers but not in the DN and T2DM groups. Insulin-induced sRAGE production was lowest in the DN group, followed by the T2DM group. The highest level of insulin-induced sRAGE production was observed in healthy volunteers. This finding indicates that sRAGE production is non-responsive to insulin in DN.

The non-responsiveness of sRAGE production to insulin treatment may be due to insulin resistance. Insulin resistance is a condition wherein insulin-dependent cells fail to absorb glucose despite adequate levels of insulin (Freeman and Pennings, 2021). As a result, patients show elevated serum levels of both insulin and glucose. Furthermore, insulin resistance is a condition commonly observed in T2DM and DN (Goyal and Jialal, 2021).

Furthermore, insulin-induced sRAGE production was also compared to measures of glycaemic control and measures of renal function. A negative correlation was observed. However, the effect size of the correlation was weak for both measures. This implies that the capacity for sRAGE production is

inversely related to the capacity for glycemic control and that decreased sRAGE levels may contribute to the deterioration of renal function.

Previous studies have mainly focused on measuring serum sRAGE levels in diabetic patients. To the best of our knowledge, this is the first study to examine the effect of insulin on sRAGE production. Previous reports have shown that serum sRAGE levels are significantly lower in diabetic patients with microvascular complications such as nephropathy and retinopathy than in diabetic patients without microvascular complications (Grossin *et al*., 2008, Derosa *et al*., 2017, Farhan and Hussain, 2019). The results of this study indicate that impairment of sRAGE production may be responsible for the reduced serum sRAGE observed in previous studies.

Inference 3: *ADAM10* gene expression is downregulated in DN

ADAM10 mRNA levels were found to be lower in DN and T2DM patients than in healthy volunteers. This finding indicates that *ADAM10* gene expression is downregulated in DN and T2DM patients. Furthermore, *ADAM10* mRNA levels in the insulin-treated samples were found to be higher than those in untreated samples in the healthy volunteers but not in DN and T2DM patients. Insulin-induced *ADAM10* gene expression was lowest in the DN group, followed by the T2DM group. The highest level of insulin-induced *ADAM10* gene expression was observed in healthy volunteers. These results indicate that *ADAM10* gene expression is not responsive to insulin in DN and T2DM patients. As seen in the case of sRAGE production, non-responsiveness to insulin may be

due to insulin resistance seen in DN and T2DM patients. This assumption is supported by the cellular studies of Hu *et al*. (2016). These authors have shown that insulin is involved in the regulation of *ADAM10* gene expression. Insulin was found to activate *ADAM10* gene expression *via* the ERK1/2 signalling pathway and upstream transcription factor 1.

The ADAM10 enzyme is involved in sRAGE production. Both *ADAM10* gene expression and sRAGE production were found to be reduced in DN and T2DM. Correlation analysis was carried out to examine the relationship between the two entities. It was found that there was a weak positive correlation between ADAM10 and sRAGE.

Furthermore, insulin-induced *ADAM10* gene expression was also compared to measures of glycaemic control and measures of renal function. A negative correlation was observed. However, the effect size of the correlation was weak for both measures. This finding indicates that the magnitude of *ADAM10* gene expression may be inversely associated with glycemic control and that decreased *ADAM10* expression may be a minor contributor to the deterioration of renal function.

Final conclusion: This study shows that the RAGE pathway is hyperresponsive to CML in diabetic patients, with a higher effect in patients with renal complications. Furthermore, sRAGE and ADAM10, which play an important role in the regulation of the RAGE pathway, were found to be reduced in DN. Together, these results support the conclusion that the RAGE pathway may be abnormally active in DN patients, probably due to poor regulation. This is the

first attempt to examine the intrinsic capacity of diabetic patients to respond to CML. These results show that diabetic patients are intrinsically prone to produce excessive inflammatory cytokines when exposed to elevated sugar levels.

CHAPTER VIII

a) The aim of this study was to evaluate the functional status of the RAGE signalling pathway in DN.

b) The study was carried out by adopting a three group observational study design. Groups I, II, and III comprised DN patients, T2DM patients and healthy volunteers, respectively. A total of 59 patients were recruited in each group.

c) The first objective of the study was to compare the levels of CML-induced *NF-κB* gene expression and TNF secretion in DN, T2DM, and healthy volunteers. This was tested by treating whole blood samples obtained from the study participants with CML for 24 hours along with a control. *NF-κB* gene expression was quantified in the treated cells by qRT–PCR. TNF secretion was quantified in the conditioned cell culture medium by ELISA. CML treatment was found to result in significantly increased expression of the *NF-κB* gene in DN ($p < 0.0001$) and T2DM ($p < 0.0001$) but not in healthy volunteers ($p = 0.08$). Furthermore, CML treatment also resulted in increased TNF secretion. The secretion was highest at 1.76 ± 0.32 ($p < 0.0001$) in the DN group, followed by 1.47 ± 0.16 ($p < 0.0001$) T2DM and 1.30 ± 0.31 ($p < 0.0001$) in the control group. These observations indicate that the RAGE pathway is hyper-responsive to CML in DN patients.

d) CML-induced *NF-κB* expression and TNF secretion were also compared to measures of glycemic control and renal function. CML-induced *NF-κB* gene expression and TNF secretion showed a positive correlation (weak magnitude) with the measures of glycemic control and renal function. Additionally, a negative correlation was observed with respect to the estimated glomerular filtration rate.

e) The second objective of the study was to compare the amounts of insulin-induced sRAGE production in DN, T2DM, and healthy volunteers. This was tested by treating whole blood samples obtained from the study participants with insulin for 24 hours along with a control. The sRAGE levels were quantified in the conditioned cell culture medium by ELISA. The average insulin-induced fold change in the sRAGE level was 1.29 in DN (p <0.0001), 1.25 in T2DM (p <0.0001) and 1.74 in the healthy volunteers (p <0.0001). The highest fold change was observed in the case of healthy volunteers when compared to DN and T2DM. This finding indicates that sRAGE production is less responsive to insulin in DN and T2DM patients than in healthy volunteers.

f) Insulin-induced sRAGE production was correlated with measures of glycemic control and renal function. A negative correlation was observed. However, the effect size of the correlation was weak for both measures.

g) The third objective of the study was to compare the amounts of insulin-induced expression of the *ADAM10* gene in DN, T2DM, and healthy volunteers but not in DN and T2DM. Insulin-treated cells from the above

experiment were used to quantify ADAM10 gene expression by qRT–PCR. Insulin treatment resulted in higher normalised expression of the *ADAM10* gene in healthy volunteers (p <0.001), whereas there was no significant change in diabetic nephropathy (p = 0.159) or T2DM (p = 0.143).

h) Insulin-induced *ADAM10* gene expression was also compared to measures of glycaemic control and measures of renal function. A negative correlation was observed. However, the effect size of the correlation was weak for both measures. This finding indicates that the magnitude of *ADAM10* gene expression may be inversely associated with glycemic control and that decreased *ADAM10* expression may be a minor contributor to the deterioration of renal function.

i) Correlation analysis was evaluated between CML-induced changes in *NF-kB* gene expression and TNF secretion, and the parameters were found to have a weak positive correlation r = 0.326, p <0.001, Pearson's correlation. These results indicate that as the expression of *NF-kB* increases, TNF secretion increases proportionally. Similarly, the correlation between insulin-induced changes in *ADAM10* gene expression and sRAGE production was found to have a weak positive correlation r = 0.3, p <0.01, Pearson's correlation. These results indicate that as the expression of *ADAM10* increases, there is a proportional increase in sRAGE production.

j) This study shows that the RAGE pathway is hyper-responsive to CML in DN patients. Furthermore, regulators of the RAGE pathway, such as sRAGE and ADAM10, were reduced in DN. Together these results support the conclusion that the RAGE pathway is abnormal in DN patients.

