



T.C.

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**EVALUATION OF THE RELATIONSHIP BETWEEN
AUTOPHAGY AND RENAL FUNCTIONS IN DIABETIC
NEPHROPATHY PATIENTS**

DOCTORATE THESIS

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In the name of Allah, the Most Gracious, the Most Merciful

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ÖZET

DIABETİK NEFROPATİ HASTALARINDA OTOFAJİNİN RENAL FONKSİYONLAR İLE İLİŞKİSİNİN DEĞERLENDİRİLMESİ

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Bu çalışma, böbrek fonksiyon bozukluğu olan tip 2 diyabet (T2DM) hastalarında serum LC3-II, BCL-2, IL-1 β , TGF- β 1, GRP-78, Cys-C ve podosin düzeylerini değerlendirmek üzere tasarlanmıştır. Çalışmaya 26'sı sağlıklı ve 150'si T2DM'li olmak üzere 176 Türk denek katılmıştır. Hastalar albümin üre oranına göre sınıflandırıldı; 88 hastada makroalbuminüri, 20 hastada mikroalbuminüri ve 42 hastada normoalbuminüri vardı. T2DM hastaları da proteinüri ve eGFR evrelerine göre üç gruba ayrıldı. İdrar albümin ekstraksiyonunun arttığı ve böbrek fonksiyonlarının bozulduğu T2DM hastalarında serum LC3-II düzeyleri artmıştı. Serum LC3-II düzeyleri ile serum BCL-2, IL-1 β , TGF- β 1, GRP-78, Cys-C ve Podocin düzeyleri arasında güçlü pozitif bir ilişki vardı. Makroproteinürisi olan DM hastalarının normoproteinürisi olan DM hastalarından ayırıcı tanısında LC3-II'nin tanısal bir biyobelirteç olarak etkinliği %75,4 idi. Böbrek fonksiyon bozukluğu olan T2DM hastalarında artan serum LC3 II düzeylerinin böbrek podosit hasarına neden olabileceği düşünüldü. Bu hastalarda serum LC3-II düzeyleri böbrek hasarının gelişimini takip etmek için yeni bir biyobelirteç olarak değerlendirilebilir.

Anahtar kelimeler: BCL-2, GRP78, DKD, LC3-II, Otofaji, Podosin, Proteinüri

ABSTRACT

EVALUATION OF THE RELATIONSHIP BETWEEN AUTOPHAGY AND RENAL FUNCTIONS IN DIABETIC NEPHROPATHY PATIENTS

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This study was designed to evaluate serum LC3-II, BCL-2, IL-1 β , TGF- β 1, GRP-78, Cys-C, and podocin levels in type 2 diabetes (T2DM) patients with renal dysfunction. 176 Turkish subjects were enrolled, of whom 26 were healthy, and 150 had T2DM. Patients were classified according to albumin urea ratio: 88 patients had macroalbuminuria, 20 patients had microalbuminuria, and 42 had normoalbuminuria. T2DM patients were also classified into three groups according to proteinuria and eGFR stages. Increased serum LC3-II levels in patients with T2DM with increased urinary albumin excretion and impaired renal functions. There was a strong relationship between serum LC3-II levels and serum BCL-2, IL-1 β , TGF- β 1, GRP-78, Cys-C, and Podocin levels. The efficiency of LC3-II as a diagnostic biomarker in the differential diagnosis of DM patients with macroproteinuria from DM patients with normoproteinuria was 75.4%. It was thought that increased serum LC3-II levels in T2DM patients with impaired renal functions may cause renal podocyte damage. In these patients, serum LC3-II levels can be evaluated as a new biomarker to follow the development of renal damage.

Keywords: Autophagy, BCL-2, GRP78, DKD, LC3-II, Podocin, Proteinuria

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LIST OF ABBREVIATION

DM	Diabetes mellitus
T1DM	Type-1 diabetes mellitus
T2DM	Type-2 diabetes mellitus
GDM	Gestational diabetes mellitus
IGT	Impaired glucose tolerance
ESRD	End-stage renal disease
DN	Diabetic nephropathy
DKD	Diabetic kidney disease
NDKD	Non-Diabetic Kidney Disease
T2DKD	Type-2 Diabetic kidney disease
T2DN	Type-2 Diabetic Nephropathy
MAPK	Mitogen-activated protein kinase
TGF- β 1	Transforming growth factor-beta1
SCs	Stromal cells
ECM	Extracellular matrix
GFR	Glomerular filtration rate
AGEs	Advanced glycation end products
ER	Endoplasmic reticulum
ERS	Endoplasmic reticulum stress
TECs	Tubular epithelial cells
kD	Kilo Daltons
eGFR	estimated glomerular filtration rate
HbA1c	glycosylated hemoglobin
Bp	blood pressure
IDF	International Diabetes Federation
ASC	apoptosis-associated spot-like protein
CHOP	C/EBP Homologous Protein (CHOP) Transcription Factor Functions in Endoplasmic Reticulum Stress-Induced Apoptosis and Microbial Infection
HK2 cells	Human kidney tubular epithelial cells

ROS	Reactive oxygen species
NADH	nicotinamide adenine dinucleotide
FADH ₂	flavin adenine dinucleotide
PARP	poly-ADP-ribose polymerase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
DAG	diacyl glycerol (DAG)
O-GlcNAc	O-linked β -Nacetyl glucosamine (O-GlcNAc),
PKC	activating protein kinase C (PKC)
NO	nitric oxide (NO),
SGLT2	sodium-glucose transport protein 2
PI3K–Akt	phosphatidylinositol 3 kinase–protein kinase B
RAS	renin-angiotensin system
ECM	extracellular matrix
ACR	albumin creatinine ratio
UACR	urine albumin-creatinine ratio
AER	albumin excretion rate
TPCR	total protein creatinine ratio
GBM	glomerular basement membrane
ECs	Endothelial cells
eGFR _{cys}	estimated glomerular filtration rate using cystatin
eGFR _{cre}	estimated glomerular filtration rate using creatinine
GSDMD	gasdermin D
PCD	programmed cell death
Apaf1	apoptotic peptidase activating factor 1 (human)
NLRP3 pyrin domain–containing-3	nucleotide-binding domain, leucine-rich–containing family,
UPR	Unfolded protein response
PERK	RNA-like endoplasmic reticulum kinase
ATF6	Activating transcription factor 6
IRE-1	inositol requiring enzyme 1
ERAD	ER-associated degradation

eIF2 kinase	pancreatic eukaryotic translation-initiation factor 2
EIF2AK3	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3
PTECs	proximal tubular epithelial cells
STZ	Streptozotocin
BUN	blood urea nitrogen
Scr	serum creatinine
FFA	free fatty acid
TG	triglycerides
HDL-C	high-density lipoprotein cholesterol
CHER	cholesterol esterase
CHOD	cholesterol oxidase
LDL-C	low-density lipoprotein cholesterol
EMSE	N-ethyl-N-(3-methylphenyl)-N-succinyl ethylenediamine
TC	total cholesterol
SARs	Selective autophagy receptors
UPS	Ubiquitin-protein system
Atgs	Autophagy-related genes
LAMP2A	lysosomal-associated membrane protein 2A
VPS34	vacuolar sorting 34 protein
EMT	epithelial-interstitial transformation
CAFs	cancer-associated fibroblasts
CVD	cardiovascular disease
HHS	hyperosmolar hyperglycemic state
DKA	diabetic ketoacidosis
MDRD	Modification of Diet in Renal Disease
CKD-EPI	Chronic Kidney Disease-Epidemiology Collaboration
WHO	World Health Organization
NAD ⁺	nicotinamide adenine dinucleotide
NICE	National Institute for Health and Care Excellence
PI3K/Akt	phosphatidylinositol 3 kinase/protein kinase B

MAPK/ERK protein kinases	mitogen-activated protein kinase/extracellular regulated
NF- κ B	nuclear factor kappa-beta
g	gram
mg	milligram
μ g	microgram
ng	nanogram
L (l)	litter
dL	deciliter
mL(ml)	milliliter
μ L(μ l)	microliter

1 Overview

Diabetes mellitus (DM) is a long-term, systemic condition marked by a lack of sufficient insulin production, stemming from either a partial or total deficiency within the body. The development of this disease leads to metabolic disorders, including carbohydrates, lipids, and proteins, which result in alterations in vitamin metabolism, water and electrolyte equilibrium, and inappropriate oxidation. Both environmental and genetic aspects enhance the progress of DM (F. Sun et al., 2019). Characterized by insulin-related defects leading to hyperglycemia (Dilworth et al., 2021). Despite several methods, no medication has demonstrated the ability to stop disease advancement efficiently (Y. Chen et al., 2019). The prolonged hyperglycemia in patients with DM is linked to chronic microvascular consequences, including retinopathy, diabetic nephropathy (DN), neuropathy, and a higher risk of cardiovascular disease (CVD) (Punthakee et al., 2018).

The kidney is the 2nd most excellent oxygen-consumer organ in the human body; the kidney exhibits a notable susceptibility to mitochondrial malfunction. Regardless of its underlying cause, the course of CKD is influenced by mitochondrial dysfunction. Furthermore, it is of utmost significance to note that hyperglycemia directly affects renal tubular cells, leading to various metabolic and cellular impairments. The development of DKD is affected by interrelated mechanisms, including the overproduction of ROS, the initiation of the apoptotic pathway, and defective mitophagy. Although renal tubular cells contain the highest concentration of mitochondria, it is important to recognize that mitochondrial damage due to diabetes can also affect podocytes, mesangial cells, and glomerular endothelial cells (P. Z. Wei & Szeto, 2019).

DKD is a frequently encountered consequence of DM and is the primary etiology in developed nations. Around 40% of DM develops, which is characterized by the presence of albuminuria, decreased GFR, or both, (Christofides & Desai, 2021) in addition to genetic predisposition, dysregulation of glycolipid metabolism, inflammatory processes, oxidative stress, impaired autophagy, endoplasmic reticulum stress, and immune system dysregulation. The condition is marked by unusual ECM buildup and a thickened glomerular basement membrane, which eventually cause tubulointerstitial fibrosis and glomerulosclerosis to develop (J. Han et al., 2021).

It is also defined by intricate interactions among multiple factors contributing to structural and functional kidney alterations. Chronic hyperglycemia plays a significant role in promoting kidney disease in individuals with DM, leading to gradual damage to the glomeruli and tubules in the kidneys, which harms the individual's health. CKD in individuals with DM is manifested by higher levels of albumin in their urine, decreased kidney function, or both. About half of T2DM patients end up with kidney disease (Siddiqui et al., 2022).

DKD subsequently emerges as the primary risk factor for renal impairment. Regrettably, a significant proportion of individuals with diabetes are not cognizant of their renal insufficiency until they encounter symptoms such as dyspnea, lethargy, anemia, and edema, which are indicative of uremic syndrome (Christofides & Desai, 2021).

The clinical diagnosis of DKD is established by identifying proteinuria, a reduction in eGFR, or the simultaneous presence of both indicators in patients with diabetes. The etiology of this disease can stem from a variety of factors, including hypertensive nephrosclerosis as well as unresolved acute renal failure (Suneja, 2021).

Patients with T2DM and increased UACR levels can also monitor the course of CKD. Higher levels of albumin in the urine and reduced eGFR are linked to an increased risk of ESRD, acute kidney injury, and the progression of diabetic kidney disease (Mcgill et al., 2022).

In order to comprehend the relationship between autophagy, renal function, and nephropathy, a common complication of diabetes, this research will evaluate autophagy. Autophagic biomarkers may be applicable to further assist patients with DN diagnosis and follow-up.

Overall, this study looked at the serum levels of LC3-II, GRP78, BCL-2, Cystatin C, IL-1 β , TGF- β 1 and Podocin in people with T2DM to see if there was a link between these markers and the presence and severity of albuminuria. Patients with macroalbuminuria displayed significantly higher serum LC3-II, GRP78, and Podocin concentrations than those with normoalbuminuria. This study found that cystatin C levels increased in advanced CKD patients. This study found that cystatin C levels increased in

advanced CKD patients. There was also a strong positive correlation between LC3-II and cystatin-C groups in all T2DM patients. However, in the comparison made on proteinuria levels, no increase was found in the macroproteinuria and microproteinuria groups. As our results reveal, cystatin-C may be insufficient to predict the development of proteinuria in patients with T2DM. In addition, as presented by our data, serum IL-1 β levels were not significantly correlated or changed in T2DM patients according to albuminuria groups and DKD stages. The LC3-II levels were positively correlated with age, ACR, BUN, Scr, Cystatin C, BCL-2, Podocin, GRP78, IL-1 β , and TGF- β 1 and negatively correlated with GFR.

Autophagy dysfunction contributes to the pathogenesis of DKD by boosting ROS production, inducing renal cell destruction and apoptosis, and mediating responses of inflammation and fibrosis. The disruption of autophagy observed during different stages of chronic kidney disease affects the glomeruli, tubules, interstitial tissues, and the vascular components of the kidneys. On the other hand, PDCN can serve as a highly reliable marker for the heightened risk of kidney damage in individuals with T2DM. As all the above points have demonstrated, serum Pdocin, GRP78, and LC3-II were sensitive biomarkers to assess the severity of DKD in patients with T2DM.

2 Literature Review

2.1 Introduction

Diabetes mellitus (DM) encompasses a variety of metabolic conditions, all of which are marked by chronic high blood sugar levels. The cause can be abnormal insulin production or varying degrees of insulin resistance, often coinciding (Schleicher et al., 2022). Macro and microvascular problems develop, mainly associated with protracted hyperglycemia, which can result in cardiovascular disorders, stroke, loss of vision, and renal failure (Asmat et al., 2016).

2.1.1 Type 1 Diabetes Mellitus (T1DM)

The autoimmune response targeting cells in the pancreas causes the destruction of β cells, leading to a shortage of insulin production in individuals with T1DM (Sami et al., 2017). Further to the deficiency of insulin release, there additionally exists a modification in the functionality of pancreatic α -cells, leading to increased glucagon release in patients with T1DM. Typically, hyperglycemia results in a decline in the secretion of glucagon. Nevertheless, in T1DM, hyperglycemia does not inhibit glucagon secretion (Ozougwu et al., 2013).

T1DM is a specific type of autoimmune diabetes that is most common in children and teenagers. It is distinguished by the collapse of the pancreas due to autoreactive T cells, leading to a complete deficiency of insulin and hyperglycemia (Pang et al., 2020).

2.1.2 Type 2 Diabetes Mellitus (T2DM)

In individuals with optimal health, insulin is released to reduce glucose production and promote the uptake of blood glucose by peripheral tissues like muscles and fat (Honardoost et al., 2016). T2DM is characterized by two main pathological issues: a defect in pancreatic β -cells leading to inadequate insulin secretion, and a decrease in insulin effectiveness due to insulin resistance (Ozougwu et al., 2013). The responsible gene for the production of the insulin receptor resides on chromosome 19. The

transmembrane and tyrosine kinase domains are part of the alpha and beta subunits of insulin encoded by this gene. Research has linked mutations affecting the insulin receptor gene to T2DM and type A insulin resistance. Type A insulin resistance is genetic, while an autoimmune disease characterizes type B insulin resistance (Ozougwu et al., 2013). High concentrations of free fatty acids (FFAs) and high blood sugar levels have been found to negatively impact the performance of β -cells. The beginning of ER stress, which results from the activation of apoptotic UPR pathways, is causing this impairment. In instances of obesity, metabolic and oxidative stress are triggered by conditions such as lipotoxicity, glucotoxicity, and glucolipotoxicity, leading to the destruction of β -cells (Galicia-garcia et al., 2020).

The prevalence of DM has increased to an epidemic level, with around 1 in 11 people worldwide being affected. Notably, in most of these cases, precisely 90% are T2DM (Sapkota et al., 2021). The most prevalent manifestation of DM is closely linked to a familial susceptibility to diabetes, advanced age, excessive body weight, and insufficient physical activity (Baynest, 2015). T2DM is a chronic medical condition characterized by abnormal glucose metabolism within the body. Recent studies have indicated a population of roughly 537 million adults afflicted with T2DM, predicting that this figure will increase to 783 million individuals by 2045 (Sehrawat et al., 2023).

Chronic complications of heart, blood vessels, eyes, kidneys, and nerves caused by prolonged hyperglycemia is a metabolic disorder identified as DM, according to the (WHO) (Galicia-garcia et al., 2020). Reaching the expected glycated hemoglobin (HbA1c) levels reduces the probability of developing macrovascular and microvascular problems. Nevertheless, it is essential to note that approximately 50% of patients can maintain their preferred HbA1c levels (Marín-peñalver et al., 2016).

2.1.3 Gestational Diabetes Mellitus (GDM)

GDM is a term used to classify women who develop DM during pregnancy based on operational factors rather than basic pathophysiology. GDM includes women who acquire T1DM during pregnancy and women who have undetected, asymptomatic T2DM that is detected throughout pregnancy. The majority of women who acquire GDM

experience the onset of the disease during the third trimester of pregnancy (Baynest, 2015). IGT complicates the mother's health and fetus growth during pregnancy. The postpartum period is marked by the gradual fading of the disorder, yet this time is also linked to a significant risk of developing Type 2 diabetes in the future (Szmuiłowicz et al., 2019).

2.2 Diagnosis of DM

Frequent hyperglycemia is a characteristic of DM and is diagnosed when a single of the following medical criteria appears:

1. Blood sugar level equal to or exceeding (126 mg/dL) in a fasting state.
2. A circulating blood sugar concentration close to or higher than (200 mg/dL) two hours after a dose of (75g) oral glucose ingestion, as determined by a glucose tolerance test.
3. The signs of hyperglycemia are spontaneous plasma blood glucose levels close to or above (200 mg/dL).
4. The criterion for diagnosing diabetes mellitus is a glycated haemoglobin (HbA1C) level equal to or higher than 6.5%.

According to the (WHO), people with fasting blood glucose levels between (110 and 125 mg/dL) are considered to have impaired fasting glucose. Furthermore, individuals whose blood glucose levels reach or exceed (140 mg/dL) but do not surpass (200 mg/dL) within two hours of consuming a (75g) oral glucose dose are diagnosed with impaired glucose tolerance (Balaji et al., 2019).

2.3 Autophagy and T2DM

In order to preserve intracellular integrity, autophagy stimulates the metabolizing of proteins and organelles that have been intracellularly wounded and are not required to be harmed or misfolded. Additionally, autophagy provides essential biosynthetic components, including amino acids, fatty acids, and ATP, which are crucial for anabolic processes. These components play a key role in maintaining cellular homeostasis and

metabolism, especially during periods of cellular nutrient deficiency (Ueno & Komatsu, 2017).

Autophagy could contribute to T2DM in a multifaceted manner and possess a context-dependent role. Recent research has shown how autophagy protects pancreatic beta cells from the cellular stress that hyperglycemia imposes. Islet amyloid polypeptide (IAPP) buildup is a hallmark of T2DM. Inhibiting autophagy in cell culture exacerbated IAPP's adverse effects. The glucose tolerance of human IAPP knock in mice with a cell-specific autophagy deficiency significantly decreased. Also, hazardous oligomers corresponding to p62 that are usually contained in p62-positive aggregates multiplied throughout the cytoplasm (Shigihara et al., 2014).

When blood sugar levels become excessively high, p53 gathers in the cytoplasm of mouse cells, hindering the process of mitophagy. In cells lacking proper autophagy, the inability to manage the UPR and ERS leads to harmful effects. Blocking autophagy in various animal models exacerbates the metabolic issues associated with hyperglycemia, obesity, and diabetes. Kosacka et al. discovered that autophagy in the adipose tissue of humans has been enhanced in obesity and T2DM and that elevated autophagy was associated with AT inflammation (Ji et al., 2019; Kosacka et al., 2015).

Recent studies conducted in T2DM human patients and mouse models have demonstrated that autophagy deficits are related to the progression of T2DM (Marrif & Al-sunousi, 2016). Numerous conditions arise from the improper regulation of autophagy, encompassing neurological diseases, inflammatory-related disorders, and the development of tumors (Levine & Kroemer, 2019).

2.4 Acute Complication of T2DM

2.4.1 Hyperosmolar Hyperglycemic State (HHS) and Diabetic Ketoacidosis (DKA)

Acute hyperglycemic problems, which involve HHS and DKA, are dangerous metabolic impacts that develop in individuals diagnosed with diabetes. HHS is identified by a state of significant hyperglycemia and hyperosmolality while lacking substantial symptoms of primary ketosis or acidosis (Balaji et al., 2019).

During periods of insufficient levels of insulin, the liver performs a process wherein it converts fatty acids into ketones, which act like intermediary components in the metabolic sequence. The occurrence of ketoacidosis is notably more frequent in individuals with Type 1 diabetes than in those with Type 2 diabetes. In patients with severe hyperglycemia, typically exceeding 300 mg/dL, the occurrence of osmotic motion causes water to be pulled out of cells and released into the circulatory system. Consequently, the kidneys respond by excreting glucose into the urine (Balaji et al., 2019). HHS typically occurs in elderly individuals who have been diagnosed with T2DM. It should be noted that HHS has a more significant death percentage, ranging from 10% to 50%, in comparison with DKA.

While it is atypical for HHS to be the initial symptom of T1DM, there is evidence for multiple reported instances of its appearance in individuals with T1DM. Nevertheless, the incidence of HHS in pediatric and teenage populations with T1DM is infrequent. It is crucial to immediately recognize and appropriately manage this disorder due to its elevated risk of death (Scott, 2015).

Differentiating between DKA and HHS just according to a patient's medical history is a difficult task because of the overlapping clinical symptoms such as alterations in consciousness, nausea, lethargy, malaise, and polyuria. Nevertheless, there are variations in their respective diagnostic criteria. The biochemical indicators used to diagnose DKA consist of several criteria: elevated blood glucose levels exceeding 200 mg/dL, a venous pH below 7.3, or a serum bicarbonate concentration lower than 15 mmol/L. Additionally, either ketonemia (presence of ketones in the blood) or ketonuria (presence of ketones in the urine) must be detected (Wolfsdorf et al., 2018).

The diagnostic criteria for HHS refer to several parameters. Several indicators define this condition: blood glucose levels surpassing 600 mg/dL, arterial pH above 7.30, venous pH higher than 7.25, serum bicarbonate concentration greater than 15 mmol/L, presence of mild ketonuria, minimal or absent ketonemia, serum osmolality practicality exceeding 320 mOsm/kg, and the manifestation of symptoms like obtundation, combativeness, or seizures, occurring in roughly half of cases (Chun et al., 2022).

2.4.2 DM and Electrolytes

Electrolytes are essential for multiple physiological processes within the human system. They contribute to maintaining acid-base equilibrium, membrane potential, muscle contraction, nerve conduction, and regulating bodily fluids. Disruptions in the maintenance of electrolyte balance can result in physiological diseases. Research studies have demonstrated that insulin can stimulate the Na^+/K^+ -ATPase enzyme. As a result, a decline in serum insulin levels causes a decrease in the functioning of Na^+/K^+ -ATPase, which leads to compromised processing of sodium (Na^+) and potassium (K^+) in the body (Eshetu et al., 2023). In addition, it limits the transport of substances between bio-membranes and complicates the intestinal epithelia's uptake of monosaccharides. DM is characterized by hyperglycemia, which leads to osmotic diuresis triggered by glucose and subsequent depletion of bodily fluids and electrolytes (Khan et al., 2019).

Hyperglycemia causes dysfunction of cell membrane receptors and channels that facilitate the movement of fluids and electrolytes from inside to outside the cell, resulting in dehydration of the cell. The dehydration circumstances induce alterations in the cellular metabolic processes, which might lead to many serious consequences. Hyperglycemia correlates with immune system suppression, inflammation, increased cardiovascular response, and blood clot formation (Reshma et al., 2020).

2.5 Chronic Complication of T2DM (DKD, DN, and ESRD)

Insufficient synthesis of insulin, inadequate compensatory insulin release, and insulin resistance are produced by chronic hyperglycemia. The strong association between T2DM and several processes, such as apoptosis, oxidative stress, inflammation, and ERS, has been widely acknowledged (K. Cui & Li, 2023). This disease is associated with both macrovascular and microvascular complications (X. Ma et al., 2023). Hyperglycemia elevates the production of ROS. These ROS molecules then start signal transduction processes and encourage the upregulation of fibrotic components like fibronectin (FN), α -smooth muscle actin (α -SMA), and collagen I (Song et al., 2020).

eGFR < 60 mL/min per 1.73 m² utilized diagnostic criteria of CKD, in addition to UACR ≥ 30 mg/g for supporting the diagnosis. CKD has emerged as a growing concern in public health, impacting a significant proportion of the global population, exceeding 13%. Prevalence rates tend to be greater in industrialized regions. Patients who have been diagnosed with diabetes face a substantially higher likelihood of developing CKD compared to those without diabetes. Specifically, those with diabetes are about twice as likely to develop CKD as their counterparts without the disease (Ariton et al., 2021).

Indeed, diabetes gives rise to significant functional and anatomical alterations throughout the nephron, impacting the glomeruli, tubules, and renal interstitium (Heyman et al., 2022). Additional clinical examination is necessary due to susceptibility to ketoacidosis, hypoglycemia, hyperkalemia, and osmotic diuretic effects (Palygin et al., 2019) .

Approximately 30% of diabetic patients experience the development of DKD. Furthermore, diabetes is becoming increasingly common worldwide. Mortality and cardiovascular disorder are strongly correlated with ESRD and stimulated by the consequences of complications with T2DM. Identifying and screening patients at risk for DKD in its early stages is essential. The kidneys are crucial in filtering waste products from the bloodstream and eliminating them as urine. As a result, urine serves as a direct indicator of renal functionality. In contrast to plasma, urine can be conveniently obtained through non-invasive means, with the proteins present in urine exhibiting stability and resistance to abrupt destruction (Ahn et al., 2020).

DKD is a condition of significant global prevalence. It is considered one of the more prevalent problems associated with DM and is the leading contributor to the development of ESKD (C. Jung & Yoo, 2022). The pathophysiology of this condition encompasses three primary components, particularly the hemodynamic, metabolic, and inflammatory axis. Clinically, DKD is characterized by consistent albuminuria, which leads to a gradual decrease in GFR (Rico-Fontalvo et al., 2023).

DN is a CKD that progresses as a consequence of DM. The pathophysiology of DN is complex, and persistent increases in albuminuria and worsening GFR exacerbate its clinical features, ultimately resulting in the development of ESRD (D. M. Zhou et al., 2022). DN represents a severe consequence associated with diabetes. The condition is

distinguished by the depletion of renal cells and subsequent substitution with ECM, ultimately resulting in glomerulosclerosis and tubulointerstitial fibrosis (Song et al., 2020). Early clinical symptoms of DN include glomerular hyperfiltration along with proteinuria. Pathological signs like mesangial dilatation, the breakdown of endothelial cells, and damage to the podocytes cause proteinuria (Y. Liu et al., 2022).

Approximately 40% of DM patients progress to DN, often identified by albuminuria, characterized by elevated levels of urine albumin and a decrease in eGFR without evidence of coexisting renal pathologies (Sapkota et al., 2021). DN undergoes a series of sequential stages, namely hyperfiltration, microalbuminuria, macroalbuminuria, and falling GFR, ultimately leading to ESKD. However, a separate study has demonstrated that the diagnostic accuracy of the ACR surpasses that of single biomarkers for DN (Mahapatra et al., 2022).

The precise etiology of DN remains uncertain. Recent studies investigating the etiology of DN have shown evidence supporting a strong correlation between DN and apoptosis. The process of apoptosis in the renal tissue of individuals with diabetes is closely linked to the presence of podocytes and mesangial cells. Notably, renal tubular epithelial cells exhibit a high association with apoptosis. The ERS route is recognized to be part of the apoptotic pathways (X. Sun et al., 2020). Until recently, there is a lack of recognized biomarkers, individually or in combination, that can accurately predict the clinical course of DN (Harkin et al., 2022).

2.5.1 Risk factors of DKD

Several elements contribute to the advancement of CKD in diabetes patients. Improving diabetes management can help prevent or mitigate various risks, including hyperglycemia, hypertension, increased BMI, and abnormal lipid levels. Furthermore, substantial research indicates that rigorous diabetes treatment effectively decreases the average rate of albuminuria, impaired kidney function, and ESRD (Thomas et al., 2015). The prevalence of DKD and progression rates are relatively unknown in individuals with T2DM compared to those with T1DM. T2DM develops at a wide range of ages; it is

difficult to determine the beginning of the disease; and there is limited availability of samples to follow-up patients with T2DM (Gheith et al., 2016).

2.5.2 Microalbuminuria

The microalbuminuria test has long been utilized as an established technique for the early identification of DKD. Typically, the early stages of DKD cause microalbuminuria resulting from increased glomerular filtration, which is due to hyperfiltration damage to the glomerular barrier or a combination of both factors (H. Wang et al., 2021).

2.5.3 Hyperglycemia

Glomerular and tubular structures are deteriorated as a consequence of chronic hyperglycemia (Siddiqui et al., 2022). Hyperglycemia was introduced as a significant trigger for DKD. The extra level of production and excretion of insulin with its resistance is commonly associated with overweight or obesity; this consequence is identified as T2DM (Thomas et al., 2015). In patients with hyperglycemia, hemoglobin undergoes glycosylation via a non-enzymatic process, forming glycosylation of hemoglobin as identified as hemoglobin A1c (HbA1c) (Thomas et al., 2015). Effective care for DN depends upon maintaining optimal glucose control. The most reliable method for assessing glycemia levels and advising treatment is the estimation of HbA1c. By controlling glycemia through HbA1c monitoring, the risk of complications related to diabetes can be significantly minimized (Lee et al., 2018).

2.5.4 Hypertension

Hypertension is a crucial determinant of the possibility of developing DN. A recent meta-analysis has identified a significant link between hypertension and the development of DN. This theory has been verified by a population-based prospective study conducted in China, which reveals that proper hypertension control may decrease ESRD risk by 23% (Hussain et al., 2021). Besides hypertension, several other factors also

play a role in worsening T2DM (Thomas et al., 2015). Hypertension remains a controversial subject, particularly its effectiveness in promoting stroke and kidney disease (Scherntaner & Scherntaner-Reiter, 2018).

2.5.5 Dyslipidemia

The presence of long-standing diabetes can lead to dysfunction of lipoprotein lipase, resulting in an elevated level of TG. This increase in TG can cause the accumulation of large fragments of VLDL that are rich in TG. Consequently, these particles give rise to the formation of VLDL particles. Therefore, even if the LDL level is not high in patients with T2DM, they typically display a majority of atherogenic VLDL particles (Palazhy & Viswanathan, 2017).

Hyperlipidemia triggers the mesangial and tubulointerstitial cells, generating cytokines and chemokines. It also promotes cell proliferation by increasing the synthesis of substances like TGF- β 1. TGF- β 1 promotes the buildup of extracellular matrix within the mesangium and tubulointerstitium (Prakash, 2012). Dyslipidemia plays a key role in the onset of DKD by causing podocyte death, attracting macrophages, and promoting excessive extracellular matrix production. Additionally, patients with DKD experience hyperglycemia and insulin resistance, which lead to hyperlipidemia (Hussain et al., 2021). Nevertheless, the level to which proteinuria or renal failure contributes to dyslipidemia caused by DKD continues to be controversial. The medical term DKD covers conventional DN that involves proteinuria and atypical DKD, which lacks proteinuria (Hirano et al., 2022).

2.5.6 Obesity

Obesity is the consequence of excess ingested calories compared to the burned amount, often due to elevated consumption of processed food, decreased exercise, and a rise in sedentary habits (Hoogeveen, 2022). Additionally, it is recognized as a definite risk factor for DM and an increase in vascular problems (Shi et al., 2023). Elevated BMI is associated with metabolic issues, which may function as a risk marker for microvascular complications (Bin Zaman et al., 2018).

Weight loss in patients with T2DM is connected to enhanced glycemic control and reduced cardiovascular risks. However, it is essential to consider that a lower BMI correlates with a greater probability of mortality. Conversely, an increased BMI is linked to a greater likelihood of disability, especially among elderly diabetic patients (Katsukawa, 2021).

2.5.7 Smoking

Smoking is a factor that damages the kidneys and the development of DKD in both T1DM and T2DM through multiple processes, such as increased oxidative stress, enhanced expression of growth factors, and impaired functioning of the endothelium (Y. C. Lin et al., 2018). It is also identified as one of the independent risk factors for the advancement and deterioration of DN. Further, it has a significant influence on the progression of DN through multiple factors, such as oxidative stress, hyperlipidemia, the accumulation of AGEs, and glomerulosclerosis. There was a direct correlation between the rate of smoking and the probability of developing DN (Hussain et al., 2021).

2.5.8 Age related to DM

The overall rise in the average age of people has been suggested to be a significant factor in the elevated incidence of diabetes, as becoming older is an essential trigger for the start of this medical disorder (Schernthaner & Schernthaner-Reiter, 2018). By 2050, the percentage of elderly patients is expected to increase from 15% to 25%. The rate of development of T2DM increases with age (W. C. Chen et al., 2018).

2.6 Pathogenesis of DKD

DKD is a complex disease due to the kidney's cellular heterogeneity and diverse physiological activities. Many pathogenic processes are activated in response to chronic hyperglycemia, which impacts glomerular endothelial cells, smooth muscle cells,

mesangial cells, and podocytes. Hemodynamic alterations lead to elevated systemic and intra-glomerular pressure, triggering vasoactive hormone pathways (Swaminathan et al., 2023).

Hyperglycemia makes it easier for PKC to start activating, which increases the production of AGEs and diacylglycerol. Research indicates that elevated PKC levels are associated with increased levels of TGF- β 1, fibronectin, and type IV collagen (Zang, 2020).

Hyperglycemia triggers various metabolic pathways, such as polyol, pentose phosphate, hexosamine, and the synthesis of DAG through de novo processes. Furthermore, the Krebs cycle generates NADH and FADH₂ from the electron donors. These additional electrons are then converted to superoxide and other ROS, causing DNA damage and triggering the DNA repair enzyme PARP activation. Consequently, PARP deactivates the enzyme GAPDH. Inactivation of GAPDH accumulates glycolytic products, then diverts to other noxious metabolic pathways such as apoptosis, hyperfiltration, endothelial injury, microvascular disease, and albuminuria. Elevated glucose flux towards the polyol pathway results in the depletion of the antioxidant glutathione, causing an oxidative stress escalation. The process above is additionally initiated by converting fructose 6-phosphate to DAG, activating PKC and O-GlcNAc transferase. Consequently, there is a consequent decline in glomerular podocytes, advancement of the mesangial matrix, and gradual development of glomerulosclerosis. (P. Z. Wei & Szeto, 2019) Patients with diabetes who have albuminuria exhibit glomerulopathy characterized by mesangial sclerosis as well as arteriolar hyalinosis (Heyman et al., 2022).

Hyperglycemia has been observed to have a detrimental effect on the generation of NO, leading to the impairment of endothelial function. Together, these factors play a role in the development of dysfunction in the endothelial cells. The organelles known as mitochondria, responsible for generating energy within cells, received recognition as the central focus of these biological processes (P. Z. Wei & Szeto, 2019).

Diabetic glomerular microangiopathy develops concurrently with microvascular changes in the retina and nerves, resulting from hyperglycemia affecting AGEs, irregular insulin levels, and increased pressure within the kidney's filtering units causing damage.

The initial insult to the GFB involves several components, including endothelial dysfunction, heightened accumulation of extracellular matrix with damaged GBM, and gradual deterioration of podocyte function (Heyman et al., 2022).

2.7 Endothelial cells and DKD

Hyperglycemia, insulin resistance, and hyperinsulinemia have been identified as separate factors that contribute to EC dysfunction. These factors lead to increased generation of ROS, activation of PKC, and triggering of inflammatory signals initiated by AGEs. Glomerular EC lesions are frequently observed in individuals diagnosed with DN, wherein EC failure gradually advances to the development of histologic glomerular sclerotic disease. Albuminuria is widely recognized as a characteristic feature of systemic vascular endothelial disease. Diabetes is considered to be a prominent contributor to both structural and functional impairments of the glycocalyx (Heyman et al., 2022).

2.8 Glomerular and DKD

GBM lesions are frequently observed in several immunological and metabolic diseases. A prominent feature of DN involves the thickening of the GBM, which coincides with the development of protein in the urine. Moreover, hyperglycemia causes disturbances in matrix regeneration through inflammatory, oxidative, and fibrosis-promoting processes (Heyman et al., 2022). Decreasing surface area of the glomerulus by enlargement of intraglomerular mesangial cells, basement membrane thickness, and effacement of podocyte foot processes as a result of DKD progression (Swaminathan et al., 2023).

2.9 Podocytes and DKD

The specialized structure of the slit diaphragm and interdigitating proteins of the glomerular epithelial cells is pivotal for the function of the GFB. The hallmark

characteristic of proteinuric renal illness, including DN, is the effacement of podocyte foot processes, which can be observed through ultrastructural abnormalities. Both experimental research and clinical observations have demonstrated a clear connection between the reduction of foot processes and the development of proteinuria (Heyman et al., 2022).

2.10 Tubular and DKD

The role of SGLT2, located in the proximal tubules of the kidneys, is crucial for reabsorbing more than two-thirds of both sodium and glucose, making it essential for physiological function. Hyperglycemia is characterized by an upregulation of SGLT2, increasing glucose reabsorption. The heightened vulnerability of proximal epithelial cells to hypoxic injury is augmented, and the production of AGEs is facilitated. Similarly, the uptake of albumin by the proximal tubules induces toxicity in the proximal epithelial cells (Swaminathan et al., 2023).

2.11 Oxidative Stress and DKD

Oxidative stress increases the production of AGEs caused by hyperglycemia in the late stages of the Maillard reaction, which is the non-enzymatic glycation of sugar and protein. Renal cell injury is produced due to the oxidative stress generated by hyperglycemia, DNA damage, lipid peroxidation, impairment of mitochondrial function, and infiltration of inflammatory cells (Swaminathan et al., 2023). In the context of diabetes, hyperglycemia, AGEs, sophisticated oxidation protein products, urine proteins, and various endogenous nephrotoxins have the potential to stimulate the activation, trans-differentiation, hypertrophy, and death of renal tubular epithelial cells (TECs). Research findings suggest that autophagy plays a crucial role in safeguarding tubular epithelial cells (TECs) from harm and apoptosis within the kidney, similar to its function in DN (C. Yang et al., 2021).

2.12 Diagnosis of DKD

The diagnosis of diabetic kidney disease depends on evaluating both the GFR and the UACR to detect the presence of albumin in the urine (Hussain et al., 2021). DKD is identified through $\text{UACR} \geq 30 \text{ mg/g}$ and a consistent $\text{eGFR} < 60 \text{ mL/min/1.73 m}^2$, measured at least 90 days apart on two separate occasions. (Rodriguez et al., 2021). Congestive heart failure, fever, physical activity, urinary tract infection, and hematuria are transient conditions that can influence the above-listed tests (Sugahara et al., 2021).

Investigated the correlation between several clinical factors and the occurrence of DKD or NDKD based on histopathological analysis in patients with T2DM. A shorter duration of diabetes mellitus was linked to an increased chance of developing NDKD, whereas a longer duration of diabetes mellitus was linked to an increased chance of developing DKD (Tan et al., 2023). Even so, it is evident that not all patients with T2DM adhere precisely to this conventional progression of renal dysfunction. According to the results from the UKPDS, 38% of the participants developed albuminuria, and 29% experienced impaired kidney function over a median follow-up period of 15 years after being diagnosed with T2DM (Fu et al., 2019).

2.13 Biomarkers in Diabetic Kidney Disease (DKD)

2.13.1 Albuminuria

Albumin, one of the main proteins in the human body's plasma, has a molecular mass (65 kDa) synthesized in the liver. In typical circumstances, a modest amount of albumin undergoes filtration within the glomerulus, although the tubules subsequently reabsorb most of it. Elevated urinary albumin excretion (UAE) is widely recognized as a reliable indicator of glomerular injury. Furthermore, it is widely recognized as a tubular dysfunction that leads to albuminuria due to reduced filtered albumin's reabsorption (Cohen-Bucay & Viswanathan, 2012).

Unusually high levels of albumin in the urine cause albuminuria, a type of proteinuria. It is an essential sign of microvascular and renal damage. Additionally, it serves as an early predictor of the onset of DN. It is important to note that urine contains

minimal traces of albumin under normal circumstances, as it primarily circulates within the blood as an essential plasma protein. Albuminuria can be categorized into three groups depending on the quantification of urine protein levels: the albumin-to-creatinine ratio (ACR) or the 24-hour albumin excretion rate (AER).

These categories are as follows:

- Normoalbuminuria, characterized by a urinary albumin level of less than 30 mg/g or less than 30 mg/24 hours.
- Microalbuminuria is characterized by a urinary albumin level ranging from 30 to 300 mg/g or 30 to 300 mg/24 hours.
- Macroalbuminuria, characterized by a urinary albumin level exceeding 300 mg/g or 300 mg/24 hours. (Ariton et al., 2021)

Albuminuria is an important indicator for determining the progression of DN. Furthermore, it indicates the difference between the leakage of albumin across the glomerular filtration barrier and the proximal tubule's capacity to reabsorb filtered albumin along the tubular lumen (Fukuda et al., 2020). In typical circumstances, the glomerular membrane effectively prevents albumin from passing through, mainly because of the albumin molecule's size and electrical charge properties. In spot urine specimens, ACR is typically quantified using units (mg albumin/g creatinine). The presence of albuminuria serves not only as an indicator of renal dysfunction but also as a well-established predictor of cardiovascular risk, applicable to both general populations and those at high risk. Several research investigations have consistently shown the link between proinflammatory cytokines and cardiovascular risk, solidifying their role as indicators in this particular domain (Fellström et al., 2021).

The diagnostic tests commonly used for DKD include albuminuria and the (eGFR), which are calculated based on creatinine levels. As mentioned before, both tests are considered standard in diagnosing DKD (C. Jung & Yoo, 2022). The parameters that should be considered are the patient's age, the duration of their diabetes, the levels of HbA1C, their BP, the presence of albuminuria, their eGFR, and the existence of additional microvascular problems (Rico-Fontalvo et al., 2023).

Non-related kidney disease factors such as intense physical activity, the menstrual cycle, hypertension, a high body temperature, and cardiovascular disease increase albuminuria (Vučić Lovrenčić et al., 2023). Various studies frequently employ different markers of clinical response, such as albuminuria, hypertension, hyperglycemia, or lipid profile levels (Rico-Fontalvo et al., 2023).

Although microalbuminuria is commonly acknowledged as one of the initial clinical diagnostic indicators for DN, numerous patients also have a drop in GFR without a corresponding change in albuminuria. Furthermore, patients with diabetes can develop severe damage to the glomeruli, even if there are no high levels of albumin in the urine (Oraby et al., 2019). Routine monitoring of albuminuria and eGFR is advised for these patients. It's important to note that a higher level of albumin in the urine may indicate other health issues. Albuminuria often signals the onset of various glomerular diseases and can be elevated in hypertensive nephrosclerosis. Furthermore, it is associated with hypertension, obesity, and vascular disease (Mcgill et al., 2022).

2.13.2 Proteinuria

An excessive amount of protein in the urine is characterized as proteinuria. It has predominantly been acknowledged as an indicator of kidney impairment and a prognostic factor for the future deterioration of renal functionality. Proteinuria is characterized by an augmented inflammatory response and impaired endothelial function, which are prominent clinical manifestations. Diabetes is commonly marked by systemic inflammation and endothelial dysfunction, both of which are closely linked to insulin resistance (Jeon & Kim, 2021).

Potential mechanisms encompass the augmentation of protein uptake by tubular cells, the activation of the complement system, and tubulointerstitial inflammation. Furthermore, there is a rise in the filtration of heme proteins that promote oxidation, growth factors that encourage fibrosis, and inflammatory cytokines, all of which are contributing to proteinuria (Jefferson et al., 2008).

As per the guidelines about the care of kidney disorders, a criterion for diagnosing the cure of renal disease is a 24-hour urine total protein level <0.5 g/day. A renal biopsy

is typically indicated when proteinuria exceeds a threshold of >1 g/day, which is regarded as a medically significant amount (Bukhari et al., 2016). Severe proteinuria characterizes nephrotic syndrome, specifically proteinuria equal to or greater than 3.5 g/day, typically referred to as nephrotic proteinuria. This condition is accompanied by hypoalbuminemia, edema, salt retention, and hyperlipidemia, as well as issues related to thromboembolism and infection (Kamińska et al., 2020).

2.13.3 Glomerular filtration Rates

GFR is a quantitative approximation of the blood volume that traverses the glomerulus per minute. Any changes to the variables mentioned in the equation below will affect GFR. These include renal blood flow, the ratio of hydrostatic pressure in the capillaries to plasma oncotic pressure, and the amount of surface area used for filtration (Zang, 2020). The Cockcroft–Gault formula, published in 1973, is widely employed for estimating the GFR by considering age, height, sex, and serum creatinine levels (Cockcroft & Gault, 1976).

Cockcroft-Gault equations:

$$\text{Clcr (ml/min)} = [(140 - \text{age in yrs}) / \text{Scr (mg/dL)}] \times (\text{weight in kg} / 72) \times (0.85 \text{ if female}).$$

Table 2.1: CKD classification according to albuminuria and eGFR

A/C ratio			eGFR (mL/min)		Characterization
A1	Normoalbuminuria	< 30(mg/g)	≥ 90	G1	Sufficient functioning of the kidneys; currently, damage to the kidneys is observed.
			60-89	G2	Damage to the renal system becomes apparent by a mildly impaired functioning of the kidneys.
A2	Microalbuminuria	30-300(mg/g)	45-59	G3a	Mild to moderately impaired functioning of the kidneys with problems in those affected (fatigue, edema, anaemia, etc.)
			30-44	G3b	
A3	Macroalbuminuria	> 300(mg/g)	15-29	G4	Significantly decreased kidney function accompanied multiple serious manifestations and impacts (such as hypertension, bone disease, etc.).
			< 15	G5	Patients experience a variety of symptoms in addition to complications that result in kidney failure.

CKD - chronic kidney disease. eGFR - estimated glomerular filtration rate. A/C ratio - urinary albumin to creatinine ratio. (Vučić Lovrenčić et al., 2023; Zang, 2020)

Patients diagnosed with T2DM who display raised levels of albuminuria tend to present with a characteristic pattern of diabetic microvascular difficulties. Conversely, those with regular albuminuria and decreased GFR are in greater danger of cardiovascular and macrovascular concerns. Furthermore, some people suffer a fast deterioration in renal function, characterized by a decrease in eGFR exceeding 5 mL/min per year (Vučić Lovrenčić et al., 2023).

2.13.4 Drawback of kidney functions Biomarkers

It has been observed that around 30% of patients diagnosed with DKD exhibit no signs of albuminuria (C. Jung & Yoo, 2022). Furthermore, it should be noted that multiplying the quantity of nephrons and the average GFR of each nephron determines the GFR. In the context of DKD, the decline in the number of nephrons can be counterbalanced by an elevation in the GFR of the remaining nephrons. The eGFR may not reflect such changes, as a drop in eGFR may only occur after each nephron surpasses its maximum filtration capacity. This phenomenon is also complicated because the expansion of the diameter of the afferent arteriole in patients with DKD can potentially elevate the GFR of individual nephrons, even in cases where there is no loss of nephrons (C. Jung & Yoo, 2022).

2.14 Molecular Biomarkers in Diabetic Kidney Disease (DKD)

2.15 Cystatin C (Cys-C)

Cystatin-C is a natural inhibitor of cysteine proteases, produced by various tissues, and found in all biological fluids. The synthesis of this substance occurs effectively throughout all nucleated cells without any discernible influence from parameters such as sex, protein intake, inflammation, or muscle mass (Taha et al., 2023).

Cys-C functions as an intrinsic inhibitor of cysteine proteinases, although with its small protein with a molecular mass of (13.3kDa). The utilization of this marker has been acknowledged as a valuable substitute for the estimation of GFR. In contrast to serum

creatinine, Cys-C is not influenced by extrarenal variables. This characteristic boosts the diagnostic use of testing serum Cys-C in evaluating kidney dysfunction and accurately representing GFR values (Swaminathan et al., 2023). It has been demonstrated to possess significant value as a biomarker for diagnosing moderate renal failure and assessing GFR, particularly in comparison with creatinine (Oraby et al., 2019).

Cys-C is a non-glycosylated protein that displays alkaline characteristics. Additionally, it is not subject to hepatic function or inflammation-induced alterations and undergoes glomerular filtration (W. Xu et al., 2019). Cys-C, a type of small plasma protein, undergoes elimination from the body through glomerular filtration and subsequent degradation via renal tubular catabolism. Conversely, the kidneys only clear a relatively lower quantity of larger proteins (Öberg et al., 2021). It is important to emphasize that the renal tubule, not the inducer Cys-C, is completely reabsorbed by the proximal tubule. As a result, it undergoes subsequent decomposition and is prevented from reentering the blood stream (W. Li et al., 2020).

Previously mentioned data indicates that the association might be modified based on ethnicity, gender, age, and disease duration. Notably, a positive correlation has been observed between prolonged disease duration and elevated levels of this particular biomarker (González et al., 2022). The kidney is the sole organ tasked with removing Cys-C from the bloodstream, and the GFR is the main factor that determines the concentration of serum Cys-C (Liao, Zhu, et al., 2022).

Recent investigations have demonstrated that serum Cys-C has superior efficacy as a marker for the decline in GFR among individuals with DM compared to Scr. A separate investigation conducted by Oddo et al. demonstrated that serum Cys-C does not exhibit superior performance compared to Scr in determining GFR among individuals with stable diabetes. However, Cys-C exhibits considerable potential as a novel biomarker for detecting and diagnosing DKD (N. Wang et al., 2022).

2.16 Autophagy

Autophagy originates from the Greek and refers to "self-eating." The trend was first identified during the latter part of the 1950s. Cells engage in three unique types of

autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Each type showcases a variety of mechanisms and functions, highlighting the complexity of cellular processes (Koch et al., 2020). Christian De Duve's introduction to lysosomes in 1955 represented a crucial milestone in the knowledge of protein catabolism and served as the foundation for the subsequent identification of autophagy. The subsequent decomposition mechanism, the UPS, which facilitates the destruction of short-lived proteins, was discovered nearly 40 years afterward (Robert et al., 2019).

There are two forms of autophagy associated with nutritional conditions. Non-selective autophagy occurs if nutrients are scarce, and selective autophagy of organelles such as mitochondria, peroxisomes, and the ER happens when nutrients are abundant (Bharath et al., 2021). ER membranes contain the capacity to promote the process of autophagosome production. Autophagosome synthesis involves four essential stages: initiation, nucleation, elongation, and closure. Proteins encoded by Atgs meticulously regulate these activities (Kume & Koya, 2015).

The autophagy process initiates the formation of new separation membranes, also known as phagophores, which occur. These phagophores grow and join together, creating double-membrane vesicles called autophagosomes. These vesicles enclose a part of the cytoplasm. The ER membrane may serve as the source of the autophagosomal membrane. The process of autophagosome development encompasses five key stages, including initiation, elongation, closure, fusion, and dissolution (Heintz & Meyer-schwesinger, 2021). Throughout the progression of this process, there is the involvement of autophagy-related genes (Atg) and proteins. The Ulk1 complex, comprising the Ulk1 Ser/Thr protein kinase, Atg13, and FIP200 (the mammalian equivalent of yeast Atg17), plays a crucial role in kickstarting the autophagy process. The activation of autophagy relies on the crucial phosphorylation of Atg13 and FIP200 by Ulk1. The process of phagophore nucleation relies on Beclin 1 (known as Atg6 in yeast), a component of the hVps34 or class III phosphatidylinositol 3-kinase (PI3 K) complex. This complex consists of hVps34, hVps15, Beclin 1, and Atg14 (Erekat, 2022; D. Yang et al., 2018).

Following the process of autophagosome elongation and closure, two interconnected ubiquitin-like conjugation systems are essential: Atg12 and LC3, which are the mammalian counterparts of the yeast Atg8. The Atg12-Atg5 conjugate forms first,

which then integrates into the Atg12-Atg5-Atg16 complex. This complex is crucial for facilitating and directing the LC3 conjugation reaction. In this reaction, the cytosolic form of LC3, termed LC3-I, conjugates with phosphatidylethanolamine through two successive ubiquitin-like events (Yamahara et al., 2013). These processes are driven by the E1-like enzyme Atg7 and the E2-like enzyme Atg3, culminating in the creation of LC3-II. Hence, LC3-II production is recognized as a marker for autophagosomes in cellular or animal research. Autophagosomes fuse with lysosomes, forming autolysosomes as they mature. The protein p62, also known as sequestosome 1, associates with LC3 and localizes to autophagosomes, where it is continuously broken down by the autophagy-lysosome pathway (Gonzalez et al., 2021; D. Yang et al., 2018).