New Knowledge Generated

This study provides the first evidence to show that the blood samples of DN patients are hyper-responsive to CML. Furthermore, this is also the first study to show that sRAGE production is impaired in DN and links the same to downregulation of the ADAM10 gene. This new information reaffirms the role of the RAGE pathway in the pathogenesis of DN and justifies its development as a drug target.

Limitations of the study

NF-κB upregulation and increased TNF secretion due to CML treatment were observed in this study. However, factors responsible for hyperresponsiveness were not explored in this study. Genetic variations may be responsible for this pattern. Genetic polymorphisms in the promoter region of the *NF-κB* and *TNF* genes have been associated with DN and T2DM. In addition, genetic variations in the *AGER* gene might also constitute a predisposing factor that leads to an abnormal inflammatory response when triggered by AGE.

The effect of CML on sRAGE production and *ADAM10* gene expression and the effect of insulin on *NF-κB* gene expression and TNF secretion were not assessed. These data would have aided in better understanding the RAGE pathway. Additionally, the effect of a combination treatment that included CML as well as insulin and its effect on the study parameters would have validated and linked the results obtained.

Diminished sRAGE production and *ADAM10* gene expression upon insulin stimulation were observed in DN and T2DM. Only *ADAM10* gene expression and not its protein level were measured. The correlation of these parameters would enhance the knowledge of the relationship between sRAGE and ADAM10.

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Annexures

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. **Dr. D.E.Gangadhar Rao, (Chairman)** Prof. & HOD of Zoology, Govt. Women's College, Kolar,
2. **Dr. Sujatha.M.P, (Member Secretary),** Assoc. Prof. of Anesthesia, SDUMC,
3. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
4. Dr. Srinivas Reddy.P, Prof. & HoD of Forensic Medicine, SDUMC
5. Dr. Prasad.K.C, Professor of ENT, SDUMC
6. Dr. Sumathi.M.E Prof. & HoD of Biochemistry, SDUMC.
7. Dr. Bhuvana.K, Prof. & HoD of Pharmacology, SDUMC
8. Dr. H.Mohan Kumar, Professor of Ophthalmology, SDUMC
9. Dr. Hariprasad, Assoc. Prof Department of Orthopedics, SDUMC
10. Dr. Pavan.K, Asst. Prof of Surgery, SDUMC
11. Dr. Talasila Sruthi, Assoc. Prof. of OBG, SDUMC
12. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC
13. Dr. Mamata Kale, Asst. Professor of Microbiology, SDUMC

No. SDUMC/KLR/IEC/218/2018-19 Date:02-01-2019

PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the study entitled **"Studies on receptor for advanced glycation end-products pathway in diabetic nephropathy"** being investigated by **Ms.Claire Gabriella Dias** (Ph.D Student), Dr.Sharath B & Dr.V.Lakshmaiah[1] in the Departments of Cell Biology and Molecular Genetics & Medicine[1] at Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study**. However, final report has to be submitted to the Ethics Committee after completion of the study for presentation in conference or for publication.

Sujatha. M.P
Member Secretary
Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

Chairman
CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College.
Tamaka, Kolar

PART I: PATIENT INFORMATION SHEET

Name of the project : Studies on Receptor for Advanced Glycation End-products pathway in Diabetic Nephropathy

Name of the Research Scholar : Ms. Claire Gabriella Dias

Name of Organization : Sri Devaraj Urs Academy of Higher Education and Research. Kolar

Diabetes is a disease involving abnormal increase in the levels of blood sugar. Some of the diabetic patients eventually develop complications of the kidneys known as nephropathy. The reason why some diabetic patients develop nephropathy is not known.The purpose of this study is to evaluate the role of RAGE pathway in the development of nephropathy in diabetic patients. The results obtained from this study will contribute towards development of therapies that can halt the advancement of nephropathy in diabetic patients.

P**articipant selection:** Patients suffering from Type 2 Diabetes with Nephropathy above 45 years of age. Pair matched patients with type 2 diabetes and no microvascular complications. Normal healthy individuals above 45 years of age will also be considered.

Voluntary Participation: Your participation in this study is entirely voluntary. There is no compulsion to participate in this study. You will be in no way affected if you do not wish to participate in the study. You are required to sign only if you voluntarily agree to participate in this study. Further you are at a liberty to withdraw from the study at any time. We assure that your withdrawal will not affect your treatment by the concerned physician in any way.

Procedure: We will be collecting a small volume of blood (3 ml) from your arm. The sample will be used for immunological analysis. Clinical and family history is also necessary.

Duration: The research will take place for about 3 years.

Risks: No drug will be tested on you. 3 ml of blood will be collected using sterile and disposable needle and syringe. Standard of care for the treatment of Type 2 diabetes with nephropathy will be given to you irrespective of your decision to enroll in the project.

Benefits: This study will be useful to understand the role of RAGE in the development of nephropathy in diabetic patients. The results gathered from this study will be beneficial in management of the diseases. Participation in this study does not involve any cost for you. Also, no monetary compensation will be paid for your participation in this study.

Storage of samples: The samples collected from you may be stored for future research projects on molecular studies on RAGE. In such an event, ethics clearance will be obtained.

Confidentiality: All information collected from you will be strictly confidential & will not be disclosed to anyone except if it is required by the law. This information collected will be used only for research. This information will not reveal your identity.

Sharing the Results: The results obtained from this study will be published in scientific/Medical Journals/Medical conferences.

For any information you are free to contact investigator. This study has been approved by the Institutional Ethics Committee & has been started only after their formal approval. The sample collected will be stored in the institute and I request you to permit us to store and use this sample for any future study.

This document will be stored in the safe locker & a copy given to you for information.

For any further clarification you are free to contact the following researchers:

Dr. V.Lakshmaiah
Professor
Department of General Medicine
SDUAHER,
Tamaka, Kolar.
Mobile: 9845209858

Claire Gabriella Dias
Ph.D Scholar
Department of Cell Biology and
Molecular Genetics, SDUAHER,
Tamaka, Kolar.
Mobile: 8095210430

PART II: INFORMED CONSENT FORM (Patients)

Note: Consent for the case group (patients with Type 2 Diabetics and nephropathy) should be obtained from the participant.

Name of Participant (Patient/Volunteer): _____

- This research study has been explained to me; I have been given the chance to discuss it and ask questions. All of my questions have been answered to my satisfaction.
- I have read each page of Patient Information Sheet or it has been read to me.
- I agree to allow access to my health information as explained in the patient information sheet. (In case of patient only)
- I agree to allow collection of 3ml blood sample and health data for the research purposes explained in the Patient Information Sheet.
- I understand that all the information collected will be kept confidentially.
- I voluntarily consent to take part in this research study.

Participant's signature or thumb impression

Date: _____

	Name	**Signature**	**Date**
Participant			
Witness 1			
Witness 2			
Person taking consent*			

If illiterate: Two literate witnesses must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

INFORMED CONSENT FORM (Healthy controls)

Note: Consent for the control group (Healthy individuals without Type 2 Diabetics and nephropathy) should be obtained from the participant.

Name of Participant (Healthy control /Volunteer): _____

- This research study has been explained to me; I have been given the chance to discuss it and ask questions. All of my questions have been answered to my satisfaction.
- I have read each page of Patient Information Sheet or it has been read to me.
- I agree to allow access to my health information as explained in the patient information sheet. (In case of patient only)
- I agree to allow collection of 3ml blood sample and health data for the research purposes explained in the Patient Information Sheet.
- I understand that all the information collected will be kept confidentially.
- I voluntarily consent to take part in this research study.