2.16.1 Macroautophagy

Macroautophagy utilizes autophagosomes, which are double-membraned vesicles that swallow cytoplasmic proteins and organelles before delivering them to the lysosome for destruction (Varela et al., 2023). Autophagosomes migrate across microtubules before fusing alongside lysosomes to form autophagolysosomes. Lysosomal enzymes destroy the material that is given after fusion with lysosomes. The outcomes of lysosomal breakdown, like amino acids, are reconstituted and utilized for protein synthesis, allowing energy that would otherwise be needed in de novo synthesis to be conserved. Thus, autophagy helps to maintain cellular energy levels and quality control. Specific macroautophagic systems, such as mitochondria and peroxisomes, are thought to be more selective in their breakdown of damaged organelles (Ghosh & Pattison, 2018). LC3-II activation coincidentally correlated with the accumulation of cargo receptors of p62 during the malnourishment of mammalian cells (Zaffagnini & Martens, 2016).

2.16.2 Microautophagy

Microautophagy does not depend on autophagosomes (Ghosh & Pattison, 2018); it is a less-selective type of autophagy that is triggered by lysosomal membrane invagination surrounding the material to be destroyed (Robert et al., 2019). Glutamine is

a major nutrient-signalling component in microautophagy that can signal upstream of or activate TOR. Glutamine deficiency mirrors TOR dysfunction by triggering the movement and activation of Gln3 into the nucleus, as well as the activation of the Rtg1p/Rtg3p heterodimeric transcription factor. Consequently, the retrograde response pathway that depends on Rtg1/2/3 operates upstream of the TOR pathway (Jian & Bao, 2012).

2.16.3 Chaperone-mediated autophagy (CMA)

Mammalian cells are the only ones that produce CMA (Ghosh & Pattison, 2018). This specific process breaks down cytosolic proteins containing a KFERQ or similar motif in their amino acid sequences. These motifs are identified by the cytosolic chaperone heat-shock protein-70 (Hsc70c or HSPA8), which unfolds the proteins and directs them to LAMP2A. LAMP2A acts as the specialized receptor for this CMA pathway. Lysosomal proteases eventually destroy proteins delivered to the lysosomal lumen, and the degradation products (amino acids) may be recycled to sustain cellular homeostasis and promote survival (Robert et al., 2019).

CMA is not associated with the development of autophagosomes or autolysosomes. Proteins decompose, while organelles remain. HSC70 and LAMP-2A proteins transport the protein loads straight into the lysosome lumen (Parzych & Klionsky, 2014). CMA differs from macroautophagy in that it transports a single protein for lysosomal breakdown one at a time. Autophagosomes engulf and disseminate mostly larger compartments for bulk cargo breakdown in macroautophagy (Ghosh & Pattison, 2018).

2.16.4 Initiation of Autophagy

Amino acid deficiency, changes in lipid metabolism (Mejlvang et al., 2018; Y. Xie et al., 2020), a reduction in calorie intake, physical activity, decreased intracellular cholesterol transportation, and the availability of protein products, injured organelles, and pathogenic microorganisms all impact autophagy (Brandt et al., 2018; Chung & Chung, 2019). Autophagy initiates with the involvement of two key components: the ULK1 complex and the PI3 Kinase Complex III. The ULK1 complex comprises FIP200,

ATG13, and ATG101. Activation of ULK1 necessitates post-translational phosphorylation and ubiquitination by the ULK1 kinase, which is regulated by mTORC1 and AMPK. The ULK1-ATG13-FIP200-ATG101 complex assesses cellular nutrient levels and triggers autophagy accordingly. Under nutrient-rich conditions, mTORC1 associates with the ULK1 complex, phosphorylating ULK1 and ATG13. Conversely, during starvation, mTORC1 dissociates, leading to dephosphorylation and activation of autophagy. ULK1 also phosphorylates and activates other autophagy proteins, such as Beclin1 (BCN1) and VPS34 (Anding & Baehrecke, 2017).

2.16.5 Nucleation

The ATG14-class III phosphatidylinositol 3-kinase complex (PtdIns3K) plays a role in phagophore nucleation and is an additional complex required for autophagy development. PtdIns3K produces PtdIns3P, which is required to nucleate a phagophore composed of PIK3C3/Vps34, PIK3R4/p150, and BCN1 (Burman & Ktistakis, 2010). By linking up with ATG14, the complex may induce traditional macroautophagy or activate the endocytic pathway by interacting with UVRAG (Lane & Mannack, 2015).

2.16.6 Elongation

Two attachment processes, including ubiquitin-like proteins, play a role in the phagophore's expansion/elongation. After attaching to the phagophore membrane, the ATG12-ATG5-ATG16L1 complex separates once autophagosomes are formed. The subsequent mechanism involves the ATG8/LC3 system (Lane & Mannack, 2015). The ATG4 protease plays a crucial role within the ATG8/LC3 system. It acts by cutting pro-LC3 to produce LC3-I, which undergoes lipidation to become LC3-II. This process contributes to the development of the extending phagophore membrane. LC3-I lipidation proceeds under a lack of diet and cellular stress (A. R. J. Young et al., 2006).

2.16.7 Selective Autophagy of Organelles

Autophagy purifies lysosomes, nuclei, endoplasmic reticulum, mitochondria, peroxisomes, and ribosomes (Amm et al., 2014). Organelle autophagy includes an initiating step followed by extremely well-organized signal transduction. The tagging of the organelle indicated for decomposition is usually the first process that triggers selective autophagy. Ubiquitination tags the target organelle, guiding its selection for autophagy. Besides ubiquitination, various other signals trigger this selective process (Khaminets et al., 2016).

Selective autophagy involves three crucial steps. Initially, it necessitates the identification of recyclable cargo. This identified cargo is then enveloped within an autophagosome. Lastly, components within the autophagosome that are not earmarked for destruction are expelled. Once the cargo is recognized, adapter proteins, or cargo recognition proteins, facilitate the transport of the tagged organelle into the phagophore. Each organelle has its own cargo recognition proteins and destruction signals. According to numerous studies, high blood sugar levels lead to dysfunction of selective autophagy and increase microvascular and macrovascular disorders (Zaffagnini & Martens, 2016).

2.16.8 Phosphorylation

Posttranslational changes such as phosphorylation and ubiquitination are widely acknowledged for controlling the function of proteins involved in autophagy and the breakdown of proteins carried by autophagy, respectively (Reggiori et al., 2012). This process is critical for initiating autophagy and is essential for controlling autophagic flux. Furthermore, it is vital to controlling SARs' capacity to connect cargo to autophagic machinery. Phosphorylation, in the vast majority of cases, affects SAR binding to Atg8/LC3/GABARAP (Gubas & Dikic, 2022).

2.16.9 Acetylation

Acetylation is an additional posttranslational alteration that was recently found to be associated with selective autophagy. Histone deacetylase 6 (HDAC6), first discovered to promote the transport of proteins misfolded to the aggresome, has recently been linked to the maturation of ubiquitin-positive autophagosomes (Reggiori et al., 2012).

Acetylation regulates LC3 proteins, which typically transfer between the nucleus and the cytoplasm. Sirt1 deacetylates LC3 at lysine 49 and 51 under deprivation, starving it of the ability to translocate from the nucleus into the cytosol and play a role in autophagy (Gubas & Dikic, 2022).

2.16.10 Autophagy and Metabolism

Impaired autophagy in hepatocyte-specific Atg7-knockout mice result in lipid droplet formation, elevated hepatic TG and cholesterol levels, and enlarged liver size. During hypoglycemia or other circumstances, such as mitochondrial malfunction or reduced cellular ATP levels, AMPK initiates a metabolic response to produce energy, boosting glucose uptake, glycolysis, and lipid catabolism (P. Lin et al., 2021).

2.16.11 Autophagy Regulation

Autophagy is regulated by four primary growth-signaling pathways that converge either at the ULK1 complex or at the Beclin1-PI3KC3-C1 complex. 1: Autophagy is triggered by AMPK under conditions of energy depletion. 2: Autophagy is suppressed by mTORC1 when nutrient and amino acid glucose supplies are abundant. 3: Autophagy is inhibited by BCL-2. 4: Autophagy is a suppressed class I (PI3K-AKT) signaling pathway upon stimulation with insulin and growth factors (Heintz & Meyer-schwesinger, 2021).

2.17 Abnormal Metabolism and Autophagy-Related Diseases

Unexpectedly, proteins are destroyed faster in the liver during starvation to produce the energy essential to compensate for the temporary low levels of nutrients. Furthermore, in Atg7-deficient hepatocytes, which are then autophagy-deficient, there is a decline in protein turnover due to the failure to synthesize autophagosomes, leading to the decomposition of proteins. These cells also accumulate protein aggregates and injured mitochondria, indicating that autophagy is required in ordinary physiological settings to eliminate harmful constituents after starvation (Komatsu et al., 2005).

According to Chung et al, hyperglycemia offers a source of energy for colorectal cancer biosynthesis, ultimately resulting in enhanced cell proliferation, EMT, and metastasis. In regular circumstances, the carbon generated from glucose and glutamine is used as power for the Krebs cycle, also known as TCA. In the cancer cell environment, where glucose is scarce, tumor cells stimulate cancer-associated fibroblasts (CAFs) to release alanine through autophagy. This alanine serves as a carbon source for the TCA cycle, aiding in the production of nonessential amino acids and lipids. Conversely, many viruses enhance autophagy to support their replication process. They utilize autophagosomal membranes as sites for viral replication and eliminate lipid droplets to generate the ATP energy essential for viral replication (P. Lin et al., 2021).

2.18 Autophagy & podocyte injury

Autophagy is crucial for maintaining post-mitotic cells, specifically neurons and podocytes. Bowman's capsule's visceral layer and the capillaries surrounding the glomerulus consist of specialized epithelial cells known as podocytes. Podocytes possess distinctive foot processes known as pedicles, which envelop the capillaries and extend inside to create a filtration slit diaphragm. The permeability of circulating blood and urine production in the glomerular filtration barrier regulates the filtration in the slit diaphragm and foot processes of podocytes (L. Zhang et al., 2020).

Podocytes regulate the glomerular filtration rate by utilizing the contraction and closure of filtration slits. Malnutrition and proteinuria develop due to acute kidney disease, a unique marker for podocyte injury. Its impairment is directly proportional to the severity of glomerulosclerosis, proteinuria, and renal damage (T. Lin et al., 2019).

Previous research on mice has indicated that podocytes demonstrate a substantial baseline level of autophagy, characterized by many autophagosomes. The decrease of the Atg5 gene, specifically in podocytes in aging mice, results in glomerulopathy. This type of disorder identifies endoplasmic reticulum stress, ubiquitinated proteins, and oxidative formations in podocytes. Ultimately, these factors contribute to the loss of podocytes, increased proteinuria, and the development of glomerulosclerosis (Yamahara et al., 2013; D. Yang et al., 2018).

The accumulation of autophagosomes, LC3, and impaired mitochondria enhance podocyte dysfunction. Regulation of autophagy in podocytes, both in rat models and in humans, may be maintained by the mTOR pathway. At the physiological level, the activity of mTOR limits the process of autophagy. Autophagosomes are crucially upheld to ensure the removal of damaged organelles, excess lipids, and proteins that linger or misfold within podocytes. This maintenance is essential for their effective function (Y.-P. Han et al., 2023; Koch et al., 2020). Boosting podocyte autophagy benefits DKD by regulating the autophagy-related (Atg) protein conjugation pathway and controlling the mTOR (Y. C. Lin et al., 2018).

2.19 Autophagy in Proximal Tubular Cells (PTC) in DN

Autophagy is a widely observed catabolic process that occurs in eukaryotes. A range of stressors can activate the process under consideration and promptly initiate a reaction aimed at eliminating dysfunctional cellular structures and protein clumps through the utilization of autophagosomes. These autophagosomes then transport the targeted materials to lysosomes for degradation. The importance of autophagy in the development of T2DM has been acknowledged, as it plays a critical role in safeguarding the structural integrity and functional capabilities of pancreatic beta cells, thereby promoting their survival (K. Cui & Li, 2023).

Autophagy serves two primary functions within the cellular context. Initially, it facilitates self-digestion during food scarcity, enabling the recycling of energy resources. Additionally, it participates in the breakdown of impaired or surplus organelles and macromolecules (Guo et al., 2020).

In addition, it is a self-preserving mechanism in reaction to stress, wherein it facilitates the degradation and recycling of internal substances to sustain optimal energy levels. Furthermore, it functions as a mechanism for ensuring quality by eliminating damaged or senescent cells in anticipation of additional destruction, which leads to several disorders (Gonzalez et al., 2021).

In diabetes, the glomerulus filters carbonyl compounds generated by AGEs, which are then reabsorbed by the proximal tubule, leading to a significant risk of tubular toxicity (W. Liu et al., 2018). The kidneys of diabetic patients generate excessive amounts of AGEs, leading to the initiation of various malfunctioning cellular activities such as apoptosis, oxidative stress, and inflammation. This process is facilitated by the interaction between AGEs and their receptor, RAGE (Erekat, 2022; N. Liu et al., 2017). Additionally, the accumulation of AGEs suppresses the beneficial effects of autophagy, which serves as a protective mechanism in the diabetic kidney (W. J. Liu et al., 2015).

In diabetes, when the autophagy-lysosomal system malfunctions, it results in the accumulation of AGEs. This increased accumulation of AGEs exacerbates lysosomal dysfunction, establishing a cycle of reinforcement. This cycle contributes significantly to the progression of tubulointerstitial inflammation and fibrosis, which are critical factors in the advancement of DN (Giraud-billoud et al., 2018).

Restoring normal autophagy in the cells lining the kidney's filtering units and reducing DN involves inhibiting the signaling of AGEs and their RAGE (Y.-P. Han et al., 2023; Yamahara et al., 2013). Numerous studies have demonstrated that autophagy insufficiency is the primary factor contributing to podocyte damage. Therefore, podocytes must maintain internal balance through autophagy to prevent podocyte injury, protein leakage, and glomerulosclerosis that occur when autophagy fails (Cai et al., 2023). Impaired autophagy has been identified as a contributing factor in the development of several metabolic disorders. Furthermore, autophagy dysfunction has been linked to significant damage to podocytes and the development of extensive proteinuria in DN (Dong et al., 2019).

Hyperglycemia induces abnormal autophagy, which facilitates the progression of DN. In particular, the lack of Atg5 in podocytes changes how their cells function, making them less able to handle the effects of stimuli caused by hyperglycemia. The elevated hyperglycemia observed in patients with diabetes leads to increased expression of the autophagy marker LC3-II in podocytes. (Ge et al., 2022). As shown by an earlier study, a decrease in autophagy activity in different organs appears in obese diabetic patients. Furthermore, the physiological significance of autophagy in the kidney has been progressively explored (Yamahara et al., 2013).

2.20 mTOR pathway

The kidneys of diabetic patients experience elevated mTOR activation, sparking inflammation and worsening renal function. Vascular endothelial growth factor can stimulate the mTOR signalling pathway through the PI3K/Akt cascade. This activation results in the suppression of autophagy by phosphorylating the downstream protein known as 70 ribosomal protein S6 kinase (p70S6K), thereby worsening DN. Although, the administration of rapamycin has been shown to alleviate these effects and restore renal function (Y.-P. Han et al., 2023).

2.21 AMPK pathway

AMPK activation hinges on phosphorylating threonine 172 (Thr172) on its α subunit. This process is tightly controlled by the AMP-to-ATP ratio, which functions as a sensitive gauge of cellular energy levels (Yu et al., 2023). This activation initiates autophagy, crucial in maintaining cellular energy balance during starvation. AMPK can phosphorylate ULK1 at two specific sites, Ser317 and Ser377. Additionally, AMPK can indirectly promote autophagy by inhibiting the activity of mTORC1, subsequently leading to the release of ULK1. The overall consequence of these processes is the inhibition of DN progression. In addition, the activation of AMPK leads to the activation of Sirtuin 1 (Sirt1) through two mechanisms: increasing the levels of cellular (NAD⁺) or phosphorylating and redistributing (GAPDH) into the nucleus, thereby releasing Sirt1. This activation of Sirt1 accelerates the autophagy process and relieves DN. AMPK activation helps break apart the beclin1/BCL-2 complex by adding a phosphate group to beclin1 at Thr388. This action initiates autophagy (Y.-P. Han et al., 2023).

Studies indicate that triggering AMPK can hinder podocyte damage, safeguard the integrity of the glomerular filtration barrier, and delay the initiation of DN (H. Gao & Wu, 2022).

2.22 Study Biomarkers

2.22.1 Microtubule-associated protein 1A/1B-light chain 3, LC3B (LC3-II)

Autophagy is a process that employs lysosomes to eliminate undesirable cellular debris, including misfolded proteins, bacteria, and viruses, while maintaining the cell's integrity (Tanida, 2011). Autophagy is a natural process that helps cells maintain homeostasis by converting useless components into beneficial molecules. Autophagy protects cells from apoptosis and facilitates their survive in low-nutrient environments (Choi et al., 2012).

The human MAP1LC3B gene produces LC3 protein, belonging to the ATG8 protein family known for its conservation. LC3 plays a pivotal role in autophagy, being crucial for choosing substrates and forming autophagosomes. LC3 stands out as the most widely employed marker for autophagosomes (Hassan et al., 2020). The mechanism of Atg8/LC3 conjugation resembles a ubiquitin-like pathway, where LC3, a homologous protein to yeast Atg8, plays a crucial role (Mohamed et al., 2019). LC3 plays a crucial role in autophagy by actively engaging in the extension, development, and merging of the autophagosome with the lysosome. The autophagosome isolation membrane contains lipidated LC3 (LC3B) at both locations (Gonzalez et al., 2021). Following a synthesis process, LC3 is subjected to proteolytic cleavage facilitated by Atg4. This process leads to the generation of LC3-I in the cytosol. Subsequently, Atg7 activates LC3-I through its binding to LC3-I via a thioester bond. Subsequently, the LC3-I that has been activated is translocated to Atg3. Upon completion of a series of biochemical transformations, LC3-I becomes bound to phosphatidylethanolamine, thereby generating LC3-II (Koch et al., 2020).

LC3-II plays a vital role in elongating and expanding the membrane of autophagosomes. Its integration into the developing autophagosome relies on the Atg5-Atg12 complex. This implies a cooperative relationship between the LC3/ATG8 and Atg5-Atg12 systems during autophagosome maturation. LC3-II stays attached to both the inner and outer membranes until the autophagosome formation is finished. On the other hand, it is noteworthy that the Arg12-Atg5-Atg16 complex often exhibits localization to the outer membrane of the autophagosome throughout its expansion phase. It is subsequently dissociated either before or quickly following the completion of

autophagosome synthesis. Therefore, LC3-II is considered the most reliable indicator for assessing the extent of autophagic activity (Heintz & Meyer-schwesinger, 2021).

Researchers have discovered that LC3II is present on the interior and exterior surfaces of phagophores, where it functions in autophagosome size regulation, membrane fusion, and the sorting of degraded cargo (Z. Zhang, Sun, et al., 2022).

The level of LC3-II, often used to gauge autophagy activation, corresponds directly to the number of autophagosomes formed. Once formed, autophagosomes merge with lysosomes, leading to the formation of autolysosomes. The p62 protein localizes to autophagosomes and undergoes degradation within autolysosomes. P62 is a useful marker for autophagic flux since it exhibits a negative correlation with autophagic activity and accumulates in autophagy-deficient cells (Erfan et al., 2021).

During autophagy, specialized membranes called phagophores expand and merge, enclosing portions of the cytoplasm within double-membrane structures known as autophagosomes. This process facilitates bulk degradation, which is crucial for eliminating damaged proteins and organelles from the cell (Yamahara et al., 2013). Research has revealed that the DKD model inhibits autophagy activity by significantly increasing the podocyte autophagy substrate protein p62 and downregulating the expression of LC3-II (X. qi Liu et al., 2022).

2.22.2 Interleukin-1 (IL-1 β)

IL-1 β has been recognized as a significant modulator of the inflammatory response in human beings (Liao, Xiao, et al., 2022). Chronic microinflammation stimulates the progression of DN, as displayed by previous studies. The phenomenon considered "microinflammation" should be separated from typical inflammatory disorders, such as furuncles and carbuncles, characterized by medical symptoms like redness, swelling, heat, and pain. The concept of "microinflammation" in DN can be identified as a form of immune-mediated inflammation. Elevated glucose levels in an environment of DN activate several inflammatory responses within the body. These responses impact DN's progression by promoting macrophage infiltration and the excessive production of inflammatory markers (Y. Wang et al., 2021).

Given that DM is a disorder characterized by inflammation that goes beyond the conventional hemodynamic and metabolic aspects, it is crucial to identify biomarkers that indicate inflammation at an early stage in order to mitigate problems associated with the disease. The timely identification of DKD, in conjunction with the enhancement of available treatment interventions, has the potential to impede the advancement of DKD to its last stages, like the requirement of renal replacement therapy, which eventually results in mortality (Rico-Fontalvo et al., 2023).

The extent of dendritic cell infiltration in the glomeruli is directly correlated with the level of proteinuria observed with the advancement of DKD. A correlation exists between mast cell infiltration of the interstitium and serum creatinine levels, but no such correlation has been observed with proteinuria. The process of mast cell degranulation and the subsequent release of IL-1 β from macrophages collectively induce the proliferation of renal fibroblasts. Conversely, IL-1 β promotes explicitly the proliferation of mesangial cells (Swaminathan et al., 2023).

Following an injury to kidney cell linings, the immune reaction intensifies due to the release of inflammatory agents through cell pyroptosis. In the typical pyroptosis process, the caspase-1 enzyme splits gasdermin D (GSDMD), activating IL-1 β and IL-18. Alternatively, in the non-standard route, caspase-11 mediates GSDMD cleavage. Cell apoptosis depends on caspase-3, caspase-8, and caspase-9. A key difference is that pyroptosis requires caspase-1, while apoptosis can occur without it. The traditional pyroptosis method involves caspase-1 activation, causing cell membrane rupture and the release of internal substances, triggering inflammation (Y. Li et al., 2022).

Bacteria, pathogens, or their endotoxins are responsible for the inflammatory process known as pyroptosis. This activation triggers caspase-1, leading to cell swelling, formation of pores in the cell membrane, membrane rupture, release of cellular contents, breakdown of DNA, inflammasome activation, and subsequent release of inflammatory substances and cellular contents. Consequently, this cascade of events induces a robust inflammatory response. This reaction induces PCD (P. Liu et al., 2021). Pyroptosis represents an immunogenic process that leads to PCD. Various cellular processes, such as swelling and lysis, lead to the release of pro-inflammatory chemicals, triggering a robust inflammatory response that causes tissue damage. The activation of caspase-1 is

critical in pyroptosis, wherein GSDMD undergoes cleavage, distinguishing this canonical pathway. GSDMD acts as a key protein in pyroptosis, functioning as an executor. When cleaved by caspases, GSDMD releases its N-terminal domain (GSDMD-NT), which binds to the cell membrane, causing the efflux of cytoplasmic contents and the subsequent release of IL-1 β and IL-18 (X. Cui et al., 2023; W. Zhao et al., 2022).

Numerous investigations have provided compelling evidence that IL-1 β plays a pivotal role as a proinflammatory cytokine in DN, actively triggering immune responses. The complex protein that triggers the activation of cytokines from the IL-1 β family is determined as NLRP3 inflammasome. A receptor resembling Nod pairs with an ASC adapter. It has been observed that extended periods of hyperglycemia may trigger this receptor-ASC combination. Moreover, decreasing the levels of this complex can alleviate inflammation in kidney tissues affected by diabetic nephropathy in individuals with T2DM (Hou et al., 2021).

The results of an experiment using Gerbil models showed that hyperglycemia stimulates human β -cells to produce and discharge IL-1 β . It triggers increased expression of the Fas receptor, activation of NF- κ B, death of β -cells, and subsequent renal failure. In a study using mice to simulate DN, injecting IL-1 β caused kidney inflammation and a gradual loss of kidney function over time (Liao, Xiao, et al., 2022).

2.22.3 Transforming growth factor-beta-1(TGF- β 1)

TGF β plays a crucial role in managing various cellular activities, including the growth and division of cells, their specialization, their ability to stick to each other, and the process of programmed cell death (J. Zhang et al., 2020). TGF- β 1 is a significant constituent of the transforming growth factor superfamily (TGF-s) and is synthesized by several cellular entities. This entity's composition consists of various interconnected proteins, including activin and bone morphogenetic proteins. TGF- β 1 is presently acknowledged as a potent fibrotic factor consisting of five isomers. Among mammals, there exist three distinct forms of TGF- β , specifically TGF- β 1, TGF- β 2, and TGF- β 3. Each isomer has a distinct pattern of distribution within various tissues. TGF- β 1 exhibits

predominant expression within the renal system, particularly in the kidney, where it is abundantly distributed in both the glomeruli and renal tubules (F. Sun et al., 2019).

Research has demonstrated that the TGF- β 1 signaling cascade is a driving force behind the advancement of renal fibrosis. When TGF- β 1 is activated, it attaches itself to the serine/threonine kinase receptors, known as TRI and TRII, which are found on the surface of the cell membrane. As a result of this interaction, the Smad 3 and Smad 2 proteins undergo phosphorylation. The phosphorylation of Smad3 initiates its complexation with Smad4, which then moves into the nucleus. This complex specifically activates genes involved in fibrosis. Conversely, Smad7 acts as an inhibitor of Smad proteins by blocking the phosphorylation of Smad2 and Smad3, leading to their degradation (Sierra-Mondragon et al., 2019; C. Yang et al., 2021).

One consequence of aging and dysfunction in the kidney is hyperglycemia, which appears by accumulating extra AGEs with podocyte damage and the death of mesangial cells, in addition to upregulating TGF- β 1 (Guo et al., 2020). DN is characterized by tubulointerstitial fibrosis due to the activation of TGF- β 1, promoted by excessive fat deposition in the kidney (W. Zheng et al., 2021). TGF- β 1 enhances the production of various extracellular matrix elements, including collagen, and concurrently hinders their breakdown. As a result, this process contributes to the mesangial matrix's expansion and the glomeruli's hypertrophy (Mohammad et al., 2023).

Moreover, a contemporary investigation has revealed that TGF- β 1 has the capability to trigger the PI3K-Akt signaling cascade. This PI3K-Akt pathway plays an essential role in various biological functions, including cell proliferation, differentiation, programmed cell death, tumor development, and the progression of the cell cycle (Ni et al., 2020). Research has demonstrated that TGF- β 1 and p38 MAPK significantly elevate apoptosis rates in renal tubular epithelial cells, podocytes, and glomeruli. The production of TGF- β 1 and p38 MAPK is triggered by ROS, which are generated due to oxidative stress and the apoptotic processes in DN (Y. Wang et al., 2022).

TGF- β 1 initiates the process of fibrogenesis and contributes to the progression of DKD by accumulating in the extracellular matrix and expanding glomerular mesangial cells. As earlier, elevated levels of TGF- β 1 in both their serum and urine specimens in

patients with T2DM were studied. Significantly, it was highest in the macroalbuminuria subgroup compared to the microalbuminuria and normoalbuminuria subgroups (Swaminathan et al., 2023).

Activating the mTOR signaling pathway leads to higher levels of profibrotic cytokines, including TGF- β 1 and connective tissue growth factor. This activation also promotes the onset of interstitial fibrosis in DKD (Yasuda-Yamahara et al., 2021). In the early stages of diabetes-related nephropathy, TGF- β 1 is believed to be a sensitive biomarker (J. Zhang et al., 2018). TGF- β 1, widely accepted as an essential promoter of DN, is vital for aberrant signaling in DN. TGF- β 1 signaling dysfunction regulates glucose flow into renal cells by upregulating GLUT-1 (Magee et al., 2017).

2.22.4 Glucose-Regulated Protein 78 (GRP78)

ERS corresponds to physiological or pathological conditions that might lead to an enhanced requirement for protein folding or interfere with the mechanisms involved. As a result, proteins begin to fold improperly within the ER lumen. ER possesses a quality control system that encompasses the UPR and ERAD to facilitate the recovery of misfolded proteins (Cybulsky, 2010). The ER luminal chaperone GRP78 makes detecting stress within the ER easier. When misfolded proteins build up in the ER lumen, GRP78 breaks apart and stimulates three ER transmembrane proteins that are important for sending stress signals (Barati et al., 2016).

IRE1 α , pancreatic eIF2 kinase, and ATF6 are ER-resident sensors regulating ERS. These sensors are liberated from the ER chaperone-binding immunoglobulin protein (BiP), also called 78 kDa GRP78, and activated in response to ER stress. Chronic ER stress induces a transition from adaptive mechanisms promoting cell survival to a response favoring PCD via various signaling pathways (H. Xie et al., 2022). It turns on the growth-inhibiting and DNA-damage-inducible gene 153 (CHOP). This activation of CHOP acts as a significant indicator of ERS (S. Xu et al., 2020).

During ERS, the protein known as GRP78 interacts with unfolded or misfolded proteins, leading to its dissociation from protein kinase R-like ER kinase (PERK). When GRP78 is no longer attached to PERK, it is easier to be trans phosphorylated, which turns

on transcript factor 4 (ATF4). ATF4 subsequently triggers the transcription of specific genes linked to the UPR, among which is the C/EBP homologous protein (CHOP). Following this, CHOP plays a crucial role in balancing pro-apoptotic and anti-apoptotic BCL-2 family proteins, which in turn activates caspases (Hartner et al., 2014; Ju et al., 2019).

The PI3K/AKT pathway influences various cellular functions, including growth, differentiation, programmed cell death, aging, and protection against oxidative stress. Research by Kasinath et al. revealed heightened PI3K/AKT activity in the renal cortex of a DN model. This observation underscores the crucial roles of both ERS and PI3K/AKT signaling in the onset and advancement of DN (Deng et al., 2022). GRP78, an ER luminal protein produced in response to ER stress, serves a distinctive protective function by preventing cell death induced by ERS (N. Ma et al., 2021). If the adaptive responses fail to reduce ERS, apoptosis is initiated by many mechanisms that remain incompletely elucidated (Y. Cao et al., 2014).

ER is a fundamental intracellular organelle responsible for regulating Ca^{2+} homeostasis, protein synthesis, and folding. The regulation of Ca^{2+} permeable channels immediately affects intracellular Ca^{2+} levels. It affects several Ca^{2+} -dependent cellular processes, such as cell division, ERS, apoptosis, and autophagy. ER stress is a disease that causes the redox balance to be thrown off and the luminal Ca^{2+} regulation to break down. Additionally, it causes a buildup of unfolded or wrongly folded proteins. UPR causes the upregulation of GRP78, an ER chaperone, which makes the ER better at folding proteins (Bektur Aykanat et al., 2021).

Throughout the progression of T2DM, lipotoxicity is observed, which leads to an accumulation of lipids within pancreatic beta cells. As a result of this accumulation of lipids, there is a reduction in the activity of genes associated with autophagy (ARGs), leading to disruption in the regular functioning of autophagy or the progression of autophagic processes. Cavener et al. conducted a study whereby they observed that the downregulation of EIF2AK3 in tissue or cells led to the manifestation of diabetes in a significant manner. Furthermore, EIF2AK3 has been identified as a protein associated with the ER, and its involvement in beta-cell death has been acknowledged (K. Cui & Li, 2023).

AGEs can activate receptors known as RAGE, which then triggers oxidative stress and impairs cellular function. This activation in the renal system also leads to oxidative stress, ERS, inflammation, and fibrotic reactions. These processes involve the stimulation of various intracellular signaling pathways like NF- κ B, MAPK/ERK, and PI3K/Akt. These pathways contribute to kidney function and structural impairment and accelerate aging (Guo et al., 2020). AGE plays a crucial role in the progression of DN. They can increase the expression of GRP78 and trigger ERS, which depends on the dosage and duration of exposure. Ultimately, this process leads to the apoptosis of podocytes (Dai et al., 2017).

ERS displays adaptability by helping cells make functional proteins and protecting them from high levels of calcium ions in the cytosol, oxidative stress, and cell death. Recent significant findings establish a connection between chronic ER stress and the development of fibrotic diseases. ERS can potentially enhance TGF- β signaling, while substances that decrease ERS can mitigate fibrosis. ER stress has been shown to affect both the epithelial and fibroblast compartments in the kidney. It can cause apoptosis, a change to the mesenchymal phenotype, and changes in how the tubular epithelium makes the ECM. The progression of CKD is increased via tubular damage (Lu et al., 2020).

2.22.5 B-cell lymphoma-2 (BCL-2)

During the progression of DN, several factors have the potential to activate apoptosis, leading to an imbalance between apoptosis and cell proliferation. Consequently, this imbalance results in an increased apoptotic rate of renal cells and subsequent renal dysfunction (Ju et al., 2019). Cellular apoptosis can be identified through the assessment of BCL-2 and BCL-2-associated X protein (BAX). BAX functions as a proapoptotic protein, while BCL-2 acts as an anti-apoptotic protein. Research has shown that activation of the p38 MAPK pathway by AGE, ROS, or similar triggers enhances BAX levels and reduces BCL-2 levels. Therefore, this pathway plays a pivotal role in mediating apoptosis (Tang et al., 2021).

When apoptotic factors intracellularly release BCL-2 family proteins, they can be moved to the mitochondria. Additionally, it stimulates programmed cell death, like

caspase 3 (He et al., 2020). BCL-2, a critical mediator, plays a significant function in autophagy and apoptosis counter-regulation. It interacts with beclin-1, a protein that promotes autophagy, affecting cellular decisions between apoptosis and autophagy. Yet, its exact function in regulating both apoptosis and autophagy is still unclear (Hamzawy et al., 2017).

The BCL-2 family plays crucial roles in apoptosis, encompassing BCL-2 as an anti-apoptotic gene and Bax as a pro-apoptotic gene, exerting pivotal roles in apoptotic signaling pathways. The upregulation of Bax facilitates the process of apoptosis, while the upregulation of BCL-2 blocks apoptosis (Ju et al., 2019). The involvement of BCL-2 in podocyte death and ECM deposition contributes to the progression of glomerulosclerosis. The process of cellular apoptosis was observed to be enhanced in mice with a DN model, along with a decrease in the expression of BCL-2 (Maiti, 2021).

2.22.6 Podocin (PDCN)

Podocytes are pivotal in regulating urinary protein loss by creating and upholding foot structures along with interposed slit-diaphragms. Podocytes play a crucial function in DN. Several factors, including the inflammatory response and oxidative stress, play roles in the development of podocyte abnormalities in diabetic patients. The presence of these anomalies impairs the ability of the podocytes to perform self-repair and reproduction. In addition, it has been observed that diabetes can lead to the separation, hypertrophy, and loss of podocyte foot processes (B. Wei et al., 2020).

DN is a renal microvascular complication that arises as a result of diabetes. It serves as the primary catalyst for the development of CKD and can lead to renal failure. DN is marked by persistent proteinuria and a progressive decline in kidney function, indicating the onset of classic glomerular disease. Podocytes, specialized glomerular epithelial cells essential for maintaining the integrity of the GFB, play a pivotal role in this process. The inadequate reparative and regenerative abilities of podocytes contribute significantly to the prognosis of DN progression (Ye et al., 2023).

The presence of podocytes is crucial for ensuring the specific operation of the GFB. Podocytes are specialized cells in the Bowman's capsule, forming a lining and encircling the capillaries. The occurrence of podocyte damage frequently leads to the manifestation of significant proteinuria (X. Xu et al., 2020). Since podocytes do not reproduce, it is crucial that their internal degradation mechanism function effectively to maintain their balance (Dong et al., 2019). Podocyte injury manifests as the withdrawal of their foot processes, detachment from the glomerular basement membrane, and irregular expression of essential proteins such as nephrin and podocin. These changes cause a range of structural abnormalities in the kidneys, including thickened glomerular basement membranes, expanded mesangial areas, and the development of glomerulosclerosis (X. Cao et al., 2019; F. Gao et al., 2018).

Presently, there is an increasing amount of empirical data suggesting that the malfunctioning of autophagy is responsible for the podocyte lesion. The ability of podocytes to undergo autophagy is significantly compromised in patients with T2DM, which plays a role in the severity of kidney damage (X. Zhang et al., 2023). The hallmark of DKD is structural damage to the glomeruli, particularly the podocytes, during the early stages. AGEs can trigger podocyte cell cycle arrest, enlargement, and apoptosis (Zhuang et al., 2021).

There is a significant connection between the damage to podocytes and the onset of glomerular sclerosis, elevated protein levels in urine, and decreased kidney function. These factors play a crucial role in the advancement of DN (H. Feng et al., 2021). Furthermore, the consequence of podocyte injury involves a decrease of podocytes inside the glomerulus, an abnormality that can be associated with apoptosis triggered by hyperglycemia. This process ultimately results in the manifestation of proteinuria and glomerular sclerosis (Q. Wang et al., 2020).

This study aims to evaluate autophagy to enhance our understanding of its association with renal function and nephropathy, a common complication of diabetes. Additionally, we aim to explore the potential use of autophagic biomarkers in assisting the diagnosis and monitoring of DN. Specifically, we investigated the utility of serum levels of LC3-II, GRP78, BCL-2, Cystatin C, IL-1 β , TGF- β 1, and Podocin for diagnosing DN, assessing their correlation with kidney function, and examining their longitudinal

changes in type 2 diabetics with renal impairment. Moreover, we used LC3-II as an autophagy biomarker, GRP-78 ER as a stress indicator, IL-1 as an inflammatory marker, TGF- β 1 to assess renal fibrosis, BCL-2 as an anti-apoptotic marker, Podocin as a podocyte damage indicator, and Cystatin C, eGFR, and UACR to stage renal failure in these patients. We also analyzed UACR values to classify normoalbuminuria, microalbuminuria, and macroalbuminuria.



3 MATERIALS AND METHODS

3.1 Selection of Patients and Formation of Study Groups

All participants attended the polyclinics of Internal Medicine at Tokat Gaziosmanpaşa University Hospital from September 2022 to April 2023. A total of 176 (86 female, 90 male) between the ages of 42 and 85 years, Turkish subjects were enrolled, of whom 26 were healthy, and 150 had type 2 diabetes mellitus. Under the light of American Diabetes Association criteria, we selected 150 patients and introduced them as type 2 DM (Olson et al., 2010). The inclusion criteria were being diagnosed with T2DM and continuing to use antidiabetic medications. Patients who were excluded from the study included those with type 1 diabetes, previously diagnosed kidney issues, cancer, pregnancy, or acute or chronic infections. The control group was randomly selected from people without any disease or medications.

Patients were classified according to urine albumin creatinine ratio (ACR): 88 patients had macroalbuminuria (ACR > 300 mg/g), 20 patients had microalbuminuria (ACR between 30 and 300 mg/g), and 42 had normoalbuminuria (ACR < 30 mg/g). According to eGFR values, T2DM patients were divided into six categories [standard to high (G1), slightly decreased (G2), slightly to moderately decreased (G3a), moderate to severely decreased (G3b), severely decreased (G4), and renal failure (G5)]. Proteinuria severity and eGFR stages were evaluated together and divided into three groups (Stage 1: Normoalbuminuria + G1/G2, Stage 2: Microalbuminuria + G3a/G3b, Stage 3: Macroalbuminuria + G5/G4). $ACR (mg/g) = \text{microalbumin in urine (mg/l)} / [\text{creatinine in urine (mg/dl)} / 100]$. $TPCR (mg/mg) = [\text{total protein in the urine (mg/l)} / 10] / \text{creatinine in urine (mg/dl)}$. eGFR was calculated by CrCl (mL/min) by Cockcroft–Gault formula with considering age, height, sex, and serum creatinine levels (Cockcroft & Gault, 1976).

LC3-II, GRP78, BCL-2, podocin, IL-1 β , and TGF- β 1 levels were comparatively analyzed. All individuals' body weight and height were measured during the recruitment process. Body weight in (kilograms (kg)/height in meters (m²)) was introduced as the simplest measurement method for Body mass index (BMI) (Keys et al., 1972). Body weight and height were measured using standard procedures between 8 and 10 a.m. after an overnight fast. The questionnaire part of the study was composed of Age, Sex, Smoking history, Lifestyle, history of Hypertension, and time since diagnosis of type 2

DM (duration of type 2 DM). Clinical data such as dyspnea, chest pain, abdominal pain, fever, edema, polyurea, and anorexia were collected about the patient groups. The ethical committee and clinical research board of Tokat Gaziosmanpaşa University granted approval for the study on (09.09, 2022), under the reference number (2022-37/51). The study adhered to the principles outlined in the Helsinki Declaration. Each participant provided informed consent prior to their involvement in the research.

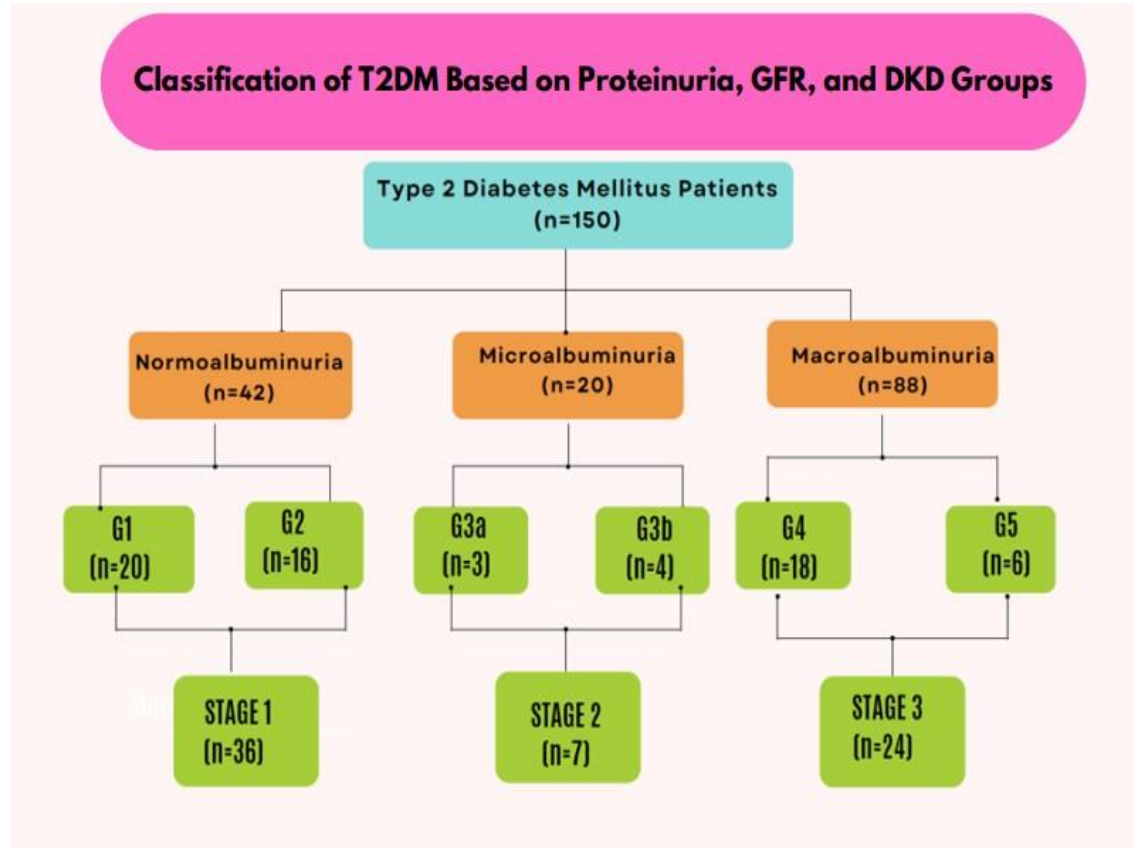


Figure 3.1: Graphical abstract

A total of 176 Turkish subjects were enrolled, of whom 26 were healthy, and 150 had type 2 diabetes mellitus. Under the light of American Diabetes Association criteria, we selected 150 patients and introduced them as type 2 DM. Patients were classified according to albumin urea ratio: 88 patients had macroalbuminuria (ACR > 300 mg/g), 20 patients had microalbuminuria (ACR between 30 and 300 mg/g), and 42 had normoalbuminuria (ACR < 30 mg/g). According to eGFR values, T2DM patients were divided into six categories [standard to high (G1), slightly decreased (G2), slightly to moderately decreased (G3a), moderate to severely decreased (G3b), severely decreased (G4), and renal failure (G5)]. Proteinuria severity and eGFR stages were evaluated together and divided into three groups (Stage 1: Normoalbuminuria+G1/G2, Stage 2: Microalbuminuria+G3a/G3b, Stage 3: Macroalbuminuria+G5/G4).

3.2 Laboratory analysis

Blood was sampled by vein puncture of antecubital veins. After 12-h dating (overnight fasting) in the morning, venous blood was taken via a straight needle attached to a tube holder loaded with the plastic 10ml Serum gel and clot activator tube, filled PET 5ml CBC tube with EDTA K2/K3 additive and filled 5ml HbA1c tube with EDTA additive respectively. First, the samples were kept at room temperature for five minutes, then centrifuged at 3000 rpm for 20 minutes. The supernatant was divided into two parts. One part was used to measure Serum Glucose, TG, LDL, HDL, Albumin, Urea, and Creatinine concentrations, following the Cobas c 6000 manufacturer's guidelines. Additionally, 3ml of serum was placed in an Eppendorf tube for further study and stored in a deep freezer at -80°C until the day of the experiment. After blood collection, levels of TGF- β 1, IL-1 β , CYS-C, GRP78, BCL-2, LC3-II, and PDCN in the serum samples will be analyzed using commercially available ELISA kits. Urine samples were collected randomly in a plastic round-bottom 10.0mL tube without additives, and measurements of Urine-Glucose, Urine-Protein, Urine-albumin, and Urine-creatinine concentrations were performed promptly according to the Cobas c 6000 manufacturer's guidelines.

Concentrations of Urine-Glucose, Urine-Protein, Urine-albumin, and Urine-creatinine were measured manually via Cobas c6000-core, Roche Glucose HK Gen.3, GLUC3, Cobas integra/cobas c systems REF.04404483190, ID.0768316, LotNo: 71884001 Cobas c Kit, Germany, and Roche Total Protein Urine Gen.3, TPUC3, Cobas integra/cobas c systems REF.03333825190, ID.0767638, LotNo: 69927101 Cobas c Kit, Germany, and Roche Tina-quant Albumin Gen.2, ALBT2, Cobas integra/cobas c systems REF.04469658190, ID.0767433, LotNo: 68768501 Cobas c Kit, Germany, and Roche Creatinine Jaffe Gen.2, CREJ2, Cobas integra/cobas c systems REF.04810716190, ID.0769282, LotNo: 72593401 Cobas c Kit, Germany. Concentrations of Serum Glucose, Albumin, TG, LDL, HDL, Urea, and Creatinine were measured manually via Cobas c6000-core, Roche Glucose HK Gen.3, GLUC3, Cobas integra/cobas c systems REF.04404483190, ID.0768316, LotNo: 71884001 Cobas c Kit, Germany, and Roche Albumin Gen.2, ALB2, Cobas integra/cobas c systems REF.03183688122, ID.0765929, LotNo: 72570901 Cobas c Kit, Germany, Roche Triglycerides, TRIGL, Cobas integra/cobas c systems REF.20767107322, ID.0767107, LotNo: 72472501 Cobas c Kit, Germany, and, and Roche LDL-Cholesterol Gen.3, LDLC3, Cobas integra/cobas c

systems REF.007005717190, ID.075657, LotNo: 68807401 Cobas c Kit, Germany, and, and Roche HDL-Cholesterol Gen.4, HDLC4, Cobas integra/cobas c systems REF.07528566190, ID.075894, LotNo: 65300701 Cobas c Kit, Germany, and Roche Urea/BUN, UREAL, Cobas integra/cobas c systems REF.04460715190, ID.0763039, LotNo: 73051101 Cobas c Kit, Germany, and Roche Creatinine Jaffe Gen.2, CREJ2, Cobas integra/cobas c systems REF.04810716190, ID.0769282, LotNo: 72593401 Cobas c Kit, Germany. The concentration of HbA1c was measured manually via Cobas c6000-core, Roche Tina-quant Hemoglobin A1c Gen.3, A1C-3, Cobas integra/cobas c systems REF.05336163190, ID.0774553, LotNo: 70879701 Cobas c Kit, Germany. CBC concentrations were automated via Sysmex, XN-1000, Fluorocell, WNR, and REF.CP-066-715, LotNo: D3009 Sysmex Kit, Fluorocell WDF, REF.CV-377-552, AA-325-279, LotNo: D3012, 1-5-1-Wakinohama-Kaigandori Chuo-ku, Kobe 651-0073, Japan.

Concentrations of TGF- β 1, IL-1 β , CYS-C, GRP78, BCL-2, LC3-II, and PDCN were measured manually via a sandwich enzyme-linked immunosorbent assay (ELISA), Bioassay Technology Laboratory Human Transforming Growth Factor β 1, TGF- β 1 ELISA Kit; Shanghai YL Biotech Cat.No.E0134Hu, LotNo:202303008, Shanghai, China, and Bioassay Technology Laboratory Human Interleukin 1 β , IL-1 β ELISA Kit; Shanghai YL Biotech Cat.No.E0143Hu, LotNo:202303008, Shanghai, China, and Bioassay Technology Laboratory Human Cystatin C, CYS-C ELISA Kit; Shanghai YL Biotech Cat.No.E1104Hu, LotNo:202303008, Shanghai, China, and Bioassay Technology Laboratory Human Glucose Regulated Protein 78, GRP78 ELISA Kit; Shanghai YL Biotech Cat.No.E3624Hu, LotNo:202303008, Shanghai, China, and Bioassay Technology Laboratory Human BCL-2-like Protein 1, BCLX ELISA Kit; Shanghai YL Biotech Cat.No.E4035Hu, LotNo:202303008, Shanghai, China, and Bioassay Technology Laboratory Human Microtubule-associated Proteins 1A/1B Light Chain 3B, MAP1LC3B ELISA Kit; Shanghai YL Biotech Cat.No.E4774Hu, LotNo:202303008, Shanghai, China, and Bioassay Technology Laboratory Human Podocin, PDCN ELISA Kit; Shanghai YL Biotech Cat.No.E5893Hu, LotNo:202303008, Shanghai, China.

The optical density was read using the EPOCH 2 (Bio Tek Instruments, Winooski, USA) ELISA plate reader at the 450 nm wavelength. Values are represented in ng/ml or pg/ml. The sensitivity was 5.11 ng/L, and the lower limit of detection for TGF- β 1 was 10 ng/L. The manufacturer reported that the coefficient of variation (CV%) was 5.5%, and

the inter-assay CV% value was < 10%. The sensitivity was 10.07 pg/mL, the lower limit of detection for IL-1 β was 20 pg/mL. The manufacturer reported that the coefficient of variation (CV%) was 4.9%, and the inter-assay CV% value was < 10%. The sensitivity was 0.02 mg/dL, and the lower limit of detection for cystatin C was 0.05mg/dL. The manufacturer reported that the coefficient of variation (CV%) was 4.3%, and the inter-assay CV% value was < 10%. The sensitivity was 0.23 ng/mL, and the lower limit of detection for human GRP78 was 0.5 ng/mL. The manufacturer reported that the coefficient of variation (CV%) was 8% and the inter-assay CV% value was < 10%. The sensitivity was 0.13 ng/mL, and the lower limit of detection for human BCL-2 was 0.3 ng/mL. The manufacturer reported that the coefficient of variation (CV%) was 5% and the inter-assay CV% value was < 10%. The sensitivity was 5.28 ng/L, and the lower limit of detection for LC3-II was 10 ng/L. The manufacturer reported that the coefficient of variation (CV%) was 5.3% and the inter-assay CV% value was < 10%. The sensitivity was 0.056 ng/mL, and the lower limit of detection for human podocin, was 0.1 ng/mL. The manufacturer reported that the coefficient of variation (CV%) was 5.6% and the inter-assay CV% value was < 10%. 96-well ELISA plates coated with specific capture antibodies (against TGF- β 1, IL-1 β , CYS-C, GRP78, BCL-2, LC3-II, and PDCN) were utilized. The immobilized antibody in standard solutions binds the target protein in a sample to the wells, and samples are pipetted into the wells. A biotinylated detection antibody that is particular to the target protein is added. Then streptavidin linked to horseradish peroxidase (HRP) is pipetted into the wells. After removing the unbound biotinylated antibody following five rounds of washing, a 3,3',5,5'-tetramethylbenzidine (TMB) substrate was introduced into the wells. The colour changed based on the amount of bound target protein. The stop solution turned colour from blue to yellow, and a microplate reader at 450 nm measured the absorbance in each well. ELISA was performed according to manufacturer's protocol.