Participant's signature or thumb impression

Date:_____

	Name	**Signature**	**Date**
Participant			
Witness 1			
Witness 2			
Person taking consent*			

If illiterate: Two literate witnesses must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

ಭಾಗ I:

ಮಧುಮೇಹವುರಕ್ತದಲಿನಸಕ್ಕರೆಮಟ್ಟದಅಸಹಜಹೆಚ್ಚಳದಒಂದುರೋಗ.ಕೆಲವುಮಧುಮೇಹರೋಗಿಗಳುಅಂತಿಮವಾಗಿನೆಫ್ರೋಪತಿಎಂದುಕರೆಯಲ್ಪಡುವಮೂತ್ರಪಿಂಡದತೊಂದರೆಯಿಂದಬಳಲುತ್ತಾರೆ.ಕೆಲವು ಸಕ್ಕರೆಖಾಯಿಲೆಯುರೋಗಿಗಳಲ್ಲಿನೆಫ್ರೋಪತಿರೋಗದಬೆಳವಣಿಗೆಯಕಾರಣತಿಳಿದಿಲ್ಲ. ಮಧುಮೇಹರೋಗಿಗಳಲ್ಲಿನೆಫ್ರೊಪತಿಯಬೆಳವಣಿಗೆಯಲ್ಲಿರೇಜ್ಪಥದಪಾತ್ರವನ್ನುಮೌಲ್ಯಮಾಪನಮಾಡುವುದು ಈಅಧ್ಯಯನದಉದ್ದೇಶವಾಗಿದೆ.ಈಅಧ್ಯಯನದಮೂಲಕಪಡೆದಫಲಿತಾಂಶಗಳುಚಿಕಿತ್ಸೆಯಅಭಿವೃದ್ಧಿಗೆಕಾರಣವಾಗುತ್ತವೆ, ಅದುಮಧುಮೇಹರೋಗಿಗಳಲ್ಲಿನನೆಫ್ರೊಪತಿಯಬೆಳವಣಿಗೆಯನ್ನುತಡೆಯಬಹುದು.

ಭಾಗವಹಿಸುವವರುಆಯ್ಕೆ:೪೦ವರ್ಷಕ್ಕಿಂತಮೇಲ್ಪಟ್ಟನೆಫ್ರಾಪತಿಯೊಂದಿಗೆ ಟೈಪ್-೨ಮಧುಮೇಹದಿಂದ ಳಲುತ್ತಿರುವ ರೋಗಿಗಳು. ಟೈಪ್ ೨ ಮಧುಮೇಹರೋಗಿಗಳಿಗೆಹೊಂದಿಕೆಯಾಗುವಮತ್ತುಯಾವುದೇಮೈಕ್ರೊವ್ಯಾಸ್ಕುಲರ್ತೊಡಕುಗಳಿಲ್ಲದ ವ್ಯಕ್ತಿಗಳು. ೪೦ ವರ್ಷಕ್ಕಿಂತಮೇಲ್ಪಟ್ಟಸಾಮಾನ್ಯಆರೋಗ್ಯಕರವ್ಯಕ್ತಿಗಳನ್ನುಸಹಪರಿಗಣಿಸಲಾಗುತ್ತದೆ.

ಸ್ವಯಂಪ್ರೇರಿತಭಾಗವಹಿಸುವಿಕೆ:ಈಅಧ್ಯಯನದಲ್ಲಿನಿಮ್ಮಭಾಗವಹಿಸುವಿಕೆಸಂಪೂರ್ಣವಾಗಿಸ್ವಯಂಪ್ರೇರಿತವಾಗಿರುತ್ತದೆ. ಈಅಧ್ಯಯನದಲ್ಲಿಭಾಗವಹಿಸಲುಯಾವುದೇನಿರ್ಬಂಧವಿಲ್ಲ. ನೀವುಅಧ್ಯಯನದಲ್ಲಿಭಾಗವಹಿಸಲುಬಯಸದಿದ್ದರೆ, ನಿಮ್ಮಚಿಕಿತ್ಸೆಗೆಯಾವುದೇರೀತಿಯಪರಿಣಾಮಬೀರುವುದಿಲ್ಲ. ಈಅಧ್ಯಯನದಲ್ಲಿಪಾಲ್ಗೊಳ್ಳಲುನೀವುಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದಸಮ್ಮತಿಸಿದರೆಮಾತ್ರನೀವುಸಹಿಮಾಡಬೇಕಾಗುತ್ತದೆ. ನೀವುಯಾವುದೇಸಮಯದಲ್ಲಿಅಧ್ಯಯನದಿಂದಹಿಂದೆಸರಿಯಲುಸ್ವಾತಂತ್ರ್ಯವಾಗಿರುತ್ತೀರಿ. ನಿಮ್ಮಹಿಂತೆಗೆದುಕೊಳ್ಳುವಿಕೆಯುವೈದ್ಯರನ್ನುಯಾವುದೇರೀತಿಯಲ್ಲಿಯಾವುದೇಚಿಕಿತ್ಸೆಯಮೇಲೆಪರಿಣಾಮಬೀರುವುದಿಲ್ಲಎಂದುನಾವುಭರವಸೆನೀಡುತ್ತೇವೆ.

ವಿಧಾನ:ನಾವುನಿಮ್ಮಕೈಯಿಂದಒಂದುಸಣ್ಣಪ್ರಮಾಣದರಕ್ತವನ್ನು (೩ ಮಿಲಿ) ಸಂಗ್ರಹಿಸುತ್ತೇವೆ. ಈ ಮಾದರಿಯನ್ನುರೋಗನಿರೋಧಕವಿಶ್ಲೇಷಣೆಗಾಗಿಬಳಸಲಾಗುತ್ತದೆ. ಕ್ಲಿನಿಕಲ್ಮತ್ತುಕುಟುಂಬದಇತಿಹಾಸವೂಅವಶ್ಯಕ.

ಅವಧಿ:ಸಂಶೋಧನೆಸುಮಾರು ೩ ವರ್ಷಗಳಕಾಲನಡೆಯುತ್ತದೆ.

ಅಪಾಯಗಳು:ನಿಮ್ಮಮೇಲೆಯಾವುದೇಔಷಧಿಪರೀಕ್ಷಿಸಲಾಗುವುದಿಲ್ಲ. ೩ ಮಿ.ಲಿರಕ್ತವನ್ನುಸೂಜಿಮತ್ತುಸಿರಿಂಜ್ಬಳಸಿಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.
ಯೋಜನೆಯಲ್ಲಿತೊಡಗಿಸಿಕೊಳ್ಳುವನಿಮ್ಮನಿರ್ಧಾರವನ್ನುಹೊರತುಪಡಿಸಿ ನೆಫ್ರೋಪತಿಇರುವಟೈಪ್ ೨ ಮಧುಮೇಹ ಹೊಂದಿರುವ ರೋಗಿಗಳಿಗೆ ಗುಣಮಟ್ಟದಚಿಕಿತ್ಸೆಯನ್ನುನೀಡಲಾಗುತ್ತದೆ.