3.3 Tools and Equipment

Table 3.3.1: Devices Used

Oven	Memmert INB 500
Incubator	Nuaire AutoFlow NU-4750
Centrifugal	Bench-top, NF800
Cooler (+4)	UGUR USS 440 MIN
Deep Freeze (-80)	NU-9483E
Microplate Reader	Organon Teknika Microwell System Auction (0024-3022309), Grays Australia
Cobas c	Cobas c6000-Core
Sysmex	Sysmex, XN-1000
Vortex	Velp Scientifica Wizard X Vortex Mixer F202A0125 w/ Sensor
Handy step micropipette	Touch
Handy step micropipette	Electronic Repetitive Pipet
Orbital shaker laboratory	IKA, Model: KS-130-B
Water Distillated (KROS)	KRS-R-250

Table 3.3.2: Chemicals Used

GLUC3	Roche, Cobas c6000 Kit
TPUC3	Roche, Cobas c6000 Kit
ALBT2	Roche, Cobas c6000 Kit
CREJ2	Roche, Cobas c6000 Kit
A1C-3	Roche, Cobas c6000 Kit
TRGIL	Roche, Cobas c6000 Kit
LDLC3	Roche, Cobas c6000 Kit
HDLC3	Roche, Cobas c6000 Kit
ALB2	Roche, Cobas c6000 Kit
UREAL	Roche, Cobas c6000 Kit
WNR	Sysmex, XN-1000 Kit
WDF	Sysmex, XN-1000 Kit
Human Transforming Growth Factor- β 1, TGF- β 1 ELISA Kit	Bioassay Technology Laboratory
Human Interleukin-1 β , IL-1 β ELISA Kit	Bioassay Technology Laboratory
Human Cystatin C, CYS-C ELISA Kit	Bioassay Technology Laboratory
Human Glucose Regulated Protein 78 ELISA Kit	Bioassay Technology Laboratory
Human BCL-2-like Protein 1, BCLX (BCL-2) ELISA kit	Bioassay Technology Laboratory
Human Microtubule-associated Proteins 1A/1B Light Chain 3B, MAP1LC3B (LC3-II) ELISA kit	Bioassay Technology Laboratory
Human Podocin, PDCN ELISA kit	Bioassay Technology Laboratory

3.4 Methods

Measurement of Human Serum and Urine Glucose, GLUC3 by Cobas c 6000 method

Hexokinase acts as an enzymatic benchmark. ATP facilitates the phosphorylation of glucose into glucose-6-phosphate through hexokinase. When NADP is present, glucose-6-phosphate dehydrogenase converts glucose-6-phosphate to gluconate-6-phosphate. Unlike other carbohydrates, this process excludes oxidation. The photometric measurement of NADPH production rate directly correlates with glucose concentration.

3.4.1 Measurement of Human Total Protein Urine TPUC3 by Cobas c 6000 method

Turbidimetric techniques employing trichloroacetic acid (TCA) or sulfosalicylic acid (SSA) precipitate proteins based on their size, yielding unstable turbidity prone to flocculation. Dye-binding agents such as Coomassie blue and pyrogallol red molybdate interact with proteins according to their amino acid composition, albeit causing potential staining of glass and plasticware. Both turbidimetric and colorimetric approaches exhibit varying sensitivities to different proteins due to their unique response mechanisms, especially towards protein fragments like Bence Jones proteins 2 and small molecules such as 1 microglobulin. To prepare the sample, it undergoes preincubation in an alkaline EDTA solution, which denatures proteins and eliminates magnesium ion interference. Introducing benzethonium chloride subsequently induces turbidity.

3.4.2 Measurement of Human Albumin ALBT2 by Cobas c 6000 method

In an immunoturbidimetric assay, antibodies against albumin interact with the antigen present in the sample, forming antigen/antibody complexes. These complexes are subsequently evaluated turbidimetrically following agglutination.

3.4.3 Measurement of Human Serum and Urine Creatinine Jaffe, CREJ2 by Cobas c 6000 method

The Jaffé method employs a kinetic colorimetric test, where creatine reacts with picrate under alkaline conditions, forming a yellow-orange compound. The rate at which the dye forms correlates directly with the concentration of creatinine in the sample. To minimize interference from substances like bilirubin, the assay utilizes a "rate-blanking" technique. Additionally, to correct for non-specific reactions caused by pseudo-creatinine chromogens in serum or plasma (such as proteins and ketones), a correction factor of 26 mol/L (0.3 mg/dL) is applied to the serum or plasma values.

3.4.4 Measurement of Human Serum Albumin ALB2 by Cobas c 6000 method

Colorimetric assay: At a pH of 4.1, albumin exhibits a strong positive charge that allows it to interact with bromocresol green (BCG), which is a negatively charged dye, resulting in the formation of a complex that is blue-green. The photometric measurement of the blue-green color's strength is directly related to the concentration of albumin in the specimen.

3.4.5 Measurement of Human Serum Triglycerides, TRIGL by Cobas c 6000 method

Eggstein and Kreutz originally required potassium hydroxide for their enzymatic triglyceride assay, involving saponification. Attempts were made to replace alkaline saponification with lipase-driven enzymatic hydrolysis. Bucolo and David utilized a mixture of lipase and protease for hydrolysis, while Wahlefeld employed a liver esterase alongside a potent lipase from *Rhizopus arrhizus*. Wahlefeld's method, pivotal in the field, involved using a lipoprotein lipase from microorganisms to hydrolyze triglycerides into glycerol. Subsequent oxidation yielded dihydroxyacetone phosphate and hydrogen peroxide, catalyzed by peroxidase. This hydrogen peroxide then reacted with 4-aminophenazone and 4-chlorophenol to produce a red dyestuff in the Trinder endpoint reaction. The measurement of the color intensity of this dyestuff by photometry directly correlates with the concentration of triglycerides.

3.4.6 Measurement of Human Serum LDL-Cholesterol, LDL C3 by Cobas c 6000 method

The enzymatic technique for assessing cholesterol esters and free cholesterol in LDL leverages the action of cholesterol esterase and cholesterol oxidase, along with surfactants that specifically solubilize LDL. These surfactants, coupled with a sugar molecule, inhibit enzymatic reactions with lipoproteins other than LDL. Consequently, cholesterol in HDL, VLDL, and chylomicrons is not measured. The generated hydrogen peroxide reacts with 4-aminoantipyrine and EMSE in the presence of peroxidase, producing a red-purple hue. The intensity of this color, measured photometrically, correlates directly with the cholesterol concentration.

3.4.7 Measurement of Human Serum HDL-Cholesterol, HDL C4 by Cobas c 6000 method

Non-HDL lipoproteins, which include LDL, VLDL, and chylomicrons, form a water-soluble complex when combined with polyanions and a detergent. This mixture prevents CHER and CHOD enzymes from acting on non-HDL lipoproteins. As a result, only HDL particles can interact with CHER and CHOD. These enzymes are used to measure HDL cholesterol levels: CHER breaks down cholesterol esters into free cholesterol and fatty acids, while the hydrogen peroxide produced then reacts with 4-aminoantipyrine and EMSE, in the presence of peroxidase, to create a colored compound. The intensity of this color is measured photometrically and is directly proportional to the cholesterol concentration.

3.4.8 Measurement of Human Serum Urea/BUN, UREA by Cobas c 6000 method

Kinetic analysis leverages the enzymes urease and glutamate dehydrogenase (GLDH). Urease catalyzes the breakdown of urea, resulting in the formation of ammonium and carbonate ions. In the subsequent reaction, ammonium reacts with 2-oxoglutarate in the presence of GLDH and the coenzyme NADH to produce L-glutamate. This process oxidizes two moles of NADH to NAD⁺ for each molecule of urea

hydrolyzed. The reduction in NADH concentration is measured photometrically and correlates directly with the urea content in the samples.

3.4.9 Measurement of Human Hemoglobin A1c, HbA1c by Cobas c 6000 method

The blood sample is diluted and mixed with TRIS buffer to remove hemoglobin from the red blood cells. A portion of the sample is placed into a reaction chamber and combined with sodium lauryl sulfate (SLS), which forms an SLS-hemoglobin complex. The total hemoglobin concentration is determined by measuring this complex at 525 nm. Another portion of the sample has its hemoglobin A1c (HbA1c) desaturated using potassium ferricyanide and sucrose laurate. The denatured HbA1c on latex particles interacts with an HbA1c antibody. The latex agglutination inhibition reaction is triggered by reacting with a synthetic antigen that binds to the HbA1c antibody. The HbA1c concentration is measured at 625 nm during this reaction. The percentage of hemoglobin A1c is calculated by dividing the HbA1c concentration by the total hemoglobin concentration.

3.5 Measurement of CBC by Sysmex Kit method

3.5.1 Fluorocell WNR examination method

The analyzer begins by taking the total volume of the blood sample. A portion of this sample is then diluted at a 1:60 ratio and treated with the Sysmex lysing reagent Lysercell WNR. After this, Fluorocell WNR is added to the mixture, and the dilution is kept at a constant temperature to help identify nucleated cells in the sample. The processed sample is then placed into a sheath flow detector, which uses forward scattered light and side fluorescence measurements to determine the white blood cell count (WBC#). Additionally, this process calculates the nucleated red blood cell count (NRBC#), basophil count (BASO#), nucleated red blood cell percentage (NRBC%), and basophil percentage (BASO%).

3.5.2 Fluorocell WDF examination method

The analyzer first collects a complete blood sample. A portion of this sample is then diluted to a ratio of 1:60 and lysed using either the Sysmex lysing reagent Lysercell WDF II* or Lysercell WDF. After lysis, Fluorocell WDF is added to the diluted sample, which is then kept at a stable temperature to identify nucleated cells. This prepared sample is subsequently passed through the sheath flow detector, which uses side scattered light and side fluorescence measurements to determine the WBC differential.

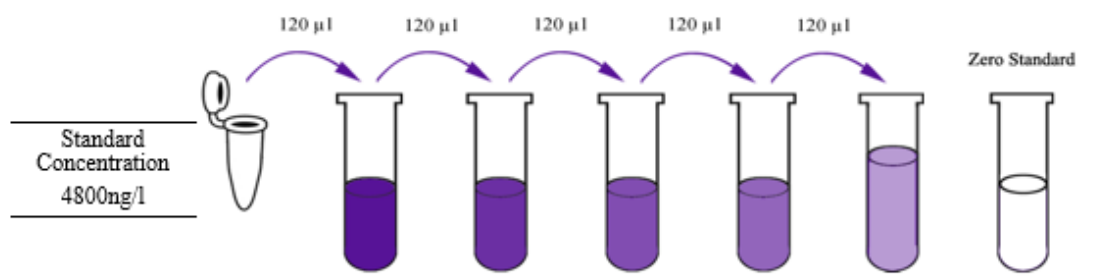
3.6 Measurement of Human Transforming Growth Factor - β 1, TGF- β 1 by ELISA method

Bioassays Laboratory Technology for the Human Transforming Growth Factor- β 1, TGF- β 1 sandwich kit (Cat. No. E0134Hu) was utilized to measure the TGF- β 1 level using the ELISA technique. First, a standard solution was made for this purpose per kit procedure. In the table below, standard solutions and dilution ratios are shown.

3.6.1 Principles of TGF- β 1 test

The kit is used to perform an ELISA (enzyme-linked immunosorbent assay). In this procedure, the plate is pre-coated with an antibody specific to human TGF- β 1. When the sample is introduced, the TGF- β 1 in it binds to these pre-coated antibodies. Next, a biotinylated antibody against human TGF- β 1 is added, which attaches to the TGF- β 1 in the sample. This is followed by the addition of streptavidin-HRP, which binds to the biotinylated antibody. After an incubation period, any unbound streptavidin-HRP is washed away. A substrate solution is then added, causing a color change that correlates with the concentration of TGF- β 1. The reaction is stopped by adding an acidic stop solution, and the absorbance is read at 450 nm.

Table 3.7: Preparation of TGF- β 1 Standards



Standard Diluent	120 μ l	120 μ l	120 μ l	120 μ l	120 μ l	120 μ l
Standard Concentration	2400ng/l	1200ng/l	600ng/l	300ng/l	150ng/l	0ng/l
Standard Numbers	5	4	3	2	1	Blank

3.6.2 TGF- β 1 Test Procedure

1. Reagents, standard solutions, and samples were prepared according to the instructions. All reagents were brought to room temperature prior to use, and the experiment was conducted at room temperature.
2. A volume of 50 μ l of the standard solution, which already contained biotinylated antibodies, was added to the standard well, eliminating the need for additional antibodies.
3. In the sample wells, 40 μ l of the sample, 10 μ l of human TGF- β 1 antibody, and 50 μ l of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.
4. After incubation, the parafilm was removed, and the plate was washed five times with a wash buffer. Each well was soaked in 300 μ l of wash buffer for 30 seconds to 1 minute per wash and then blotted onto paper towels.
5. Following the washes, 50 μ l of substrate solution A and 50 μ l of substrate solution B were added to each well. The plate was covered with fresh parafilm and incubated in the dark at 37°C for 10 minutes.
6. The blue color instantly became yellow when each well-received 50 μ l of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

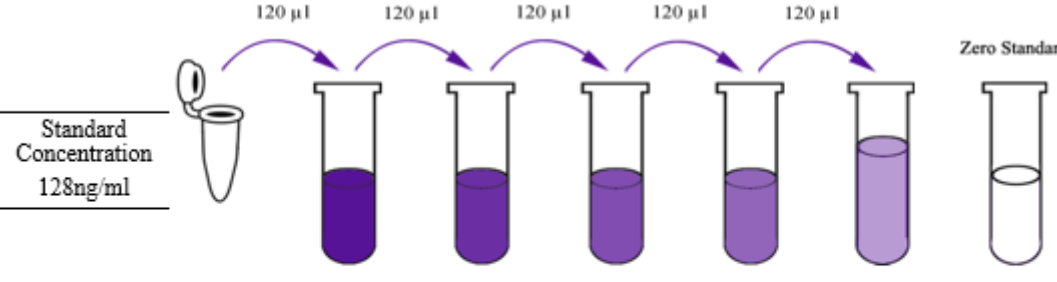
3.7 Measurement of Human Glucose Regulated Protein 78 by ELISA method

Bioassays Laboratory Technology for the Human (GRP78) sandwich kit (Cat. No. E3624Hu) was utilized to measure the GRP78 level using the ELISA technique. First, a standard solution was made for this purpose per the kit procedure. In the table below, standard solutions and dilution ratios are shown.

3.7.1 Principles of GRP78 test

This kit performs an ELISA or enzyme-linked immunosorbent assay. Human GRP78 antibody has been pre-coated on the plate. Upon addition of GRP78 from the specimen forms a binding interaction with the antibodies coated on the wells. Human GRP78 antibody that has been biotinylated is then added, and this antibody binds to GRP78 in the sample. The addition of streptavidin-HRP then binds the biotinylated GRP78 antibody. Unbound Streptavidin-HRP is eliminated during a washing step after incubation. Following the addition of the substrate solution, color changes according to the concentration of Human GRP78. Adding an acidic stop solution stops the process, and absorbance is measured at 450 nm.

Table 3.8: Preparation of GRP78 Standards



Standard Concentration 128ng/ml	120 µl	120 µl	120 µl	120 µl	120 µl	Zero Standard
Standard Diluent	120µl	120µl	120µl	120µl	120µl	120µl
Standard Concentration	64ng/ml	32ng/ml	16ng/ml	8ng/ml	4ng/ml	0ng/ml
Standard Numbers	5	4	3	2	1	Blank

3.7.2 GRP78 Test Procedure

1. All reagents, standard solutions, and samples were prepared according to the given instructions. They were allowed to reach room temperature before use. The experiment was conducted at room temperature.
2. 50µl standard was added to the standard well. Since the standard solution already contains biotinylated antibodies, no additional antibody was added to the standard well.
3. In the sample wells, 40µl of the sample, 10µl of human GRP78 antibody, and 50µl of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.
4. Remove the parafilm and wash the plate with a wash buffer five times. Wells were soaked in a 300µl wash buffer for 30 sec. to 1 minute per wash. It was blotted onto paper towels.
5. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. a fresh parafilm was placed over the plate and incubated for 10 min at 37 °C in the dark.
6. The blue color instantly became yellow when each well-received 50µl of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

3.8 Measurement of Human Interleukin -1 β , IL-1 β ELISA method

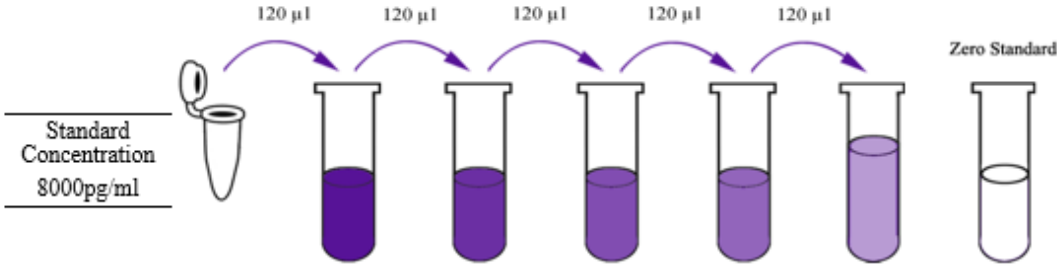
The Human Interleukin -1 β , IL-1 β sandwich kit from Bioassays Laboratory Technology (Cat. No. E0143Hu) was used to test the IL-1 β level using the ELISA method. Per the kit's instructions, a standard solution was created for this purpose. Standard solutions and dilution ratios are displayed in the table below.

3.8.1 Principles of IL-1 β test

An ELISA, or enzyme-linked immunosorbent assay, is performed using this kit. Human IL-1 β antibody has been pre-coated on the plate. The sample's IL-1 β binds to the

coated antibodies in the wells when introduced. A human IL-1 β antibody that has been biotinylated is then added, and this antibody binds to the sample's IL-1 β . The next step is to add streptavidin-HRP, which binds the biotinylated IL-1 β antibody. Unbound Streptavidin-HRP is eliminated following incubation through washing. After that, the substrate solution is added, and depending on how much human IL-1 β is present, the color changes. After stopping the process with an acidic stop solution, absorbance is measured at 450 nm.

Table 3.9: Preparation of IL-1 β Standards



Standard Diluent	120 μ l	120 μ l	120 μ l	120 μ l	120 μ l	120 μ l
Standard Concentration	4000pg/ml	2000pg/ml	1000pg/ml	500pg/ml	250pg/ml	0pg/ml
Standard Numbers	5	4	3	2	1	Blank

3.8.2 IL-1 β Test Procedure

1. All reagents, standard solutions, and samples were prepared according to the given instructions. They were allowed to reach room temperature before use. The experiment was conducted at room temperature.
2. 50 μ l standard was added to the standard well. Since the standard solution already contains biotinylated antibodies, no additional antibody was added to the standard well.
3. In the sample wells, 40 μ l of the sample, 10 μ l of human IL-1 β antibody, and 50 μ l of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.

4. Remove the parafilm and wash the plate with a wash buffer five times. Wells were soaked in a 300µl wash buffer for 30 sec. to 1 minute per wash. It was blotted onto paper towels.
5. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. a fresh parafilm was placed over the plate and incubated in the dark for 10 min at 37 °C.
6. The blue color instantly became yellow when each well-received 50µl of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

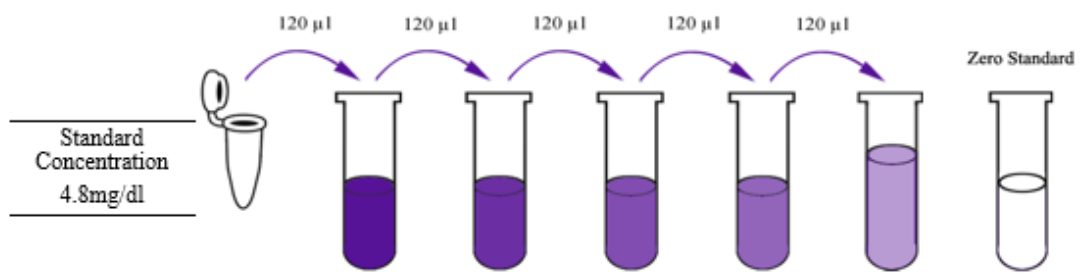
3.9 Measurement of Human Cystatin C, CYS-C ELISA method

The ELISA technique using the Human Cystatin C, CYS-C sandwich kit from Bioassays Laboratory Technology (Cat. No. E1104Hu) assessed the CYS-C level. A standard solution was first developed for this purpose by the kit's instructions. The table below shows dilution ratios and standard solutions.

3.9.1 Principles of CYS-C test

An ELISA, or enzyme-linked immunosorbent assay, is carried out using this kit. The plate has been pre-coated with a human CYS-C antibody. When introduced, the coated antibodies in the wells are bound by the sample's CYS-C. Then, a biotinylated human CYS-C antibody, which binds to the sample's CYS-C, is added. The biotinylated CYS-C antibody is then bound by streptavidin-HRP, added next. After incubation, unbound Streptavidin-HRP is removed by washing. The substrate solution is then added, and the color changes based on the quantity of human CYS-C present. At 450 nm, absorbance is measured following the application of an acidic stop solution to the process.

Table 3.10: Preparation of CYS-C Standards



Standard Diluent	120µl	120µl	120µl	120µl	120µl	120µl
Standard Concentration	2mg/dl	1mg/dl	0.5mg/dl	0.25mg/dl	0.125mg/dl	0mg/dl
Standard Numbers	5	4	3	2	1	Blank

3.9.2 CYS-C Test Procedure

1. All reagents, standard solutions, and samples were prepared according to the given instructions. They were allowed to reach room temperature before use. The experiment was conducted at room temperature.
2. No additional antibody was added to the standard well after adding the 50µl standard because the standard solution already contains biotinylated antibodies.
3. In the sample wells, 40µl of the sample, 10µl of human CYS-C antibody, and 50µl of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.
4. After removing the parafilm, the plate was washed five times with a wash buffer. Wells were soaked for 30 sec. to 1 minute in a 300µl wash buffer for each wash. It was blotted onto paper towels.
5. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. a fresh parafilm was placed over the plate and incubated for 10 min at 37 °C in the dark.
6. The blue color instantly became yellow when each well-received 50µl of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

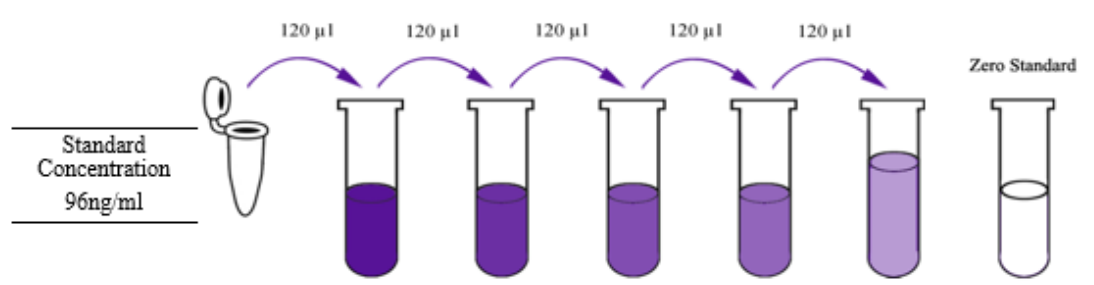
3.10 Measurement of Human BCL-2-like Protein 1, BCLX ELISA method

The Human BCL-2-like Protein 1, BCL-2 sandwich kit from Bioassays Laboratory Technology (Cat. No. E4035Hu) was used to measure the BCL-2 level using the ELISA method. For this reason, a standard solution was initially created based on the kit's instructions. Dilution ratios and standard solutions are displayed in the table below.

3.10.1 Principles of BCL-2 test

An ELISA, or enzyme-linked immunosorbent assay, is performed using this kit. The human BCL-2 antibody was pre-coated on the plate. When the sample is added, the BCL-2 in the wells binds to the coated antibodies. Then, a human Bcl-2 antibody that has been biotinylated is included, and this antibody binds to the BCL-2 in the sample. Streptavidin-HRP is subsequently added, and this binds the biotinylated BCL-2 antibody. Streptavidin-HRP that is not bound is eliminated by washing after incubation. The substrate solution is added, and the color varies depending on how much human BCL-2 is present. After the process has been stopped using an acidic solution, absorbance at 450 nm is measured.

Table 3.11: Preparation of BCL-2 Standards



Standard Diluent	120µl	120µl	120µl	120µl	120µl	120µl
Standard Concentration	48ng/ml	24ng/ml	12ng/ml	6ng/ml	3ng/ml	0ng/ml
Standard Numbers	5	4	3	2	1	Blank

3.10.2 BCL-2 Test Procedure

1. All reagents, standard solutions, and samples were prepared according to the given instructions. They were allowed to reach room temperature before use. The experiment was conducted at room temperature.
2. The standard solution already contains biotinylated antibodies, so no extra antibody was added to the standard well after adding the 50µl standard.
3. In the sample wells, 40µl of the sample, 10µl of human BCL-2 antibody, and 50µl of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.
4. After removing the parafilm, the plate was washed five times with a wash buffer. For each wash, wells were soaked for 30 sec. to 1 minute in a 300µl wash buffer. The plate was blotted with paper towels.
5. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. a fresh parafilm was placed over the plate and incubated for 10 min at 37 °C in the dark.
6. The blue color instantly became yellow when each well-received 50µl of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

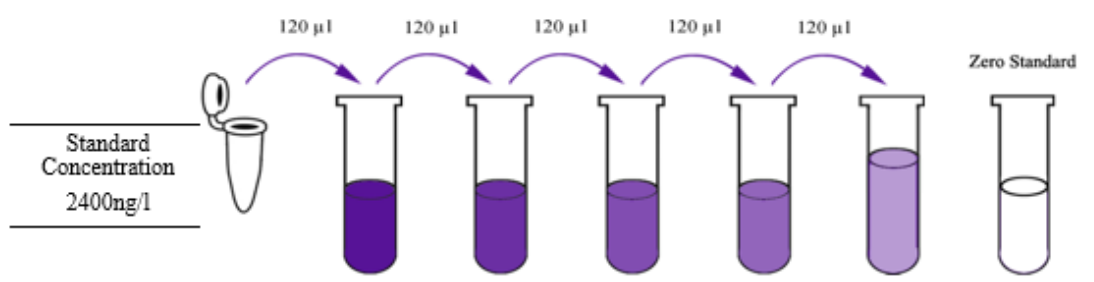
3.11 Measurement of Human Microtubule-associated Proteins 1A/1B Light Chain 3B, MAP1LC3B (LC3-II) ELISA method

The ELISA method was used to measure the LC3-II level using the Measurement of Human Microtubule-associated Proteins 1A/1B Light Chain 3B, MAP1LC3B (LC3-II) sandwich kit from Bioassays Laboratory Technology (Cat. No. E4774Hu). As a result, a standard solution was first developed based on the kit's instructions. The table below shows dilution ratios and standard solutions.

3.11.1 Principles of LC3-II test

An ELISA, or enzyme-linked immunosorbent assay, is performed with this kit. The plate has been pre-coated with human LC3-II antibodies. When a sample is put into the wells, the LC3-II binds to the coated antibodies. Then, a biotinylated human LC3-II antibody, which binds to the LC3-II in the sample, is added. Following that, streptavidin-HRP is added, which binds the biotinylated LC3-II antibody. After incubation, any unbound streptavidin-HRP is washed away. The substrate solution is then added, and the color fluctuates based on the concentration of human LC3-II. After stopping the process with an acidic solution, absorbance at 450 nm is measured.