ಪ್ರಯೋಜನಗಳು:
ದಯಾಬಿಟಿಕ್ರೋಗಿಗಳಲ್ಲಿನೆಫ್ರೋಪತಿಯಬೆಳವಣಿಗೆಯಲ್ಲಿರೇಜ್ಪಾತ್ರವನ್ನುಅರ್ಥಮಾಡಿಕೊಳ್ಳಲುಈಅಧ್ಯಯನವುಉಪಯುಕ್ತವಾಗಿದೆ.
ಈಅಧ್ಯಯನದಿಂದಸಂಗ್ರಹಿಸಿದಫಲಿತಾಂಶಗಳುರೋಗಗಳನಿರ್ವಹಣೆಗೆಅನುಕೂಲಕರವಾಗಿರುತ್ತದೆ.
ಈಅಧ್ಯಯನದಲ್ಲಿಭಾಗವಹಿಸುವಿಕೆಯುನಿಮಗೆಯಾವುದೇವೆಚ್ಚವನ್ನುಒಳಗೊಂಡಿರುವುದಿಲ್ಲ. ಅಲ್ಲದೆ, ಈಅಧ್ಯಯನದಲ್ಲಿನಿಮ್ಮಪಾಲ್ಗೊಳ್ಳುವಿಕೆಗೆಯಾವುದೇವಿತ್ತೀಯಪರಿಹಾರವನ್ನುಪಾವತಿಸಲಾಗುವುದಿಲ್ಲ.

ಮಾದರಿಗಳಶೇಖರಣೆ:ಭವಿಷ್ಯದಸಂಶೋಧನಾಯೋಜನೆಗಳಿಗಾಗಿನಿಮ್ಮರಕ್ತದಮಾದರಿಯಿಂದವಂಶವಾಹಿವಸ್ತುಗಳನ್ನುಸಂಗ್ರಹಿಸಬಹುದು.ಅಂತಹಸಂದರ್ಭದಲ್ಲಿ,
ಎಥಿಕ್ಸ್ಕಮಿಟಿಯಿಂದಅನುಮತಿಪಡೆಯುವಮೊದಲುಅದನ್ನುಪಡೆಯಬಹುದು.

ಗೋಪ್ಯತೆ:ವೈಯಕ್ತಿಕಗುರುತಿನಬಗ್ಗೆಎಲ್ಲಾಮಾಹಿತಿಯನ್ನೂಗೌಪ್ಯವಾಗಿರಿಸಲಾಗುವುದುಮತ್ತುಕಾನೂನಿನಅಗತ್ಯವನ್ನುಹೊರತುಪಡಿಸಿಯಾರಿಗೂಅದನ್ನುಬಹಿರಂಗಪಡಿಸಲಾಗುವುದಿಲ್ಲ.
ಯೋಜನೆಯಶೋಧಕರುಮಾತ್ರಗುರುತಿನವಿವರಗಳಿಗೆಪ್ರವೇಶವನ್ನುಹೊಂದಿರುತ್ತಾರೆ.

ಫಲಿತಾಂಶಗಳನ್ನುಹಂಚಿಕೆ:ಈಅಧ್ಯಯನದಿಂದಪಡೆದಫಲಿತಾಂಶಗಳುವೈಜ್ಞಾನಿಕ / ವೈದ್ಯಕೀಯಜರ್ನಲ್ / ಮೆಡಿಕಲ್ಸಮ್ಮೇಳನಗಳಲ್ಲಿಪ್ರಕಟವಾಗುತ್ತವೆ.

ಯಾವುದೇಮಾಹಿತಿಗಾಗಿನೀವುತನಿಖೆದಾರರನ್ನುಸಂಪರ್ಕಿಸಲುಮುಕ್ತವಾಗಿರುತ್ತೀರಿ.
ಈಅಧ್ಯಯನವುಸಾಂಸ್ಥಿಕನೀತಿಶಾಸ್ತ್ರಸಮಿತಿಯಿಂದಅನುಮೋದಿಸಲ್ಪಟ್ಟಿದೆಮತ್ತುಅವರಔಪಚಾರಿಕಅನುಮೋದನೆಯನಂತರಮಾತ್ರಪ್ರಾರಂಭಿಸಲ್ಪಟ್ಟಿದೆ.
ಸಂಗ್ರಹಿಸಿದಮಾದರಿಯನ್ನುಇನ್ವಿಟ್ರೊಟ್ನಲ್ಲಿಸಂಗ್ರಹಿಸಲಾಗುವುದುಮತ್ತುಭವಿಷ್ಯದಅಧ್ಯಯನಕ್ಕಾಗಿಈಮಾದರಿಯನ್ನುಸಂಗ್ರಹಿಸಲುಮತ್ತುಬಳಸಲುನಮಗೆಅನುಮತಿಸಲುನಾನುವಿನಂತಿಸುತ್ತೇನೆ.

ಈಡಾಕ್ಯುಮೆಂಟ್‌ಅನ್ನುಸುರಕ್ಷಿತಲಾಕರ್ನಲ್ಲಿಮತ್ತುಮಾಹಿತಿಗಾಗಿನಿಮಗೆನೀಡಿದಪ್ರತಿಯನ್ನುಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.

ಯಾವುದೇಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿನೀವುಈಕೆಳಗಿನಸಂಶೋಧಕರನ್ನುಸಂಪರ್ಕಿಸಲುಮುಕ್ತವಾಗಿರುತ್ತೀರಿ:

ಡಾ. ವಿ. ಲಕ್ಷ್ಮಯ್ಯ
ಪ್ರೊಫೆಸರ್
ಜನರಲ್‌ಮೆಡಿಸಿನ್‌ಶಾಸ್ತ್ರವಿಭಾಗ,
ಶ್ರೀ ದೇವರಾಜ್ ಅರಸ್ ಮೆಡಿಕಲ್ ಕಾಲೇಜು,
ತಮಕಾ, ಕೋಲಾರ.
ಮೊಬೈಲ್: ೯೮೪೫೫೨೨೯೮೫

ಕ್ಲೇರ್‌ಗೆಬ್ರಿಯೆಲ್ಲಾಡಯಾಸ್
ಪಿ ಎಚ್ ಡಿವಿದ್ಯಾರ್ಥಿ
ಸೆಲ್ಬಯಾಲಜಿಮತ್ತುಮಾಲಿಕ್ಯೂಲರ್ಜೆನೆಟಿಕ್ಸ್‌ವಿಭಾಗ,
ಶ್ರೀ ದೇವರಾಜ್ ಅರಸ್ ಮೆಡಿಕಲ್ ಕಾಲೇಜು,
ತಮಕಾ, ಕೋಲಾರ.
ಮೊಬೈಲ್: ೮೦೯೫೫೨೧೧೪೩

ಭಾಗ ಖಖ:

ತಿಳಿವಳಿಕೆಯಸಮ್ಮತಿನಮೂನೆ (ರೋಗಿಗಳು)

ಗಮನಿಸಿ: ಕೇಸ್ಟ್ರೂಪ್ (ಟೈಪ್ ೨ ಮಧುಮೇಹಮತ್ತುನೆಫ್ರೋಪಥಿರೋಗಿಗಳು) ಒಪ್ಪಿಗೆಪಡೆಯುವವರುಭಾಗವಹಿಸುವವರಿಂದಪಡೆಯಬೇಕು.

ಪಾಲ್ಗೊಳ್ಳುವವರಹೆಸರು (ರೋಗಿಯ / ಸ್ವಇಚ್ಛೆಯಿಂದ ಪಾಲ್ಗೊಳ್ಳುವವರು): _____

•ಈಸಂಶೋಧನಾಅಧ್ಯಯನವನ್ನುನನಗೆವಿವರಿಸಲಾಗಿದೆ;
ಇದನ್ನುಚರ್ಚಿಸಲುಮತ್ತುಪ್ರಶ್ನೆಗಳನ್ನುಕೇಳಲುನನಗೆಅವಕಾಶನೀಡಲಾಗಿದೆ.
ನನ್ನಎಲ್ಲಾಪ್ರಶ್ನೆಗಳಿಗೆನನ್ನತೃಪ್ತಿಗೆಉತ್ತರನೀಡಲಾಗಿದೆ.

•ನಾನುರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯಪ್ರತಿಪುಟವನ್ನುಓದಿದ್ದೇನೆಅಥವಾನನಗೆಓದಲಾಗಿದೆ.

ರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯಲ್ಲಿವಿವರಿಸಿದಂತೆನನ್ನಆರೋಗ್ಯಮಾಹಿತಿಯನ್ನು
ಪಡೆಯಲುನಾನುಒಪ್ಪುತ್ತೇನೆ. (ರೋಗಿಗೆಮಾತ್ರ)

•ರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯಲ್ಲಿವಿವರಿಸಿರುವಸಂಶೋಧನಾಉದ್ದೇಶಗಳಿಗಾಗಿ ೩
ಮಿ.ಲಿರಕ್ತಮಾದರಿಮತ್ತುಆರೋಗ್ಯದಡೇಟಾಸಂಗ್ರಹಣೆಯನ್ನುಕೊಡಲುನಾನುಒಪ್ಪುತ್ತೇನೆ.

•ಸಂಗ್ರಹಿಸಿದಎಲ್ಲಾಮಾಹಿತಿಯನ್ನುಗೌಪ್ಯವಾಗಿಇರಿಸಲಾಗುವುದುಎಂದುನಾನುಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

•ನಾನುಈಸಂಶೋಧನಾಅಧ್ಯಯನದಲ್ಲಿಪಾಲ್ಗೊಳ್ಳಲುಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದಸಮ್ಮತಿಸುತ್ತೇನೆ.

ಭಾಗವಹಿಸುವವರಸಹಿಅಥವಾಹೆಬ್ಬೆರಳುಗುರುತು

ದಿನಾಂಕ: _____

ಅನಕ್ಷರಸ್ಥನಾಗಿದ್ದರೆ: ಎರಡುಸಾಕ್ಷರತಾಸಾಕ್ಷಿಗಳುಸಹಿಹಾಕಬೇಕು (ಸಾಧ್ಯವಾದರೆ,ಈವ್ಯಕ್ತಿಯನ್ನುಸಹಭಾಗಿಯುಆರಿಸಬೇಕುಮತ್ತುಸಂಶೋಧನಾತಂಡಕ್ಕೆಯಾವುದೇಸಂಪರ್ಕವನ್ನುಹೊಂದಿಲ್). ಅನಕ್ಷರಸ್ಥರುಭಾಗವಹಿಸುವವರುತಮ್ಮಹೆಬ್ಬೆರಳು-ಮುದ್ರಣವನ್ನೂಸಹಒಳಗೊಂಡಿರಬೇಕು.

ಪಾಲ್ಗೊಳ್ಳುವವರಿಗೆಸಮ್ಮತಿರೂಪದನಿಖರವಾದಓದುವಿಕೆಗೇನಾನುಸಾಕ್ಷಿಯಾಗಿದ್ದೇನೆಮತ್ತುಪ್ರಶ್ನೆಗಳನ್ನುಕೇಳಲುವ್ಯಕ್ತಿಗೆಅವಕಾಶವಿದೆ. ವ್ಯಕ್ತಿಯುಮುಕ್ತವಾಗಿಒಪ್ಪಿಗೆನೀಡಿದ್ದಾನೆಎಂದುನಾನುದೃಢೀಕರಿಸುತ್ತೇನೆ.

	ಸಾಕ್ಷಿ ೧	ಸಾಕ್ಷಿ ೨
ಹೆಸರು		
ಸಹಿ		
ದಿನಾಂಕ		

ರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯನ್ನುಪಾಲ್ಗೊಳ್ಳುವವರಿಗೆನಾನುಓದಿದ್ದೇನೆಮತ್ತುವಿವರಿಸಿದ್ದೇನೆಮತ್ತುಈ[illegible]ಳ ಪ್ರತಿಯನ್ನುಅವರಿಗೆನೀಡಲಾಗಿದೆ.

ಸಮ್ಮತಿಯನ್ನುತೆಗೆದುಕೊಳ್ಳುವವ್ಯಕ್ತಿಯಹೆಸರು _____

ಒಪ್ಪಿಗೆತೆಗೆದುಕೊಳ್ಳುವವ್ಯಕ್ತಿಯಸಹಿ _____

ದಿನಾಂಕ: _____

<u>ತಿಳಿವಳಿಕೆಯಸಮ್ಮತಿನಮೂನೆ (ಆರೋಗ್ಯಕರವ್ಯಕ್ತಿಗಳು)</u>

ಗಮನಿಸಿ: ಆರೋಗ್ಯಕರವ್ಯಕ್ತಿಗಳಒಪ್ಪಿಗೆಪಡೆಯುವವರುಭಾಗವಹಿಸುವವರಿಂದಪಡೆಯಬೇಕು.

ಪಾಲ್ಗೊಳ್ಳುವವರಹೆಸರು (ಆರೋಗ್ಯಕರವ್ಯಕ್ತಿ): _____

•ಈಸಂಶೋಧನಾಅಧ್ಯಯನವನ್ನುನನಗೆವಿವರಿಸಲಾಗಿದೆ;
ಇದನ್ನುಚರ್ಚಿಸಲುಮತ್ತುಪ್ರಶ್ನೆಗಳನ್ನುಕೇಳಲುನನಗೆಅವಕಾಶನೀಡಲಾಗಿದೆ.
ನನ್ನಎಲ್ಲಾಪ್ರಶ್ನೆಗಳಿಗೆನನ್ನತೃಪ್ತಿಗೆಉತ್ತರನೀಡಲಾಗಿದೆ.

•ನಾನುರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯಪ್ರತಿಪುಟವನ್ನುಓದಿದ್ದೇನೆಅಥವಾನನಗೆಓದುತ್ತಿದ್ದೇನೆ.

ರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯಲ್ಲಿವಿವರಿಸಿದಂತೆನನ್ನಆರೋಗ್ಯಮಾಹಿತಿಗೆಪ್ರವೇಶವನ್ನುಅನುಮತಿಸಲುನಾನು ಒಪ್ಪುತ್ತೇನೆ. (ರೋಗಿಗೆಮಾತ್ರ)

•ರೋಗಿಯಮಾಹಿತಿಹಾಳೆಯಲ್ಲಿವಿವರಿಸಿರುವಸಂಶೋಧನಾಉದ್ದೇಶಗಳಿಗಾಗಿ ಇ
ಮಿಲಿಯನ್ರಕ್ತಮಾದರಿಮತ್ತುಆರೋಗ್ಯದಡೇಟಾಸಂಗ್ರಹಣೆಯನ್ನುಅನುಮತಿಸಲುನಾನುಒಪ್ಪುತ್ತೇನೆ.

•ಸಂಗ್ರಹಿಸಿದಎಲ್ಲಾಮಾಹಿತಿಯನ್ನುಗೌಪ್ಯವಾಗಿಇರಿಸಲಾಗುವುದುಎಂದುನಾನುಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

•ನಾನುಈಸಂಶೋಧನಾಅಧ್ಯಯನದಲ್ಲಿಪಾಲ್ಗೊಳ್ಳಲುಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದಸಮ್ಮತಿಸುತ್ತೇನೆ.

ಭಾಗವಹಿಸುವವರಸಹಿಅಥವಾಹೆಬ್ಬೆರಳುಗುರುತು

ದಿನಾಂಕ: _____

ಅನಕ್ಷರಸ್ಥನಾಗಿದ್ದರೆ: ಎರಡುಸಾಕ್ಷರತಾಸಾಕ್ಷಿಗಳುಸಹಿಹಾಕಬೇಕು (ಸಾಧ್ಯವಾದರೆ,ಈವ್ಯಕ್ತಿಯನ್ನುಸಹಭಾಗಿಯುಆರಿಸಬೇಕುಮತ್ತುಸಂಶೋಧನಾತಂಡಕ್ಕೆಯಾವುದೇಸಂಪರ್ಕವನ್ನುಹೊಂದಿಲ್ಲ). ಅನಕ್ಷರಸ್ಥರುಭಾಗವಹಿಸುವವರುತಮ್ಮಹೆಬ್ಬೆರಳು-ಮುದ್ರಣವನ್ನೂಸಹಒಳಗೊಂಡಿರಬೇಕು. ಪಾಲ್ಗೊಳ್ಳುವವರಿಗೆಸಮ್ಮತಿರೂಪದನಿಖರವಾದಓದುವಿಕೆಗೇನಾನುಸಾಕ್ಷಿಯಾಗಿದ್ದೇನೆಮತ್ತುಪ್ರಶ್ನೆಗಳನ್ನುಕೇಳಲುವ್ಯಕ್ತಿಗೆಅವಕಾಶವಿದೆ. ವ್ಯಕ್ತಿಯುಮುಕ್ತವಾಗಿಒಪ್ಪಿಗೆನೀಡಿದ್ದಾನೆಎಂದುನಾನುದೃಢೀಕರಿಸುತ್ತೇನೆ.

	ಸಾಕ್ಷಿ ೧	ಸಾಕ್ಷಿ ೨
ಹೆಸರು		
ಸಹಿ		
ದಿನಾಂಕ		

ತಿಳುವಳಿಕೆಯುಳ್ಳ

ಮಾಹಿತಿಹಾಳೆಯನ್ನುಪಾಲ್ಗೊಳ್ಳುವವರಿಗೆನಾನುಓದಿದ್ದೇನೆಮತ್ತುವಿವರಿಸಿದ್ದೇನೆಮತ್ತುಈಒಂದುಪ್ರತಿಯನ್ನುಅವರಿಗೆನೀಡಲಾಗಿದೆ.

ಸಮ್ಮತಿಯನ್ನುತೆಗೆದುಕೊಳ್ಳುವವ್ಯಕ್ತಿಯಹೆಸರು _____

ಒಪ್ಪಿಗೆತೆಗೆದುಕೊಳ್ಳುವವ್ಯಕ್ತಿಯಸಹಿ _____

ದಿನಾಂಕ: _____

PATIENT PROFORMA

Patient Data	
Patient's Name	
IP/OP Number	
Age	
Gender	□M □F
Contact number	
Address	
Clinical Signs And Symptoms	
Hypertension	□Yes □ No □ Unknown
Obese	□Yes □ No □ Unknown
Weight loss/ Fatigue	□Yes □ No □ Unknown
Polyuria	□Yes □ No □ Unknown
Polyphagia	□Yes □ No □ Unknown
Polydipsia	□Yes □ No □ Unknown
Laboratory Investigations	
Random Blood sugar(mg/dL)	
FBS (mg/dL)	
eGFR (ml/min)	
Blood Urea Nitrogen (mg/dL)	
Serum Creatinine (mg/dL)	

Publications

1. Dias CG, Venkatswamy L, Balakrishna S. Reduced sRAGE Production and *ADAM10* Gene Expression in Peripheral Blood Samples of Diabetic Nephropathy Patients. Journal of Clinical & Diagnostic Research. 2022 Jan 1;16(1).

2. Dias CG, Venkatswamy L, Balakrishna S. Diabetic nephropathy patients show hyper-responsiveness to N6-carboxymethyllysine. Brazilian Journal of Medical and Biological Research. (Under review)

Presentations

1. Dias CG, Venkatswamy L, Balakrishna S. RAGE pathway is dysregulated in diabetic nephropathy. Poster session presented at: 88th Annual meeting of the Society of biological chemists, India (SBCI-2019) and conference on Advances at the Interface of Biology & Chemistry; 2019 Oct 31-Nov 3; DAE convention centre, Anushaktinagar, Mumbai.

2. Dias CG, Venkatswamy L, Balakrishna S. Advanced glycation end-product induced production of tumor necrosis factor in diabetic nephropathy. Oral session presented at: ABGCON 2021, National virtual conference and workshop; 2021 Aug 27-28; Sri Balaji Medical college and Hospital, Chennai.

Masterchart

Group I: Diabetic nephropathy

Patient ID	Age	Sex	FBS	HbA1c	eGFR	BUN	S.Cr	*NF-kB* 2^(-ddct)	TNF Fold change	*ADAM10* 2^(-ddct)	sRAGE Fold change
A1	50	M	129	6.12	28	24	2.7	2.0	1.19	0.86	1.53
A2	62	M	140	6.5	13	55	3.7	2.4	1.46	0.98	1.58
A3	45	F	137	6.4	15	32	3.6	0.5	1.96	0.39	1.21
A4	65	M	145	6.67	28	68	2.5	3.3	1.66	0.68	1.37
A5	51	F	155	7	14	53	3.7	4.3	1.52	1.37	1.57
A6	47	F	163	7.3	23	67	2.5	3.2	2.01	1.07	1.43
A7	53	F	128	6.1	21	25	2.6	4.1	2.30	0.74	1.38
A8	66	M	157	7.1	13	66	4.6	0.3	1.28	1.51	1.34
A9	44	M	244	10.1	19	78	3.9	4.7	1.65	1.76	1.21
A10	67	M	162	7.3	13	54	3.6	0.2	1.75	0.059	1.39
A11	52	F	127	6	21	23	2.5	4.6	1.97	1.11	1.30
A12	47	F	166	7.4	27	64	2.2	1.8	1.47	0.11	1.32
A13	49	F	135	6.3	21	46	2.7	1.6	1.64	1.87	1.39
A14	45	F	132	6.2	22	35	2.6	2.3	1.88	1.09	1.27
A15	65	F	145	6.7	19	63	2.7	1.7	2.20	0.62	1.37
A16	58	M	152	6.9	14	56	4.6	3.3	1.48	0.39	1.40
A17	63	M	133	6.3	25	25	2.8	3.2	1.33	0.099	1.36
A18	59	F	136	6.4	14	29	3.7	2.1	2.38	1.22	1.19
A19	67	M	147	6.7	18	54	3.5	0.9	1.51	0.036	1.39
A20	51	F	159	7.2	19	68	2.9	0.0	1.89	0.039	1.13
A21	52	M	162	7.3	20	51	3.5	8.2	1.82	0.65	1.39
A22	45	M	171	7.6	20	75	3.6	3.4	1.47	0.24	1.37