Table 3.12: Preparation of LC3-II Standards



Standard Diluent	120µl	120µl	120µl	120µl	120µl	120µl
Standard Concentration	1200ng/l	600ng/l	300ng/l	150ng/l	75ng/l	0ng/l
Standard Numbers	5	4	3	2	1	Blank

3.11.2 LC3-II Test Procedure

1. All reagents, standard solutions, and samples were prepared according to the given instructions. They were allowed to reach room temperature before use. The experiment was conducted at room temperature.
2. Because the standard solution already contains biotinylated antibodies, no additional antibody was added to the standard well after the 50µl standard was added.
3. In the sample wells, 40µl of the sample, 10µl of human LC3-II antibody, and 50µl of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.

4. After removing the parafilm, the plate was washed five times with a wash buffer. Wells were immersed in a 300µl wash buffer for 30 sec. to 1 minute for each wash. Paper towels were used to blot the plate.
5. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. a fresh parafilm was placed over the plate and incubated for 10 min at 37 °C in the dark.
6. The blue color instantly became yellow when each well-received 50µl of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

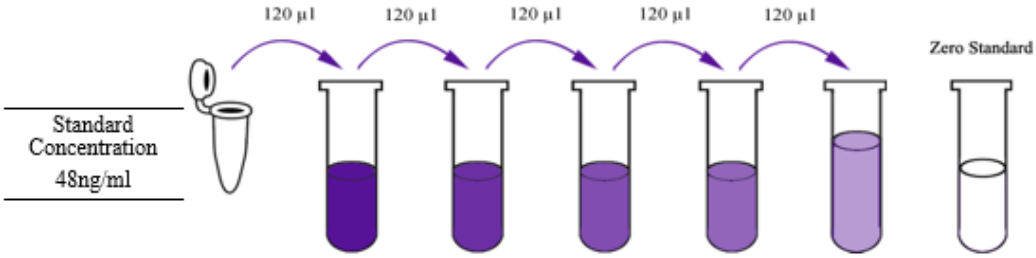
3.12 Measurement of Human Podocin, PDCN ELISA method

The ELISA method measured the PDCN level using the Bioassays Laboratory Technology Measurement of Human Podocin, PDCN sandwich kit (Cat. No. E5893Hu). As a result, a standard solution was first generated by the instructions provided within the kit. Dilution ratios and standard solutions are shown in the table below.

3.12.1 Principles of PDCN test

This kit is an ELISA (Enzyme-Linked Immunosorbent Assay). Human PDCN antibodies have been pre-coated on the plate. PDCN is introduced to the sample and binds to antibodies coated on the wells. The biotinylated Human PDCN Antibody is added to the sample and binds to PDCN. Streptavidin-HRP, which binds to the biotinylated PDCN antibody, is then added. During the washing stage after incubation, unbound Streptavidin-HRP is rinsed away. After that, the substrate solution is added, and the color develops by the amount of Human PDCN. After the process has been stopped using an acidic solution, absorbance at 450 nm is measured.

Table 3.13: Preparation of PDCN Standards



Standard Diluent	120µl	120µl	120µl	120µl	120µl	120µl
Standard Concentration	24ng/ml	12ng/ml	6ng/ml	3ng/ml	1.5ng/ml	0ng/ml
Standard Numbers	5	4	3	2	1	Blank

3.12.2 PDCN Test Procedure

1. All reagents, standard solutions, and samples were prepared according to the given instructions. They were allowed to reach room temperature before use. The experiment was conducted at room temperature.
2. The standard solution already contained biotinylated antibodies, so no extra antibody was added to the standard well after adding the 50µl standard.
3. In the sample wells, 40µl of the sample, 10µl of human PDCN antibody, and 50µl of streptavidin-HRP were thoroughly mixed without the blank control well. The plate was wrapped in parafilm and incubated at 37 °C for 60 min.
4. After removing the parafilm, the plate was washed with a wash buffer five times. For each wash, wells were immersed in a 300µl wash buffer for 30 sec. to 1 min. The plate was blotted with paper towels.
5. 50µl of substrate solution A was added to each well, followed by 50µl of substrate solution B. a fresh parafilm was placed over the plate and incubated in the dark for 10 min at 37 °C.
6. The blue color instantly became yellow when each well-received 50µl of Stop Solution.
7. The OD value for each well was computed Within 10 min. of setting up the microplate reader to 450 nm and injecting the stop solution.

3.13 Statistical analysis

The Kolmogorov-Smirnov test examined data distribution patterns. Non-parametric distributed information was displayed in median and min-max, while parametric distributed data was represented as mean $\pm$ SD. Categorical and ordinal variables were presented as numbers and percentages. Variables with typical distributions have been contrasted using a one-way ANOVA accompanied by a Tukey HSD post-hoc test, while variables with non-parametric data distributions were evaluated. Distributions were compared using the Kruskal-Wallis test and the Mann-Whitney U test. Correlations between LC3-II concentrations were analyzed by Spearman correlation analysis. A correlation coefficient of r 0.70–0.89 was considered strong, and $r = 0.40$ –0.69 was moderate (Schober et al., 2018). Categorical data were stated as percentages and frequencies and subsequently compared using the Chi-square test. The marker values utilized receiver operating characteristic (ROC) assessment to assess the biomarker's efficacy to distinguish between macro- and normo-proteinuria and the clinical importance of LC3-II in the investigated groups. An ultimate estimate of a biomarker's or diagnostic test's accuracy is the area under the ROC curve (AUC). Software packages SPSS version 18 (SPSS Inc., Chicago, USA) and MedCalc version 15.0 (MedCalc Software, Ostend, Belgium) were used for statistical analyses.

4 Results and Interpretations

Within the scope of this thesis, Table 4.1 displays the demographic and clinical characteristics of a total of 176 (90 male and 86 female) Turkish subjects enrolled, of whom 26 (13 male and 13 female) were healthy subjects, and 150 had T2DM. Patients were classified according to albumin creatinine ratio (ACR): 88 patients had macroalbuminuria (ACR > 300 mg/g), 20 patients had microalbuminuria (ACR between 30 and 300 mg/g), and 42 had normoalbuminuria (ACR < 30 mg/g).

Table 4.1: Subject demographic and clinical characteristics

	Macroproteinuria N:88	Microproteinuria N:20	Normoproteinuria N:42	Control N:26	P value
Age, years (median)	64.3±9.4	65.6 ±11.83	67.7 ±10.25	59.3±10.5	0.248
Sex (Male/Female)	44/44	14/6	19/23	13/13	0.322
Body mass index (kg/m ²)	31.67 ±5.9	30.3±4.2	30.33±4.64	28.13±5.22	0.057
Duration of T2DM	16.55±10.12 ^{a,b}	14.26 ±9 ^b	10.22±8.5	-	0.011 *
Smoking history, N (%)	50 (56.8%)	9 (45.0%)	30 (71.4%)	16 (61.5%)	0.209
Life style					0.008 ***
Active, N (%)	52 (59.1%)	13 (65.0%)	32 (76.2%)	24 (92.3 %)	
Sedentary, N (%)	36 (40.9%)	7 (35.0%)	10 (23.8%)	2 (7.7%)	
Hypertension, N (%)	24 (27.3%) ^b	7 (35.0%)	19 (45.2%)	-	<0.001 **
Worsening hypertension, N ratio	11 (12.5%)	2 (10.0%)	2 (4.8%)	-	0.170
Clinical Symptoms					
Dyspnea, N (%)	36 (40.9%)	6 (30.0%)	11 (26.2%)	-	0.068

Chest pain, N (%)	12 (13.6%)	1 (5.0%)	6 (14.3%)	-	0.381
Abdominal pain, N (%)	28 (31.8%) ^{a,b}	3 (15.0%)	8 (19.0%)	-	0.015 ***
	4 (4.5%)	-	2 (4.8%)	-	
Fever, N (%)	51 (58.0%) ^{a,b}	7 (35.0%)	17 (40.5%)	-	0.532
Edema, N (%)	59 (67.0%) ^a	7 (35.5%)	24 (57.1%)	-	0.006 ***
Polyuria, N (%)	4 (4.5%)	1 (5.0%)	6 (14.3%)	-	<0.00 1***
Anorexia, N (%)					0.182

Data are median (min-max), M $\pm$ SD (mean $\pm$ Standard deviation), and N (number); Percentage (%).

*One-way ANOVA, **Kruskal-Wallis Test, ***Chi-square test, ^aSignificantly different from microalbuminuria at $p < 0.05$ level, ^bSignificantly different from normoalbuminuria at $p < 0.05$ level, ^cSignificantly different from healthy subjects at $p < 0.05$ level.

Table 4.1 summarizes the following according to the aforementioned classified groups: Age/years (M $\pm$ SD) was nonsignificant among the patients with macroalbuminuria, microalbuminuria, normoalbuminuria, and the healthy groups (64.3 ± 9.4 , 65.6 ± 11.83 , 67.7 ± 10.25 , 59.3 ± 10.5 , respectively), with ($p > 0.05$). There were nonsignificant differences displayed among macro, micro, normoalbuminuria, and control groups for BMI (M $\pm$ SD): (31.67 ± 5.9 , 30.3 ± 4.2 , 30.33 ± 4.64 , and 28.13 ± 5.22 , respectively) with ($p > 0.05$). Sex (male/female): No significant difference was shown (44/44, 14/6, 19/23, and 13/13, respectively) for macro, micro, normoalbuminuria, and the control groups ($p > 0.05$). In addition, a nonsignificant difference was found in smoking history (%) among macro, micro, normoalbuminuria, and control groups (56.8%, 45.0%, 71.4%, and 61.5%, respectively) with ($p > 0.05$). Moreover, the duration of T2DM is significantly related to patients with T2DM by comparing the (M $\pm$ SD) of macro, micro, and normoalbuminuria (16.55 ± 10.12 , 14.26 ± 9 , and 10.22 ± 8.5 , respectively), with ($p < 0.011$). Furthermore, lifestyle: active/sedentary: (59.1%)/(40.9%), (65.0%)/(35.0%), (76.2%)/(23.8%), and (92.3%)/(7.7%) for macro, micro,

normoalbuminuria, and control groups were significantly decreased and increased, respectively, with a ($p<0.008$) Further, hypertension (27.3%, 35.0%, and 45.2%) between diabetes patients with macro, micro, and normoalbuminuria, was nonsignificant with ($p>0.05$). As well as worsening hypertension ratios, they were not significantly different among T2DM patients: (12.5%), (10.0%), and (4.8%), respectively, for macro, micro, and normoalbuminuria groups, with ($p>0.05$).

Besides this, clinical symptoms such as dyspnea (%), chest pain (%), fever (%), and anorexia (%) were resulted as nonsignificant by comparison of macro, micro, and normoalbuminuria in patients with T2DM as (49.9%, 30.0%, 26.2%, 13.6%, 5.0%, 14.3%, 4.5%, 0.0%, 4.8%, 4.5%, 5.0%, and 14.3% respectively) with ($p>0.05$). Although abdominal pain (%), edema (%), and polyuria (%): (31.8%, 15.0%, 19.0%, 58.0%, 35.0%, 45.0%, 67.0%, 35.5%, and 57.1% respectively) with ($p<0.05$), which significantly correlated with T2DM and related to the progression of DKD as demonstrated by (Figure 4.1).

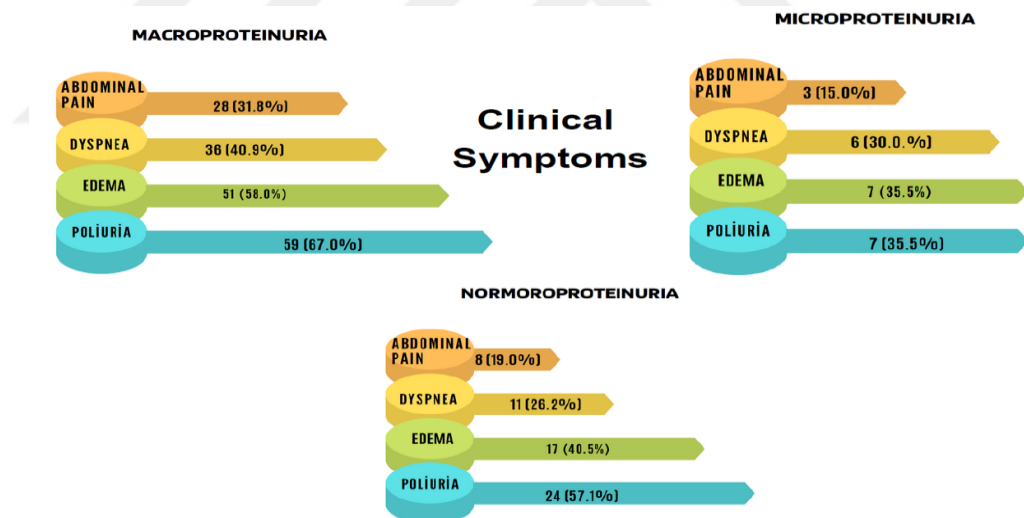


Figure 4.1: Clinical Symptoms of Patient groups

Comparison of frequency rates of clinical symptoms in patient groups: Edema (Macroproteinuria>microproteinuria), polyuria, and abdominal pain (Macroproteinuria>microproteinuria and normoproteinuria). Categorical data were stated as percentages and frequencies and subsequently compared using the Chi-square test and n (%) indicator. $P<0.05$ was considered for being significant.

Table 4.2: Biochemical characteristics of the groups

Parameters	Macroalbuminuria N:82	Microalbuminuria N:20	Normoalbuminuria N:42	Healthy N:26	P value
HbA1c (%)	8.6 (5.4-24.31) ^c	8.5 (5.4-12.3) ^c	7.6 (5.2-15.2) ^c	5.5 (4.7-6.3)	<0.001*
FBG (mg/dL)	170.3 (56.0-460.8) ^c	167.6 (89.0-354.0) ^c	147.2 (82.1-527.4) ^c	94.9 (78.0-124.0)	<0.001*
Triglyceride (mg/dL)	173.0 (50.9-722.6) ^c	152.0 (72.6-848.5) ^c	162.6 (53.0-1296.1) ^c	126.5 (45.6-292.8)	0.028*
HDL-C (mg/dL)	40.0 (14.5-97.5) ^{b,c}	42.5 (22.8-55.0) ^{b,c}	45.2 (27.3-72.1) ^c	52.8 (28.1-102.3)	0.001*
LDL-C (mg/dL)	121.75±51.56 ^{a,b}	95.5±41.2 ^{b,c}	122.4±37.7 ^c	140.9±37.1	0.002**
Hemoglobin (g/dL)	11.93±2.55 ^{a,b,c}	13.35±2.04 ^{b,c}	13.61±1.73	14.35±1.78	<0.001**
Albumin (mg/dL)	4.06 (2.08-5.18) ^{a,b,c}	4.55 (2.56-4.88) ^{b,c}	4.61 (3.86-5.1)	4.64 (4.1-5.1)	<0.001*
BUN (mg/dL)	24.70 (9.40-97.40) ^{a,b,c}	20.55(10.30-97.88,50) ^{b,c}	15.25 (10.50-29.60) ^c	14.90 (7.40-24.20)	<0.001*
Scr (mg/dL)	1.37 (0.53-7.74) ^{a,b,c}	1.08 (0.62-4.40) ^{b,c}	0.81 (0.58-1.37) ^c	0.76 (0.53-1.10)	<0.001*
eGFR(ml/min)	58.0±33.26 ^{b,c}	65.84±35.79 ^{b,c}	88.67±21.29 ^c	104.68±23.53	<0.001**
Cys-C (mg/dL)	0.507 (0.30-3.31)	0.495 (0.25-3.37)	0.443 (0.02-2.42)	0.525 (0.33-1.58)	0.161*
TPCR (mg/mg)	1.74 (0.42-8.90) ^{a,b,c}	0.231 (0.10-0.84) ^{b,c}	0.99 (0.06-0.36) ^c	0.84 (0.05-0.27)	<0.001*
ACR (mg/g)	1001.2 (304.55-7617.71) ^{a,b,c}	81.93 (29.86-286.17) ^{b,c}	9.93 (1.44-26.98) ^c	5.28 (2.39-141.86)	<0.001*
Urine glucose (mg/dL)	69.5 (2.0-2423.0) ^c	25.5 (3.0-2351.0) ^c	774 (2.0-2364.0) ^c	7.5 (3.0-16.0)	<0.001*

Data are median (min-max) or mean±SD, N (number), and (%) percentage. * Kruskal-Wallis Test, **One-way ANOVA, ^aSignificantly different from microalbuminuria at $p < 0.05$ level, ^bSignificantly different from normoalbuminuria at $p < 0.05$ level, ^cSignificantly different from healthy subjects at $p < 0.05$ level. ACR: Urine microalbumin/creatinine ratio. BUN: Blood Urea Nitrogen. Cys-C: Cystatin-C. eGFR: estimating glomerular filtration rate. FBG: Fasting Blood Glucose. HbA1c: Glycated hemoglobin. HDL-C: High-Density Lipoprotein. LDL: Low-Density Lipoprotein. Scr: Serum Creatinine. TPCR: Urine total protein/creatinine ratio.

Biochemical characteristics as illustrated in (Table 4.2): eGFR significantly decreased by comparing macroalbuminuria to normoalbuminuria, macroalbuminuria to healthy groups, as well as normoproteinuria to healthy groups and microproteinuria with healthy groups ($p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.006$, respectively). There were

significant increases in HbA1c, FBG, triglyceride, BUN, Scr, TPCR, and ACR, and urine glucose of T2DM patients with albuminuria compared to the healthy group. In contrast, significant decreases were found in eGFR, albumin, haemoglobin, LDL-C, and HDL-C of T2DM patients with albuminuria compared to the healthy group. In addition, significant increases were found in BUN, Scr, TPCR, and ACR in the macroalbuminuria group compared to the microalbuminuria and normoalbuminuria groups. In patients with T2DM exhibiting macroalbuminuria, microalbuminuria, and normoalbuminuria, eGFR and serum albumin levels were significantly lower than those observed in the control group.

While a nonsignificant difference in Cys-C was found between patient groups and controls ($p>0.05$), as presented in Table 4.2 with Figure 4.3, G.

Table 4.3: Comparison of the study parameters of the groups

Parameters	Macroalbuminuria N:88	Microalbuminuria N:20	Normoalbuminuria N:42	Healthy N:26	P * value
BCL-2 (ng/mL)	11.59 (8.03-88.95)	12.22 (3.37-86.55) ^b	11.0 (1.08-55.85)	12.99 (8.90-36.38)	0.004
LC3-II (ng/L)	299.56 (238.31-1652.7) ^{b,c}	284.25 (175.71-1678.94) ^{b,c}	224.29 (54.29-1527.14)	238.57 (147.14-799.29)	<0.001
Podocin (ng/mL)	5.618 (0.71-30.99) ^b	5.689 (1.56-30.29)	5.05 (1.04-27.32)	5.90 (3.77-18.09)	0.022
IL-1 β (pg/mL)	837.6 (621.6-5463.6)	1026.8 (629.6-5403.6)	942.8 (253.8-5063.8)	948.8 (647.8-2955.8)	0.087
TGF- β 1 (ng/L)	601.78 (205.67-3074.56) ^b	605.11 (475.67-3006.78) ^b	503.44 (125.67-2442.33)	589.55 (416.78-1611.22)	0.025
GRP78 (ng/mL)	17.08 (0.0-87.37) ^{b,c}	16.689 (9.10-85.31) ^{b,c}	14.41 (2.45-76.52)	14.17 (10.21-43.44)	<0.001

Data are median (min-max). *Kruskal-Wallis Test, ^aSignificantly different from microalbuminuria at $p < 0.05$ level, ^b Significantly different from normoalbuminuria at $p < 0.05$ level, ^cSignificantly different from healthy subjects at $p < 0.05$ level. LC3-II: Microtubule-associated protein 1A/1B-light chain 3. IL-1 β : Interleukin-1 β . TGF- β 1: Transforming Growth Factor- β 1. GRP78: Glucose-Regulated Protein 78.

The levels of LC3-II were significantly higher in the macroalbuminuria and microalbuminuria groups compared to the normoalbuminuria and control groups ($p < 0.001$). Podocin caused a substantial increase in macroalbuminuria compared to normoalbuminuria ($p = 0.022$). Similarly, there were notable disparities in TGF- β 1 levels, with substantial elevations observed in the macroalbuminuria and microalbuminuria groups in comparison to the normoalbuminuria group ($p = 0.025$). BCL-2 expression was significantly elevated in individuals with microalbuminuria compared to those with normoalbuminuria ($p = 0.004$). Furthermore, there were no significant differences seen for IL-1 β ($p = 0.087$). GRP78 levels were also significantly increased in both macro- and

microalbuminuria compared to normoalbuminuria and control groups ($p<0.05$), as shown by and Figure 4.2: A, Figure 4.3: E, Figure 4.2: D, C, B, and Figure 4.3: F, respectively.

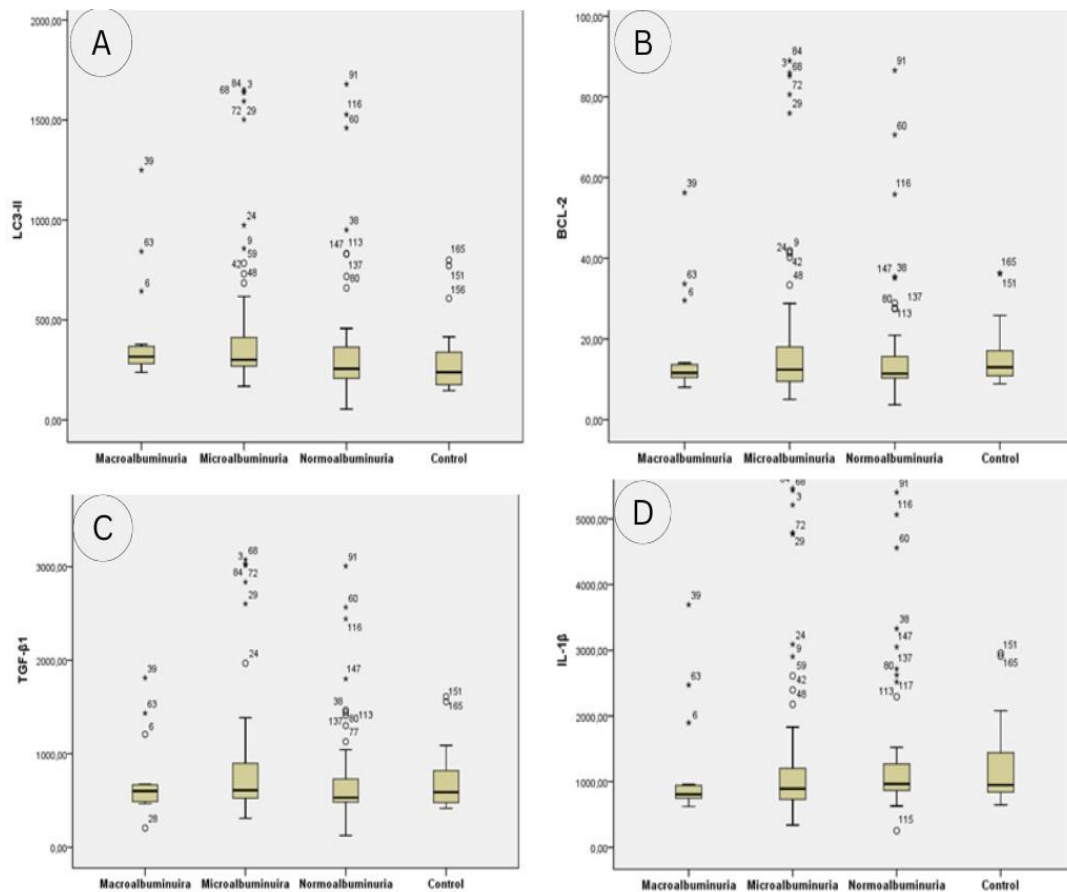


Figure 4.2: Comparison of parameters by proteinuria groups

Comparison of serum LC3-II, BCL-2, TGF- β 1, and IL-1 β levels of study groups organized by A, B, C, and D according to proteinuria severity with each other and the healthy group. The comparisons were done by the Mann-Whitney U test and median (min.-max.) indicator. $P<0.05$ was considered to be significant.

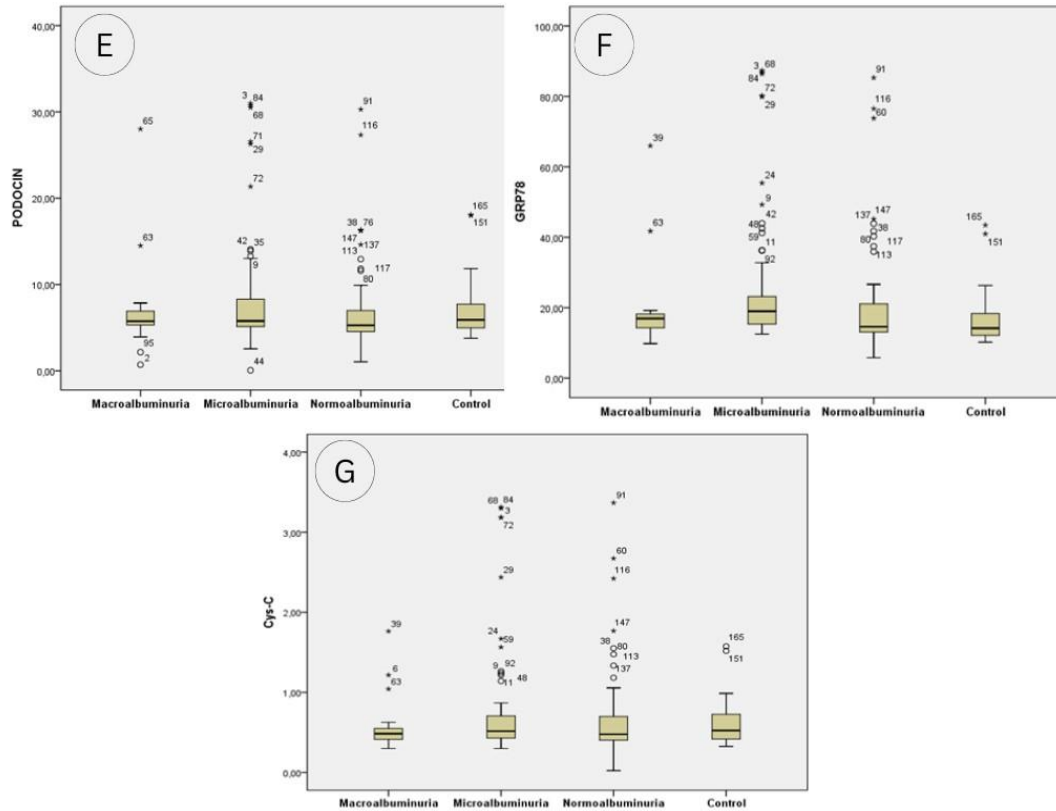


Figure 4.3: Comparison of parameters by proteinuria groups cont...