A23	65	F	142	6.6	13	46	3.8	0.8	1.64	0.29	1.26
A24	59	F	136	6.4	21	39	2.6	2.8	1.97	0.31	1.20
A25	61	M	144	6.6	19	51	3.5	4.1	1.78	1.80	1.53
A26	48	F	129	6.1	19	27	2.9	2.0	1.84	0.00	1.19
A27	55	M	131	6.2	28	47	2.6	3.3	2.35	0.01	1.32
A28	51	F	147	6.7	11	50	4.5	7.2	1.60	0.00	1.34
A29	63	M	162	7.3	21	46	3.2	0.1	1.55	0.01	1.49
A30	50	F	189	8.2	15	69	3.5	5.6	2.11	1.13	1.12
A31	66	M	158	7.1	26	66	2.6	1.4	1.19	1.05	1.53
A32	46	F	136	6.4	15	32	3.6	5.9	1.46	0.59	1.58
A33	59	F	139	6.4	14	39	3.6	2.4	1.96	1.44	1.21
A34	56	F	130	6.4	10	29	4.7	0.2	1.66	1.32	1.37
A35	45	M	171	7.6	14	75	3.6	4.7	1.52	0.47	1.57
A36	51	F	157	7	21	53	2.7	7.0	2.01	1.11	1.43
A37	47	M	138	7.6	21	47	3.5	0.5	2.30	0.38	1.38
A38	61	F	145	8.1	12	62	4	3.5	1.28	1.66	1.34
A39	65	F	156	7.1	20	73	2.6	4.7	1.65	1.35	1.21
A40	55	M	143	8	17	51	3.9	3.4	1.75	1.06	1.39
A41	51	F	177	7.8	19	24	2.9	4.4	1.97	0.73	1.30
A42	48	F	182	8	18	36	3.1	0.3	1.47	1.54	1.32
A43	57	M	162	7.3	16	39	4.2	5.1	1.64	1.81	1.39
A44	52	F	154	7	15	44	3.6	0.2	1.88	1.052	1.27
A45	49	F	143	6.6	13	70	3.9	4.9	2.20	1.09	1.37
A46	50	M	132	6.2	25	33	3.6	1.9	1.19	1.09	1.53
A47	52	F	189	8.2	17	29	3.1	1.6	1.46	1.93	1.58
A48	61	M	178	7.8	26	48	2.7	2.4	1.96	1.10	1.21
A49	53	M	167	7.4	20	74	3.5	1.8	1.66	1.60	1.37

A50	46	F	156	7.1	18	56	3.1	3.5	1.52	1.38	1.57
A51	47	M	145	6.7	15	25	3.7	3.4	2.01	0.09	1.43
A52	55	F	134	6.3	19	29	2.8	0.4	2.30	1.41	1.38
A53	61	F	192	8.3	13	31	3.7	2.6	1.28	0.69	1.34
A54	57	M	183	8	29	42	2.5	3.6	1.65	1.39	1.21
A55	63	F	174	7.7	18	55	2.9	2.3	1.75	1.07	1.39
A56	54	F	167	7.4	12	71	4.3	2.9	1.97	1.75	1.30
A57	58	M	158	7.1	26	69	2.7	0.3	1.47	1.47	1.32
A58	63	F	149	6.8	26	47	2.1	4.3	1.64	1.71	1.05
A59	49	M	173	7.6	25	28	3	0.1	1.88	3.68	1.13

Group II: Type II diabetics

Patient ID	Age	Sex	FBS	HbA1c	eGFR	BUN	S.Cr	*NF-kB* 2^(-ddct)	TNF Fold change	*ADAM10* 2^(-ddct)	sRAGE Fold change
B1	62	M	145	6.7	100	12.4	0.8	7.9	1.36	1.73	1.59
B2	45	F	157	7.1	113	15.9	0.6	2.0	1.38	0.46	1.42
B3	65	M	178	7.8	95	19.2	0.9	1.5	1.31	2.17	1.11
B4	51	F	183	8	105	16.7	0.7	6.6	1.51	0.76	1.22
B5	47	F	126	6	107	11.7	0.7	2.0	1.58	0.15	1.10
B6	53	F	135	6.3	76	17.6	0.9	1.9	1.55	1.93	1.23
B7	66	M	128	6.12	102	14.0	0.7	0.5	1.56	1.11	1.16
B8	44	M	144	6.7	108	13.2	0.9	1.2	1.24	1.36	1.15
B9	67	M	132	6.2	97	11.0	0.8	1.2	1.44	1.56	1.64
B10	52	F	129	6.12	108	18.7	0.6	4.8	1.21	0.44	1.07
B11	47	F	167	7.4	111	14.3	0.6	0.8	1.71	0.40	1.15
B12	49	F	163	7.3	106	15.5	0.7	0.4	1.42	0.47	1.14
B13	45	F	168	7.5	93	16.8	0.8	0.0	1.61	1.64	1.20
B14	65	F	155	7	82	19.1	0.8	1.2	1.44	0.21	1.15
B15	58	M	137	6.4	103	14.2	0.8	2.7	1.34	0.21	1.28
B16	63	M	189	8.2	96	17.2	0.9	8.1	1.28	1.76	1.37
B17	59	F	150	6.8	103	18.4	0.6	0.9	1.79	0.46	1.19
B18	67	M	128	6.1	101	13.6	0.7	1.9	1.38	0.41	1.11
B19	51	F	166	7.4	89	17.8	0.8	2.7	1.32	0.52	1.17
B20	52	M	178	7.8	106	11.0	0.8	1.5	1.61	2.80	1.60
B21	45	M	153	7	116	16.0	0.7	2.8	1.58	1.11	1.18
B22	65	F	129	6.12	96	16.3	0.7	0.0	1.77	1.36	0.97

B23	59	F	137	6.4	103	18.3	0.6	5.4	1.36	0.62	1.32
B24	61	M	150	6.8	101	13.2	0.8	0.0	1.47	1.56	1.52
B25	48	F	146	6.7	111	10.3	0.6	2.7	1.37	0.44	1.22
B26	55	M	155	7	101	6.4	0.9	0.3	1.74	0.40	1.12
B27	51	F	192	8.3	89	18.5	0.8	2.3	1.43	0.47	1.42
B28	63	M	166	7.4	99	14.7	0.8	1.9	1.12	1.64	1.31
B29	50	F	167	7.4	109	16.0	0.6	0.5	1.36	0.21	1.59
B30	54	M	150	7.3	109	16.3	0.7	2.3	1.38	2.66	1.42
B31	56	F	129	7.2	101	18.3	0.7	3.7	1.31	0.21	1.11
B32	47	F	153	6.4	91	13.2	0.8	1.8	1.51	2.93	1.22
B33	56	F	186	8.1	105	10.3	0.6	1.9	1.58	0.05	1.10
B34	45	M	160	7.2	116	6.4	0.7	8.8	1.55	1.78	1.23
B35	51	F	135	6.3	89	18.5	0.8	2.1	1.56	0.44	1.16
B36	47	M	144	6.6	106	14.7	0.9	1.5	1.24	2.26	1.15
B37	61	F	158	7.1	102	16.0	0.6	7.3	1.44	0.74	1.64
B38	65	F	153	7	82	18.1	0.8	2.0	1.21	0.14	1.07
B39	55	M	163	7.3	109	13.5	0.7	1.9	1.71	1.99	1.15
B40	51	F	202	8.7	109	16.3	0.6	0.5	1.42	1.11	1.14
B41	48	F	174	7.7	111	17.5	0.6	1.2	1.36	1.38	1.59
B42	57	M	175	7.7	103	12.9	0.8	1.2	1.38	1.60	1.42
B43	52	F	158	7.1	108	16.9	0.6	5.2	1.31	0.43	1.11
B44	49	F	135	6.3	90	10.5	0.8	0.7	1.51	0.38	1.22
B45	50	M	161	7.2	108	15.2	0.8	0.4	1.58	0.45	1.10
B46	52	F	169	7.5	77	15.5	0.9	7.1	1.55	1.68	1.23
B47	61	M	145	6.7	105	17.4	0.7	2.0	1.56	0.47	1.16
B48	53	M	123	5.9	110	12.5	0.7	1.5	1.24	2.09	1.15
B49	46	F	130	6.2	92	9.8	0.8	6.0	1.44	0.77	1.64

B50	47	M	143	6.6	114	16.3	0.7	1.9	1.21	0.16	1.07
B51	55	F	139	6.5	106	17.6	0.6	1.8	1.71	1.86	1.15
B52	61	F	147	6.7	84	18.1	0.8	0.5	1.42	1.10	1.14
B53	57	M	182	8	107	13.5	0.7	1.2	1.61	1.34	1.20
B54	63	F	158	7.1	101	16.3	0.6	1.2	1.44	1.53	1.15
B55	54	F	159	7.2	103	17.5	0.7	4.5	1.34	0.46	1.28
B56	58	M	143	6.6	99	12.9	0.9	0.8	1.28	0.42	1.37
B57	63	F	123	5.9	101	16.9	0.6	0.4	1.61	0.49	1.20
B58	49	M	145	6.7	108	10.5	0.8	6.0	1.44	0.77	1.15
B59	49	F	135	6.3	90	10.5	0.8	1.5	1.24	2.09	1.15

Group III: Healthy volunteers

Patient ID	Age	Sex	FBS	HbA1c	eGFR	BUN	S.Cr	*NF-kB*	TNF	*ADAM10*	sRAGE
								2^(-ddct)	Fold change	2^(-ddct)	Fold change
C1	62	M	89	4.9	104	14.0	0.7	0.50	1.02	2.00	1.73
C2	45	F	95	5	109	19.0	0.7	0.47	1.04	2.36	2.08
C3	65	M	98	4.7	95	7.5	0.9	0.47	1.07	3.32	1.89
C4	51	F	87	4.9	105	12.4	0.7	0.44	1.39	4.35	1.73
C5	47	F	94	5	79	15.9	0.9	1.88	1.08	3.18	1.68
C6	53	F	97	4.6	88	19.2	0.8	0.43	1.05	0.24	1.60
C7	66	M	86	4.9	106	16.7	0.6	0.49	1.05	0.21	1.84
C8	44	M	93	4.3	122	11.7	0.6	2.33	1.26	0.22	1.78
C9	67	M	76	4.5	101	17.6	0.7	1.02	1.83	1.84	2.25
C10	52	F	82	4.8	89	14.0	0.8	0.99	2.28	1.56	1.85
C11	47	F	91	4.8	91	13.2	0.8	0.96	1.30	2.33	1.79
C12	49	F	90	4.6	90	11.0	0.8	1.23	1.70	1.75	1.40
C13	45	F	85	5	80	18.7	0.9	0.75	1.69	3.23	1.80
C14	65	F	98	4.3	100	14.3	0.6	0.41	1.73	2.14	1.71
C15	58	M	76	4.6	107	15.5	0.7	0.75	1.08	3.41	1.45
C16	63	M	86	4.3	99	16.8	0.8	0.60	1.01	1.35	1.77
C17	59	F	77	4.2	85	19.1	0.8	1.38	1.04	1.18	1.54
C18	67	M	74	4.9	101	14.2	0.7	2.51	1.09	2.63	2.23
C19	51	F	95	5	105	17.2	0.7	0.79	1.40	2.57	1.54
C20	52	M	96	4.6	116	18.4	0.6	0.97	1.30	2.76	1.57
C21	45	M	84	4.1	111	13.6	0.8	0.65	1.03	1.11	1.91
C22	65	F	72	4.3	100	17.8	0.6	0.65	1.05	1.40	1.46

C23	59	F	78	4.4	74	11.0	0.9	0.96	1.13	1.83	1.83
C24	61	M	80	4.8	105	16.0	0.7	0.20	1.73	2.81	1.62
C25	48	F	92	5.1	91	16.3	0.8	0.58	1.38	1.53	2.37
C26	55	M	99	4.9	105	18.3	0.8	1.10	1.23	1.22	2.42
C27	51	F	95	4.7	105	13.2	0.7	0.90	1.24	2.17	1.21
C28	63	M	87	4.2	104	10.3	0.7	0.34	1.43	0.42	1.66
C29	50	F	74	4.3	109	6.4	0.6	1.34	1.39	0.76	1.58
C30	49	F	78	5	90	18.5	0.8	0.16	1.04	0.63	1.81
C31	56	M	96	4.7	113	14.7	0.6	1.44	1.08	0.41	1.68
C32	59	F	89	4.8	74	16.0	0.9	0.00	1.05	0.55	1.60
C33	56	F	91	4.4	86	18.1	0.8	0.58	1.05	0.29	1.84
C34	45	M	80	4.8	111	13.5	0.8	1.40	1.26	0.45	1.78
C35	51	F	90	4.4	109	16.3	0.6	2.62	1.83	0.28	2.25
C36	47	M	81	4.3	114	17.5	0.7	0.78	2.28	0.73	1.85
C37	61	F	78	4.7	98	12.9	0.7	0.97	1.30	0.84	1.79
C38	65	F	89	4.7	82	16.9	0.8	0.63	1.70	0.36	1.40
C39	55	M	87	4.7	114	10.5	0.6	0.63	1.69	0.37	1.80
C40	51	F	88	4.3	105	15.2	0.7	0.96	1.73	0.34	1.71
C41	48	F	76	4.5	91	15.5	0.8	0.19	1.08	0.90	1.45
C42	57	M	82	4.6	100	17.4	0.9	0.56	1.01	0.70	1.77
C43	52	F	84	5	108	12.5	0.6	1.11	1.04	0.53	1.54
C44	49	F	97	4.5	90	9.8	0.8	0.89	1.09	0.34	2.23
C45	50	M	83	4.4	112	16.3	0.7	0.32	1.40	0.66	1.54
C46	52	F	81	4.9	108	17.6	0.6	0.61	1.08	0.45	1.68
C47	61	M	93	4.1	110	20.1	0.6	1.35	1.05	0.59	1.60
C48	53	M	72	4.5	115	14.9	0.6	2.39	1.05	0.33	1.84
C49	46	F	82	4.2	91	18.1	0.8	0.80	1.26	0.48	1.78

C50	47	M	73	4.1	114	19.3	0.7	0.97	1.83	0.31	2.25
C51	55	F	70	4.8	106	14.3	0.6	0.66	2.28	0.75	1.85
C52	61	F	90	4.8	98	18.7	0.7	0.66	1.30	1.17	1.79
C53	57	M	91	4.4	100	11.6	0.9	0.96	1.70	0.40	1.40
C54	63	F	80	4	101	16.8	0.6	0.22	1.69	2.45	1.80
C55	54	F	68	4.2	88	17.1	0.8	0.59	1.73	0.38	1.71
C56	58	M	74	4.3	107	19.2	0.7	1.10	1.08	0.91	1.45
C57	63	F	76	4.7	83	13.9	0.8	0.90	1.01	0.72	1.77
C58	49	M	87	4.7	105	10.8	0.9	0.36	1.04	0.56	1.54
C59	53	M	72	4.5	115	14.9	0.6	2.39	1.05	0.33	1.84

Recommendations

This study evaluated the functional status of the RAGE pathway in DN. The sensitivity of the RAGE pathway was evaluated by comparing the AGE-induced expression of *NF-κB* and secretion of proinflammatory cytokines. The RAGE pathway was found to be hyperresponsive to AGEs. The regulatory aspect of the RAGE pathway was also evaluated by comparing the insulin-induced gene expression of *ADAM10* and sRAGE levels. *ADAM10* expression and sRAGE production were found to be reduced in DN. Together, these results support the conclusion that the RAGE pathway may be abnormal in DN. These observations suggest that the RAGE pathway may be a target for the development of novel therapeutics that can halt the progression of diabetes into nephropathy. One approach would be to ameliorate the hypersensitivity of RAGE by developing an anti-RAGE inhibitor. Such inhibitors may be based either on a small molecule or a monoclonal antibody. Another approach would be to increase the catalytic activity of ADAM10 by developing an appropriate enzyme activator. However, another approach would be to supplement the reduction in plasma sRAGE by developing recombinant sRAGE. Overall, this study encourages modulation of the RAGE pathway for the benefit of diabetic patients.