Comparison of serum Podocin, GRP78, and Cys-C levels of study groups organized by E, F, and G according to proteinuria severity with each other and the control group. The comparisons were done by the Mann-Whitney U test and median (min.-max.) indicator. P<0.05 was considered to be significant.

Table 4.4: Crosstab of study groups according to proteinuria and GFR stage

GFR Stages	Proteinuria Groups			
Creatinine Clearance (mL/min)	Macroalbuminuria N:88	Microalbuminuria N:20	Normoalbuminuria N:42	Total
G 5 (≥ 15)	6 (4.0%)	1 (0.7%)	0 (0%)	7 (4.7%)
G4 (15-29)	18 (12.0%)	2 (1.3%)	0 (0%)	20 (13.3%)
G3b (30-44)	11 (7.3%)	4 (2.7%)	0 (0%)	15 (10.0%)
G3a (45-59)	13 (8.7%)	3 (2.0%)	6 (4.0%)	22 (14.7%)
G2 (60-89)	25 (16.7%)	3 (2.0%)	16 (10.7%)	44 (29.3%)
G1 (≤ 90)	15 (10.0%)	7 (4.7%)	20 (13.3%)	42 (28.0%)

The projected GFR is classified through the following six groups: normal to high (G1), mildly to declined (G2), mildly to moderately declined (G3a), moderately to severely declined (G3b), severely decreased (G4), and renal failure (G5).

Table 4.4: displays the classification of DKD stages by comparing eGFR to proteinuria groups.

Table 4.5: Comparison of study parameters according to diabetic kidney disease stages

	Stage 1 N:36	Stage 2 N:7	Stage 3 N:24	P * value
eGFR (ml/min)	105.97 (89.09-135.0) ^{a,b}	43.35 (31.55-59.99) ^b	25.07 (8.84-29.46)	<0.001
ACR (mg/g)	1.17 (0.14-2.46) ^{a,b}	6.52 (2.99-20.48) ^b	2058.41 (331.86-7617.71)	<0.001
Cystatin-C (mg/dL)	0.40 (0.32-1.18) ^{a,b}	0.57 (0.36-3.37)	0.49 (0.30-3.31)	0.002
LC3-II (ng/L)	216.42 (151.43-717.86) ^{a,b}	284.29 (257.86-1678.94)	317.06 (168.31-1638.31)	<0.001
BCL-2 (ng/mL)	10.84 (1.08-27.53) ^{a,b}	12.41 (10.50-86.55)	12.40 (5.57-88.95)	0.012
Podocin (ng/mL)	4.58(3.68-14.61) ^b	5.51 (1.56-30.29)	5.78 (0.71-30.99)	0.040
IL-1 β (pg/mL)	929.80 (625.80-2715.80)	1112.80 (711.60-5403.60)	933.60 (621.60-5463.60)	0.397
TGF- β 1 (ng/L)	485.66 (391.22-1443.44) ^{a,b}	650.67 (487.89-3006.78)	630.11 (417.89-3029.00)	0.001
GRP-78 (ng/mL)	13.68 (2.45-43.89) ^{a,b}	18.44 (14.28-85.31)	17.27 (9.81-87.37)	<0.001

Stage 1: Normoalbuminuria+G1/G2, **Stage 2:** Microalbuminuria+G3a/G3b, **Stage 3:** Macroalbuminuria+G5/G4. Data are median (min-max) and N (number). * Kruskal-Wallis Test, ^a Significantly different from stage 2 at $p < 0.05$ level, ^b Significantly different from stage 3 at $p < 0.05$ level.

As demonstrated by Table 4.5, there were significant increases in serum Cystatin C, LC3-II, BCL-2, PDCN, GRP-78, and TGF- β 1 levels in stage 3 with severely high levels of ACR and severely low eGFR compared to stage 1 ($p < 0.05$). Similarly, there were significantly higher levels of serum cystatin C, LC3-II, BCL-2, GRP-78, and TGF- β 1 in stage 2 with medium-high ACR and medium-low eGFR compared to stage 1 ($p < 0.05$). In addition, according to the present data, IL-1 β was not significantly correlated with DKD stages ($p = 0.397$).

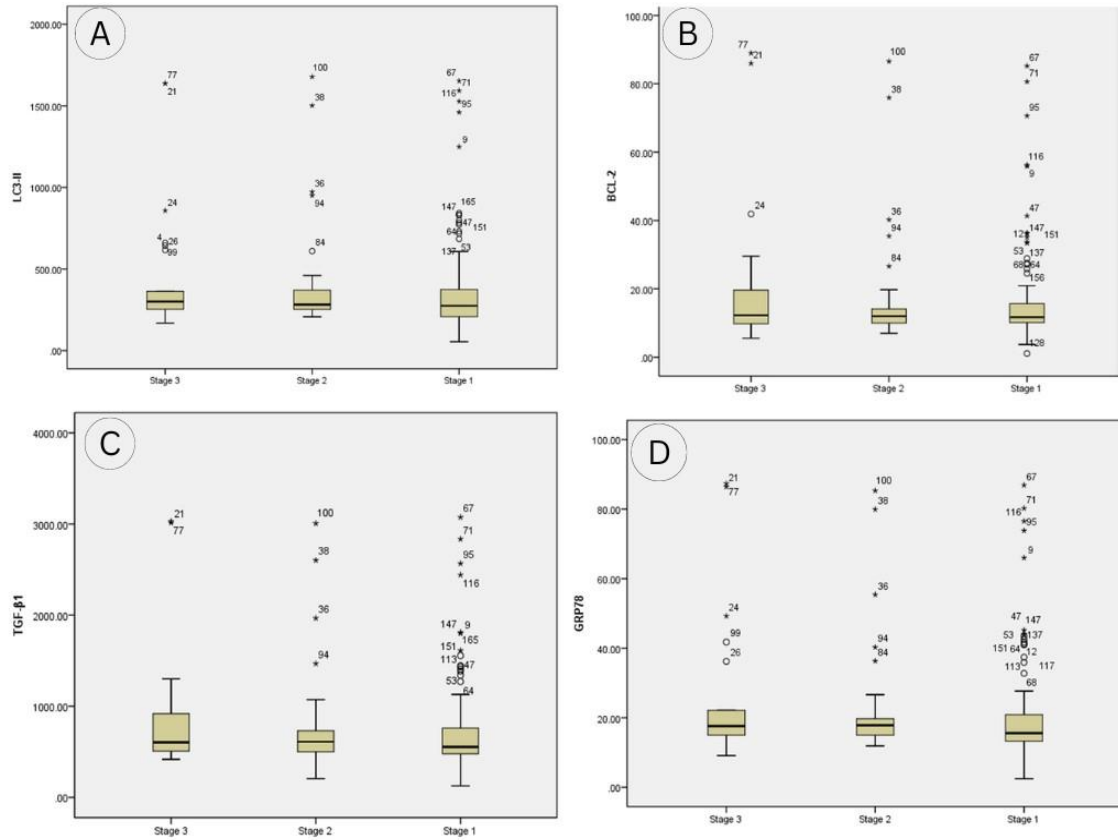


Figure 4.4: Comparison of study parameters by DKD stages

Comparison of working parameters of study groups organized according to proteinuria severity and eGFR stages (Stage 1: Normoalbuminuria+G1/G2, Stage 2: Microalbuminuria+G3a/G3b, Stage 3: Macroalbuminuria+G5/G4): A) LC3-II, B) BCL-2, C) TGF-β1, and D) GRP78 levels significantly increased in stages 3 and 2 compared to stage 1 ($p<0.001$), ($p=0.012$), ($p=0.001$), and ($p<0.001$) respectively. The comparisons were done by the Mann-Whitney U test and median (min.-max.) indicator. ($P<0.05$) was considered for being significant.

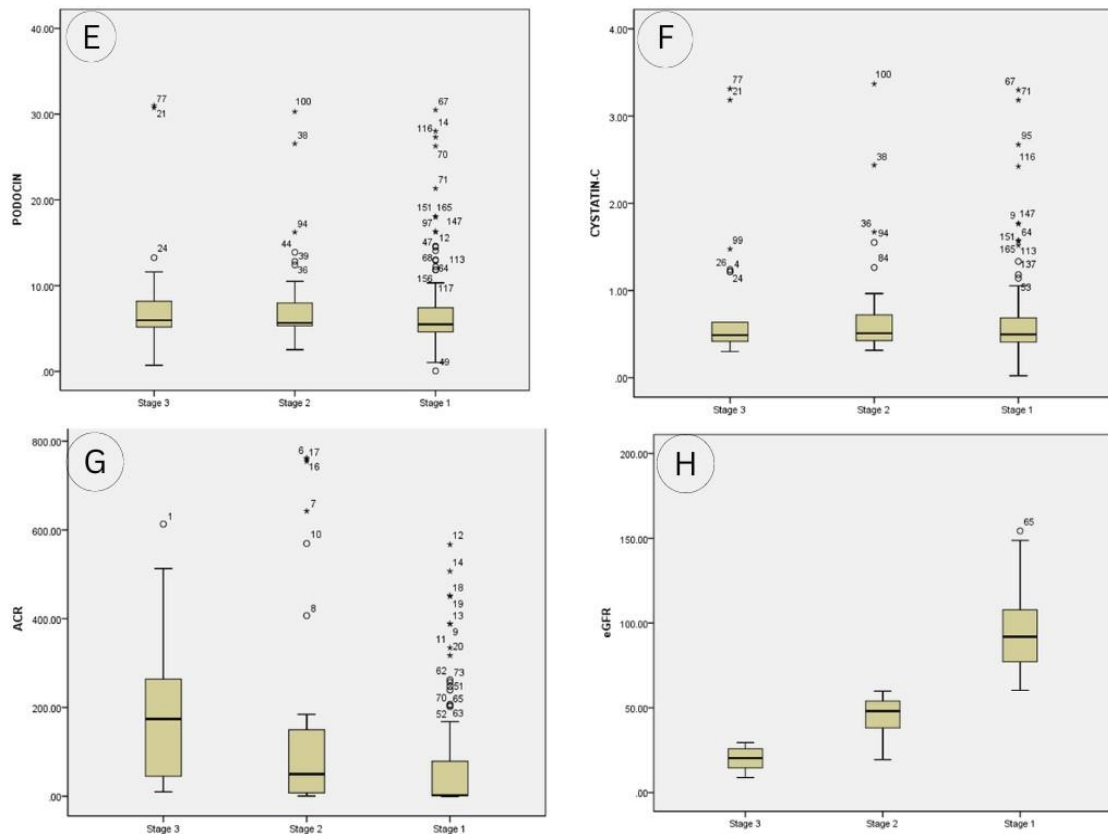


Figure 4.5: Comparison of study parameters by DKD stages cont....

Comparison of working parameters of study groups organized according to proteinuria severity and eGFR stages (Stage 1: Normoalbuminuria+G1/G2, Stage 2: Microalbuminuria+G3a/G3b, Stage 3: Macroalbuminuria+G5/G4): E) Podocin levels were significantly increased in stage 3 versus stage 1 ($p < 0.040$), F) Cystatin-C levels were significantly higher in stage 2, and 3 compared to stage 1 ($p = 0.002$), and G) ACR levels were significantly increased in stage 2, and 3 compared to stage 1, and in stage 3 compared to stage 2 ($p < 0.001$). H) eGFR levels were significantly increased in stages 2, and 3 compared to stage 1, and in stage 3 compared to stage 2 ($p < 0.001$). The comparisons were done by the Mann-Whitney U test and median (min.-max.) indicator. ($P < 0.05$) was considered for being significant.

Table 4.6: Univariate correlations between various parameters and LC3-II levels.

Parameters	Macroalbuminuria <i>r</i> (<i>P</i> value)	Microalbuminuria <i>r</i> (<i>P</i> value)	Normoalbuminuria <i>r</i> (<i>P</i> value)	Healthy <i>r</i> (<i>P</i> value)	All participants <i>r</i> (<i>P</i> value)
Age (years)	0.154 (0.152)	-0.086 (0.719)	0.212 (0.178)	0.164 (0.425)	0.172 (0.025)*
DM duration (years)	0.075 (0.409)	-0.009 (0.968)	-0.008 (0.960)	-	0.143 (0.087)
BMI (kg/m ²)	-0.092 (0.394)	-0.178 (0.454)	-0.176 (0.264)	-0.318 (0.113)	-0.068 (0.378)
FBG (mg/dL)	-0.018 (0.871)	-0.051 (0.830)	-0.139 (0.378)	-0.310 (0.124)	0.056 (0.470)
HbA1c (%)	-0.031 (0.726)	-0.166 (0.486)	-0.046 (0.770)	-0.195 (0.341)	0.084 (0.274)
eGFR (ml/min)	0.059 (0.588)	-0.321 (0.167)	-0.274 (0.079)	-0.217 (0.287)	-0.235 (0.002)*
ACR (mg/g)	0.096 (0.373)	0.263 (0.264)	0.132 (0.406)	0.133 (0.516)	0.381 (<0.001)*
Triglyceride (mg/dL)	-0.138 (0.199)	-0.412 (0.071)	0.080 (0.615)	0.093(0.653)	-0.009 (0.910)
LDL cholesterol (mg/dL)	-0.017 (0.875)	-0.056 (0.816)	0.132 (0.405)	0.105 (0.611)	-0.003 (0.873)
HDL cholesterol (md/dL)	0.195 (0.069)	0.310 (0.183)	-0.312 (0.044)*	-0.264 (0.193)	-0.050 (0.513)
Hemoglobin (g/dL)	0.013 (0.902)	0.050 (0.833)	-0.135 (0.394)	0.322 (0.109)	-0.078 (0.303)
Albumin (mg/dL)	0.060 (0.578)	-0.157 (0.508)	0.199 (0.207)	-0.017 (0.935)	-0.132 (0.081)
BUN (mg/dL)	0.006 (0.959)	0.193 (0.414)	0.192 (0.224)	0.093 (0.653)	0.286 (<0.001)**
Scr (mg/dL)	-0.135 (0.211)	0.333 (0.151)	0.155 (0.328)	0.435 (0.026)*	0.181 (0.016)*
SYN-C (mg/dL)	0.733 (<0.001)**	0.691 (0.001)**	0.648 (<0.001)**	0.829 (<0.001)**	0.750 (<0.001)**
BCL-2 (ng/mL)	0.859 (<0.001)**	0.882 (<0.001)**	0.706 (<0.001)**	0.881 (<0.001)**	0.791 (<0.001)**
Podocin (ng/mL)	0.599 (<0.001)**	0.489 (0.029)*	0.709 (<0.001)**	0.916 (<0.001)**	0.681 (<0.001)**
GRP-78 (ng/mL)	0.675 (<0.001)**	0.824 (<0.001)**	0.995 (<0.001)**	0.869 (<0.001)**	0.796 (<0.001)**
IL-1 β (pg/mL)	0.769 (<0.001)**	0.593 (<0.001)**	0.738 (<0.001)**	0.956 (<0.001)**	0.639 (<0.001)**
TGF- β 1 (ng/L)	0.722 (<0.001)**	0.788 (<0.001)**	0.676 (<0.001)**	0.907 (<0.001)**	0.779 (<0.001)**

FBG refers to fasting blood glucose, HbA1c stands for hemoglobin A1c, ACR represents albumin/creatinine ratio, eGFR is the estimated glomerular filtration rate, BUN is for Blood Urea Nitrogen, *r* coefficient refers to correlation, Scr represents Serum creatinine, and BMI stands for body mass index.

* *P* value <0.05 significant correlation as assessed by Spearman's correlation method

** *P* value <0.001 significant correlation as assessed by Spearman's correlation method

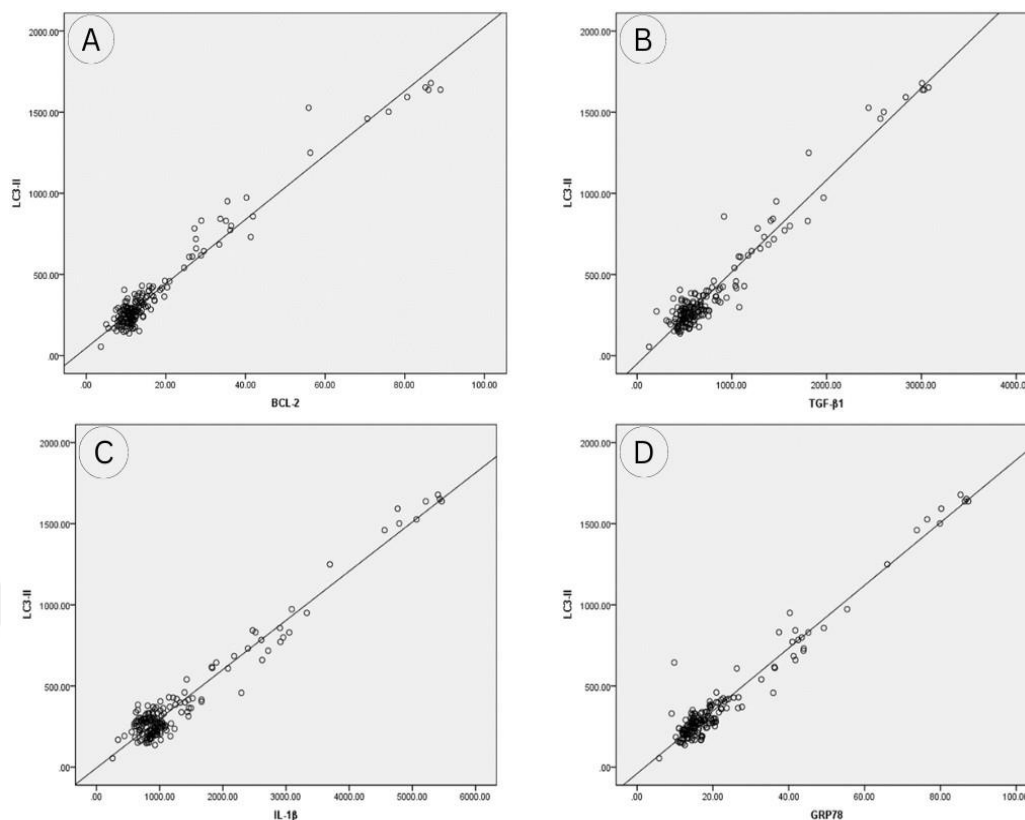


Figure 4.6: Relationship between serum LC3-II levels and study parameters in T2DM patients with impaired renal functions

Illustrates the relationship between serum LC3-II levels and study parameters in T2DM patients with impaired renal functions: A) relationship with LC3-II and BCL-2 ($r=0.791$, $p<0.001$). B) LC3-II relationship with TGF- β 1 ($r=0.779$, $p<0.001$). C) Relationship between LC3-II and IL-1 β ($r=0.639$, $p<0.001$). D) Relationship between LC3-II and GRP78 ($r=0.796$, $p<0.001$). Correlations between LC3-II concentrations were analyzed by Spearman correlation analysis. A correlation coefficient of $r=0.70$ – 0.89 was considered strong, and $r=0.40$ – 0.69 was moderate.

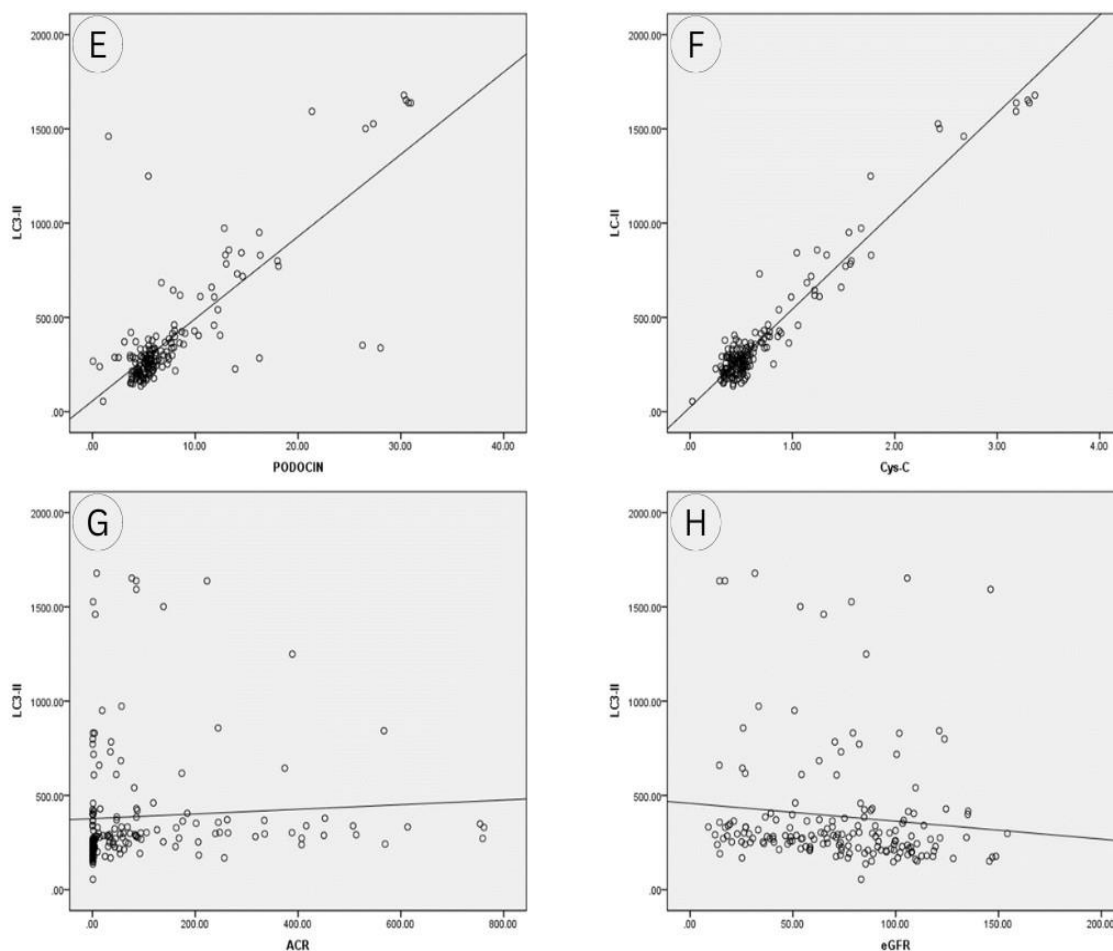


Figure 4.7: Relationship between serum LC3-II levels and study parameters in T2DM patients with impaired renal functions cont....

The relationship between serum LC3-II levels and study parameters in T2DM patients with impaired renal functions: E) Relationship between LC3-II and Podocin ($r=0.681$, $p<0.001$) F) Relationship between LC3-II and Cys-C ($r=0.750$, $p<0.001$) G) Relationship between LC3-II and ACR ($r=0.381$, $p<0.001$) H) LC3-II relationship with eGFR ($r=-0.235$, $p=0.002$). Correlations between LC3-II concentrations were analyzed by Spearman correlation analysis. A correlation coefficient of $r=0.70$ – 0.89 was considered strong, and $r=0.40$ – 0.69 was moderate.

As shown in Table 4.6 and Figures 4.6 and 4.7, there were univariate correlations between various parameters and LC3-II levels. In all participants, there was a significant positive and strong correlation between LC3-II and Cystatin C, BCL-2, IL-1 β , Podocin, GRP-78, and TGF- β 1 ($r=0.750$, $p<0.001$; $r=0.791$, $p<0.001$; $r=0.639$, $p<0.001$; $r=0.681$, $p<0.001$; $r=0.796$, $p<0.001$; $r=0.779$, $p<0.001$, respectively). However, a strong positive correlation was found only between BCL-2 and LC3-II levels in patients with T2DM (macroproteinuria: $r=0.859$, $p = 0.001$; microproteinuria: $r=0.882$, $p<0.001$; normoproteinuria: $r=0.706$, $p<0.001$). The positive relationship between LC3-II and Cystatin C, IL-1 β , and TGF- β 1 in patients with T2DM increases depending on the severity of proteinuria. However, non-significant correlations were found among LC3-II and T2DM duration, age, BMI, FBG, HbA1c, TG, LDL-C, HDL-C, Hb, albumin, BUN, Scr, and ACR levels in these patient groups. Additionally, a non-significant relationship was found between LC3-II and eGFR in patients with proteinuria with type 2DM; HDL-C was moderately significant in normoproteinuria with a p-value of 0.044.

Figure 4.8 presents ROC analyses for LC3-II, detailing optimal cut-off points and diagnostic characteristics. Through this evaluation, we identified enhanced positivity thresholds that improve the efficiency of this autophagy biomarker. The MedCalc software's ROC analysis determined the appropriate cut-off settings. Its diagnostic performance in differentiating LC3-II macroproteinuria patients from normo-proteinuria patients at AUC = 0.764, $P<0.001$, cut-off ≥ 270.04 ng/ml, 76.3% sensitivity, 77.1% specificity, 61.2% NPV, 84.4% precision, and 75.4% efficiency values were obtained.

As revealed by Figure 4.9, DKD progress is suppressed by activating LC3-II, which is responsible for activating BCL-2 and inhibiting the cytokine IL-1 β .

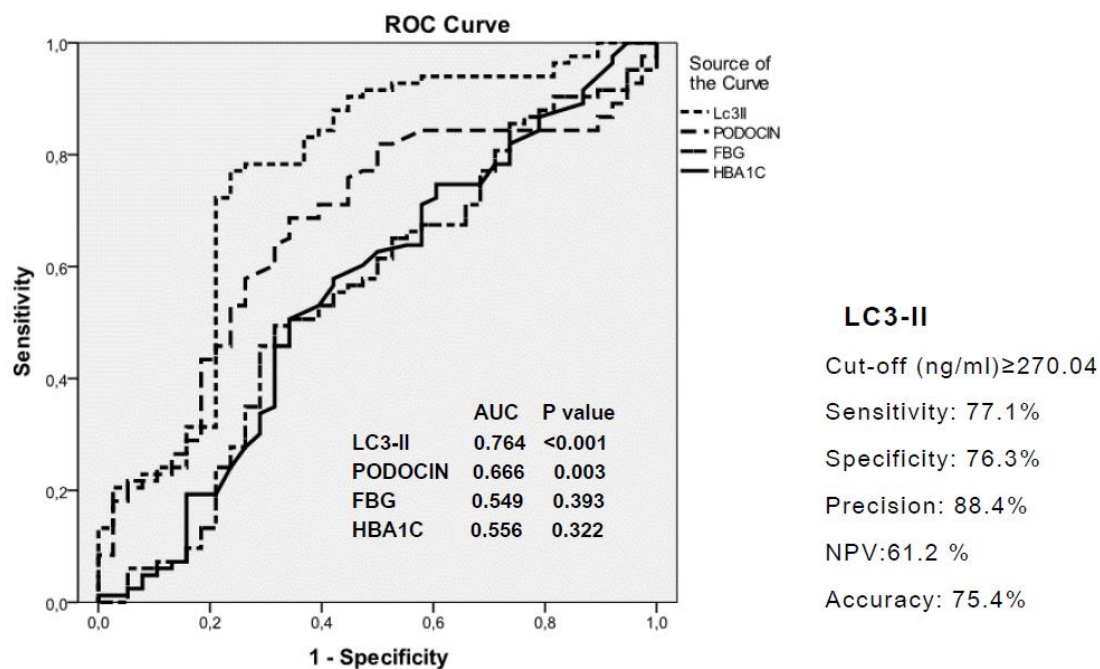
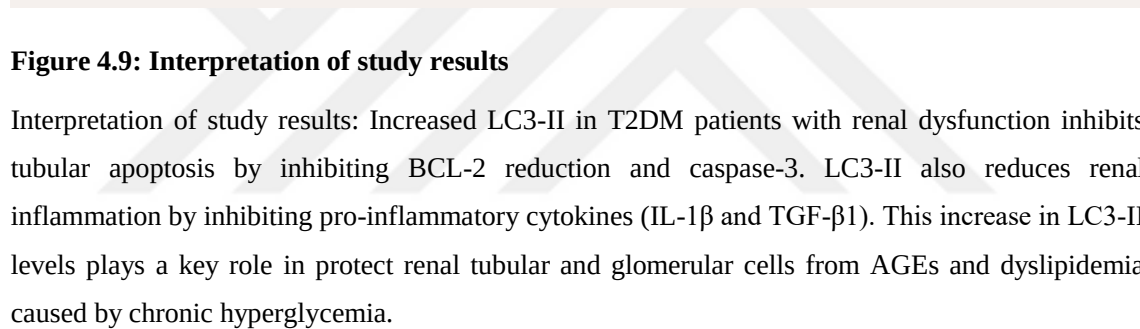


Figure 4.8: ROC analysis

Receiver operating characteristic (ROC) analysis of serum LC3-II, Podocin, FBG, and HBA1C in diagnosing severe proteinuria in T2DM patients with impaired renal function: LC3-II had the highest area under the curve (AUC) value (AUC=0.764, $p<0.001$). The cut-off value for LC3-II is ≥ 270.04 ng/ml; Sensitivity was 77.1%, specificity 76.3%, precision 88.4%, negative predictive value (NPV) 61.2%, and accuracy 75.4%. The cut-off for LC3-II was chosen according to Youden index.



Interpretation of study results: Increased LC3-II in T2DM patients with renal dysfunction inhibits tubular apoptosis by inhibiting BCL-2 reduction and caspase-3. LC3-II also reduces renal inflammation by inhibiting pro-inflammatory cytokines (IL-1 β and TGF- β 1). This increase in LC3-II levels plays a key role in protect renal tubular and glomerular cells from AGEs and dyslipidemia caused by chronic hyperglycemia.

5 Discussion and Conclusion:

DM is a complex disease characterized by various pathophysiological pathways (Gonzalez et al., 2021). It is forecasted that the instances of T2DM will increase from 415 million to 642 million by the year 2040 (Sanabria-de la Torre et al., 2022). Hyperglycemia is a situation that leads to a state of hypercoagulability. Hypercoagulability reduces blood flow, triggering renal ischemia and hypoxia by promoting oxidative stress, cell death, and the growth of mesangial cells, which eventually lead to the progression of DN (B. Zhang et al., 2020). DN is identified by the appearance of glomerular hypertrophy, reduced glomerular filtration, proteinuria, and renal fibrosis. It is acknowledged as a chronic consequence of DM, which has the potential to result in renal function loss ultimately (Huang et al., 2021). Although many patients with T2DM develop DN-absent albuminuria, using this dependable biomarker is essential (Erekat, 2022; Samsu, 2021).

DN is a major long-term vascular complication linked to DM, playing a key role in the onset of ESRD. In the absence of adequate medical intervention, DN can ultimately result in the development of glomerular sclerosis, proteinuria, and perhaps even renal failure (F. Sun et al., 2019).

Additionally, a considerable number of DM patients experience the progression of kidney disease to ESRD (Y. Shen et al., 2020). While the exact leading mechanisms of the progression of DN remain incompletely elucidated, it is plausible that DN could be linked to disruptions in glucose-lipid metabolism, inflammation, ERS, or autophagy. Podocytes are crucial in serving as a barrier for glomerular filtration (X. Xu et al., 2020).

Demographic and clinical parameters calculated according to ACR groups. We found that age, BMI, smoking history, dyspnea, chest pain, fever, and anorexia were not significantly altered among macro, micro, normoalbuminuria, and the control group as shown by our findings. The duration of T2DM, sedentary lifestyle, abdominal pain, edema, and polyuria significantly increased in the macro- and microalbuminuria groups compared to the normoalbuminuria group. These findings are consistent with those determined by (B. A. Young et al., 2016).

Hypertension remains a controversial subject, particularly its effectiveness in promoting stroke and kidney disease over 50% of patients with T2DM have a sedentary

lifestyle (D. Li et al., 2022). Hypertension showed a significant increase in macroalbuminuria compared to normoalbuminuria, as shown by the present data.

In order to reduce the risks associated with DKD and coronary artery disease, diabetes patients need to adopt modifications to their lifestyles, such as restricting sodium and protein intake, engaging in frequent physical activity and weight loss, and quitting smoking (Y. C. Lin et al., 2018).

Multiple investigations have confirmed that the decline in renal function, is directly caused by renal tubular atrophy resulting from apoptosis and the loss of renal tubular cells (Y. Wang et al., 2022). Inflammatory and fibrotic responses observed in patients with diabetes are mainly attributed to hyperglycemia and eventually contribute to the progression of DN (L. Wang et al., 2021). Relying merely on one marker for probability stratification in disease development poses numerous inherent limitations. Human, biological, and analytical variability challenges hinder the utilization of single markers (C. Jung & Yoo, 2022).

Hyperglycemia is associated with endothelial damage and leads to various vascular problems (Y. H. Shen et al., 2018). Maintaining an HbA1c level of about 7.0% is vital for avoiding or postponing the microvascular problems caused by diabetes, like renal failure. Nevertheless, multiple controlled studies have shown that decreasing blood glucose levels failed to significantly reduce the development of ESRD or prevent the decline of the functioning of the kidneys (Lee et al., 2018). To prevent the advancement of T2DM to DKD, HbA1c, and BMI levels should be kept under 7.0% and 25 kg/m², respectively, as revealed by retrospective research (Nakanishi et al., 2019).

The above-mentioned studies agreed with our findings, which showed significantly increased levels of serum HbA1c and FBS in patient groups compared to healthy groups.

Hyperlipidemia frequently appear in DM, resulting in elevated levels of cholesterol and triglycerides. LDL-cholesterol oxidation is linked to the progression of DN (R. Zhang et al., 2019). Dyslipidemia in DKD patients may be worsened by hyperglycemia and insulin resistance. There was a direct correlation between the levels of lipoproteins and the incidence of DKD. A study conducted in Nigeria revealed a

significant positive link between the ACR and TG (Hussain et al., 2021). Research on patients diagnosed with type 2 diabetes mellitus found a link between high total cholesterol, LDL, and TG levels and the progression of nephropathy.

Nevertheless, proteinuria levels or renal failure contributing to dyslipidemia caused by DKD continue to be controversial. (Hirano et al., 2022). Additionally, utilizing BMI as an indicator to determine the diagnosis of CKD in individuals with T2DM poses complications. DM not only exhibits muscular atrophy but also undergoes excess fat accumulation. BMI is not considered a dependable indicator for CKD in patients with T2DM (Bin Zaman et al., 2018).

In agreement with earlier stated research, our lipid profile results displayed that TG was significantly elevated in patient groups compared to the control group, and HDL-C levels in macro- and microalbuminuria were significantly declined compared to the control group. LDL-C levels were significantly lower in T2DM patient groups compared to healthy groups.

DM patients are typically more susceptible to the impacts of anemia. Al-Khoury et al. documented that Hb levels are 1 g/dL lower in patients with DM than those without DM at each stage of CKD, and this study was in agreement with our findings (Al-Khoury et al., 2006).

As demonstrated by our findings, serum albumin was significantly decreased in macro- and microalbuminuria compared to normoalbuminuria and controls, as well as non-significantly correlated to the LC3-II. Similar results were documented in the literature (Bilgin et al., 2021; S. Liu et al., 2024; Viswanathan et al., 2004).

Diabetic patients often experience ongoing exposure to various metabolic and hemodynamic hazards, which can lead to the development of DKD through many pathophysiological mechanisms (Ahn et al., 2020). It is crucial to conduct regular albuminuria screenings in patients with T2DM to identify the presence of CKD and track its advancement over time (Mcgill et al., 2022). At the level of the glomerulus, several factors can contribute to an increased leakage of albumin in the glomerular apparatus. These factors include hyperfiltration, thickening of the basement membrane, endothelial dysfunction, effacement or effusion of podocytes, as well as their detachment, and the presence of hyalinosis and glomerular sclerosis (Heyman et al., 2022).

Ginsberg et al., and Kristal et al., have established a reference value of 0.2 mg/mg for the Protein/Creatinine ratio and a value of 3.5 mg/mg for the differential diagnosis of nephrotic proteinuria. The 2014 guidelines published by the (NICE) in the UK underlined the necessity of creating standardized criteria for proteinuria. The maximum threshold identified was approximately 0.15 grams/day, corresponding to a Protein/Creatinine ratio of 0.15 mg/mg (Kamińska et al., 2020).

The pathophysiology of DN is complicated, resulting from the combined influence of various causes and mechanisms. Excessive proteinuria can induce tubular cell destruction, impair the integrity of the podocyte diaphragm, and lead to the disappearance of foot processes (Z. Zhang, Hu, et al., 2022). BUN, Scr, ACR, and TPCR were also significantly higher in normo-, micro-, and macroalbuminuric patients than in control groups. Similar results were obtained (H. Feng et al., 2021; Luan et al., 2023; Nna et al., 2021). ACR was significantly increased in stages 3 and 2 compared to stage 1 according to DKD stages, as shown by our results.

Notably, patients diagnosed with DN and exhibiting high levels of proteinuria have a pronounced loss in GFR, with an estimated rate of impairment ranging from 46 to 60 mL/min/1.73 m² each year (Guo et al., 2020). A significant decline of around 70% in eGFR was observed in DN patients compared to the control group and those with type T2DM (Harkin et al., 2022). Based on ACR classification, eGFR levels significantly increased in macro- and microalbuminuria compared to normoalbuminuria and the control group, and in normoalbuminuria compared to controls. eGFR was significantly declined in stages 3 and 2 compared to stage 1 according to DKD stages, as presented by our results.

Cys-C is ubiquitously present in diverse tissues and exhibits consistent amounts in the serum under physiological circumstances. Various factors, including dietary patterns, physical activity levels, muscle mass, age, gender, racial background, and inflammatory conditions, unalter Cys-C production (W. Xu et al., 2019).

Blood Cys-C levels were found to be significantly greater in those with DN compared to those without DN, as shown by (Thipsawat, 2021). Serum Cys-C concentrations were higher in T2DM with eGFRs less than 60 ml/min compared to T2DM

with eGFRs greater than 60 ml/min and healthy groups. However, no link was seen between Cys-C and HbA1C (Javanmardi et al., 2015). HbA1C, Cys-C, and TG quantities were significantly increased in patients with T2DM compared with healthy subjects, as predicted by (Araújo et al., 2020; C. Y. Jung & Yoo, 2022; L. Li et al., 2017).

Nevertheless, it is essential to emphasize that the Cys-C level was shown to be elevated primarily in patients with macroalbuminuria (D. Zhang et al., 2019). Cys-C is a biomarker for the initial stages of renal dysfunction characterized by inflammation (Piko et al., 2021). Also, Samsu et al. found a strong positive correlation between serum Cys-C and albuminuria. (Samsu et al., 2019). Prior research has provided evidence indicating that Cys-C can be an early biomarker of DKD compared to the eGFR and UACR (N. Wang et al., 2022). The association between Cys-C and the decline in eGFR, as well as its predictive value for the advancement of DN, has been established in previous studies (Thipsawat, 2021)

In contrast to our research findings, Jeon et al. observed increased serum Cys-C levels during the transition from normoalbuminuria to macroalbuminuria (Sapkota et al., 2021). A study conducted by Norlund et al. showed no significant variations by gender in plasma and serum Cys-C levels. A study conducted by Finney et al. demonstrated that slight variations were seen within genders, thus suggesting the use of a unique reference interval for each gender (N. Wang et al., 2022). In an additional study performed by Harkin et al., it was found that there were no significant variations in the levels of Cys-C in blood circulation between the control group and T2DM. However, patients diagnosed with DN reported Cys-C values that were twice as elevated (Harkin et al., 2022).

The precise mechanisms linking Cys-C and DN remain incompletely comprehended and require further investigation for full elucidation (B. Feng et al., 2022). Multiple investigations have reported that serum Cys-C levels increased earlier than creatinine levels (Dejenie et al., 2023). Prior research found that, in contrast to a control group, diabetic individuals' serum Cys-C levels were significantly higher and their eGFR was significantly lower (Taha et al., 2023). The level of Cys-C in T2DM correlates with eGFR alone, and it rises in T2DM with normoalbuminuria and decreasing GFR (Pelle et al., 2022). It has been shown that Cys-C is an early renal marker in diabetic patients and has been accepted as a potential marker of kidney dysfunction (Assal et al., 2013). Even

though serum Cys-C has been studied, multiple research investigations have concluded that it is not superior to Scr in identifying renal sickness (Arceo et al., 2019).

Our results revealed that serum Cys-C level was non-significantly related to T2DM patients with proteinuria groups but significantly increased in stages 3 and 2 compared to stage 1, and these results align with other studies (Araújo et al., 2020; C. Y. Jung & Yoo, 2022; L. Li et al., 2017).

Autophagy is the process by which cells participate in self-phagocytosis, a crucial degradation mechanism that facilitates the elimination of damaged organelles and detrimental chemicals within the lysosome (X. Xu et al., 2020). This process is significant for cellular equilibrium maintenance, survival, differentiation, and development (L. Chen et al., 2021).

Autophagy is essential in maintaining blood glucose levels in renal epithelial cells, influencing podocytes, proximal tubular, mesangial, and endothelial cells. However, in the case of hyperglycemia, the impairment of autophagy can contribute to the beginning and advancement of DKD (Cai et al., 2023). Dysfunction of autophagy plays a role in the injury of podocytes, and it has been determined that autophagy is suppressed in a hyperglycemic environment (X. qi Liu et al., 2022).

Because T2DM is a diverse condition, including numerous pathophysiological pathways, utilizing merely one marker for the development of DKD comes with multiple disadvantages, including analytical, individual, and biological instabilities (C. Jung & Yoo, 2022). A boost in autophagy biomarkers, which include ATG5 in subcutaneous and visceral adipose tissue, was previously detected in patients with T2DM (Changotra et al., 2022).

Short-term exposure to urinary proteins led to a rise in autophagic activity in renal tubules. This was indicated by an increase in autophagic flux, measured through the transit of LC3-II in the presence of a lysosomal inhibitor. The findings suggest heightened production of autophagosomes, resulting in the accumulation of autophagic vacuoles (H. J. Zheng et al., 2020). However, it is widely understood that autophagosome

accumulation does not always imply autophagy induction and may represent restricted autolysosome maturation (W. Liu et al., 2018).

LC-3 associated phagocytosis is crucial in removing dying cells to avert excessive pro-inflammatory cytokine production (Nakashima et al., 2021). Huang et al. have revealed that Sirt1 promotes LC-3 nucleocytoplasmic transport via LC-3 deacetylation, which promotes starvation-induced autophagy. Also, Fang et al. found that hyperglycemia stopped basal autophagy in cultured podocytes by lowering the expression level of Beclin1, Atg5-12, and LC-3. They also found that rapamycin fixed the wrong autophagy caused by hyperglycemia and protected podocytes from damage caused by hyperglycemia. Kitada et al. found that the expression of the LC-3 gene in the plasma of DN patients was much lower than in T2DM and healthy groups (Kitada et al., 2017).

The goal of this investigation was to evaluate serum LC3-II levels in T2DM patients with renal insufficiency by introducing a gold biomarker with a higher correlation between the presence and severity of albuminuria and a decline in GFR.

According to the ACR classification, serum levels of LC3-II significantly increased in macro- and microalbuminuria compared to normoalbuminuria and the healthy group, as shown by our results. As per the DKD stage classifications, serum LC3-II levels were significantly elevated in stages 3 and 2 compared to stage 1, as declared by our findings. This discovery aligns with findings from other research (Barutta et al., 2023; Gou et al., 2016). In contrast to our findings, decreased levels of serum LC3-II were obtained (Yassin et al., 2021). Additionally, univariant correlations of LC3-II with T2DM patients by proteinuria groups showed highly significant correlations with Cys-C, BCL-2, PODCN, GRP78, IL-1 β , and TGF- β 1.

IL-1 β performs an essential role in initiating and regulating the inflammatory response by boosting the aggregation and adhesion of inflammatory cells and activating the subsequent inflammatory cascade (Ding et al., 2021). The significance of the innate proinflammatory cytokine IL-1 β was recently documented in inducing a metabolic shift from oxidative phosphorylation to glycolysis in kidney SCs, hence facilitating proximal tubule impairment and fibrosis (Tziastoudi et al., 2022). Research findings have indicated that upon exposure to the GSDMD-N-terminal, the phospholipid structure becomes

embedded within the inner membrane of the cell, forming minuscule pores. Subsequently, water enters the cell, causing cellular swelling and eventual cell membrane rupture. This process ultimately results in the significant release of numerous pro-inflammatory mediators inside the cell (Y. Li et al., 2022).

IL-1 β is elevated in both the serum and kidneys of individuals with DN and plays a significant role in the advancement of DN (Rico-Fontalvo et al., 2023). IL-1 β is a growth factor that functions in an autocrine way. It has been shown through experiments to play a role in stimulating the growth of glomerular mesangial cells (Wu et al., 2021). Specifically, IL-1 β promotes the proliferation of mesangial cells and the accumulation of matrix (Z. C. Zheng et al., 2020).

Multiple studies have shown that inflammation significantly contributes to the accelerated progression of DN. Numerous factors, including prolonged hyperglycemia, obesity, and ROS, can activate the NLRP3 inflammasome, a crucial component in preserving innate immunity. These variables then activate pro-inflammatory agents like IL-1 β , leading to the development of kidney dysfunction in diabetes patients (H. Feng et al., 2021). Another investigation revealed the expression and secretion of IL-1 β were elevated in HK-2 cells induced by hyperglycemia in a manner that depended on the duration of exposure (Hou et al., 2021).

Inflammation plays a crucial role in the progression of DN, as displayed by multiple studies. Additionally, Niknami et al. reported that patients with DN had significantly higher levels of IL-1 β compared to diabetic patients without nephropathy (Liao, Xiao, et al., 2022). Inflammatory biomarkers like IL-1 β have been utilized for assessing DKD advancement (C. Y. Jung & Yoo, 2022). Patients with DN had higher levels of the proinflammatory cytokine IL-1 β (Araújo et al., 2020). The carrier proteins that produce LC-3B protein capture the cytokine IL-1 β from the fluid within the cell and fuse with the outer membrane surrounding the cell to allow the release of IL-1 β via secretory autophagy (Gonzalez et al., 2021).

There wasn't a big difference in IL-1 between T2DM patients and controls based on proteinuria and DKD stages. However, we did find a link between patient groups and healthy groups based on proteinuria with LC3-II, and the link got stronger as the proteinuria got worse. Contrary to our findings, IL-1 β is enhanced in patients with DN

compared to non-DN, as shown in other studies (S. W. Jung & Moon, 2021; Lei et al., 2019). According to the prior-documented literature, we can conclude the importance of IL-1 β , which plays a role in stimulating the growth of glomerular mesangial cells and activating pro-inflammatory agents like IL-1 β , which induce kidney dysfunction in patients with DM.

Upon exposure of the GSDMD-N-terminal, the phospholipid structure becomes embedded within the inner membrane of the cell, and water enters the cell, causing cellular swelling and eventual rupture of the cell membrane to stimulate apoptosis. Based on our findings, it may be that elevated serum LC3-II suppressed pro-inflammatory factors like IL-1 β .

Hyperglycemia elevates ROS and oxidative free radicals. Raised levels of ROS trigger an inflammatory reaction, impair the regular operation of vascular endothelial cells, increase the production of TGF- β 1 and ECM proteins, and ultimately result in the progression of renal fibrosis (Wu et al., 2021). The occurrence of tubulointerstitial injury precedes glomerular injury in the progression of DN. The primary factors contributing to DN are oxidative stress-induced damage and excessive TGF- β 1/Smads signaling pathway stimulation (Du et al., 2018).

Autophagy plays a crucial role in regulating TGF- β levels and crucially mitigating kidney fibrosis. This mechanism involves the breakdown of mature TGF- β through autophagy, which is implicated in diabetic glomerulosclerosis and the excessive buildup of fibrotic materials in DN (Guo et al., 2020). Glomerulosclerosis and tubulointerstitial fibrosis result from excessive production of extracellular matrix proteins in renal cells, which is triggered by the overexpression of TGF- β 1 (L. Wang & Li, 2020). Dai et al. proposed that apoptosis in renal tubular epithelial cells induced by TGF- β 1 is primarily mediated through MAPK pathway activation rather than the Smad pathway.

Furthermore, Wu et al. demonstrated that TGF- β 1 triggers apoptosis in mouse glomerular podocytes by activating the p38 MAPK pathway (Y. Wang et al., 2022). TGF- β 1 is a signal that promotes fibrosis by inducing the conversion of fibroblasts into myofibroblasts, cells associated with fibrotic tissue (Prakash, 2012).

The increase in ROS triggers an inflammatory reaction, impairs the typical functioning of vascular endothelial cells, enhances the production of TGF- β 1 and ECM proteins, and ultimately results in the development of kidney fibrosis. TGF- β 1 is a cytokine that promotes fibrosis and functions as a critical pathogenic component in DN (Wu et al., 2021). Qiao et al. and Mou et al. showed that people with DM and DN have higher levels of TGF- β 1 in their serum and urine (T. Zhou et al., 2018).

The serum level of TGF- β 1 in patients with microalbuminuria was shown to be significantly elevated compared to patients with normoalbuminuria, and macroalbuminuria was also significantly higher compared to patients with microalbuminuria (T. Zhou et al., 2018). TGF- β 1 signaling is a crucial factor in the development of fibrosis (R. Liu et al., 2020). Recent reliable data indicated that renal expression of TGF- β is elevated in DKD. TGF- β is widely recognized as a significant pro-fibrotic factor contributing to glomerulosclerosis and interstitial fibrosis development in DKD and other chronic nephropathies (Guiteras et al., 2019). Glomerulosclerosis and tubulointerstitial fibrosis result from excessive production of extracellular matrix proteins in renal cells, which is triggered by the overexpression of TGF- β 1 (L. Wang & Li, 2020).

Furthermore, serum TGF- β 1 increased significantly in T2DM patients with macroalbuminuria compared to the normoalbuminuria group. TGF- β 1 is a crucial mediator in the progression of kidney disorders, particularly DN, and it controls cell proliferation and death (Kitada et al., 2017). In the early stages of DN, TGF- β 1 was thought to be an accurate biomarker. Hyperglycemia increases TGF- β 1 expression, which is thought to be the most critical cytokine promoting glomerulosclerosis and tubulointerstitial fibrosis. According to Xie's findings, serum TGF- β 1 levels in T2DM patients with microalbuminuria were considerably higher than in T2DM patients with normoalbuminuria and healthy groups (J. Zhang et al., 2018). Instead of increasing glomerular filtration, reduction of tubular reabsorption of albumin tends to be the primary cause of albuminuria in patients with T2DM, as mentioned earlier (Chang et al., 2016).

Serum levels of TGF- β 1 exhibited a significant increase in T2DM patients with macroalbuminuria and microalbuminuria compared to normoalbuminuria, based on our findings from the current study. This finding is in agreement with other studies (Kitada et al., 2017; J. Zhang et al., 2018; T. Zhou et al., 2018). In addition to the DKD stages,

serum TGF- β 1 levels were significantly higher in stages 2 and 3 compared to stage 1. The level of TGF- β 1 displayed a significant correlation with LC3-II, as demonstrated by our results.

The ER stress response within mammalian cells consists of three primary branches, including the PERK, IRE1, and ATF6 pathways. In reaction to ERS, the pERK pathway promptly reduces protein translation. On the other hand, the IRE1 pathway boosts the expression of factors involved in ER-associated degradation, which helps decompose unfolded proteins. Additionally, the ATF6 pathway enhances the expression of ER chaperones, such as GRP78/BiP and calreticulin, to facilitate the refolding of unfolded proteins accumulated within the ER (Z. Gao et al., 2018).

The susceptibility of islet β -cells to changes in the ER is significant. ERS in patients with DM is mainly caused by factors such as high nutritional intake and higher levels of FFA, which are predominantly influenced by hyperglycemia. Chronic stimuli can trigger ERS in renal tubular epithelial cells or other renal cells, which may lead to apoptosis. ERS can also result in damage to glomerular epithelial cells, leading to the development of proteinuria (X. Y. Sun et al., 2016). ER is known to be active during periods of stress, and an increase often follows this activation in the production of GRP78 (Deng et al., 2022). GRP78 and p-ERK are valuable indicators of ERS, while caspase-12 functions as a marker for ERS-induced apoptosis (Z. Gao et al., 2018).

Upregulation of chaperone proteins is a cellular mechanism that is associated with UPR. These chaperone proteins play a crucial role in facilitating the correct folding of proteins. GRP78, a chaperone protein, has been specifically identified as an inducer of DN (Hartner et al., 2014). Previous studies conducted by Chen, J. et al. showed that AGEs can induce premature senescence of RTECs through the activation of the GRP78-dependent ATF4/p16 and p21 signaling pathways in DN (J. Chen et al., 2022).

Higher concentrations of GRP78 promote defense against misfolded proteins by inhibiting their aggregation, enhancing their typical translocation, and starting the UPR pathway (Tian et al., 2018). The assemblage of unfolded or misfolded proteins in the lumen of the ER causes ERS, which can be seen in diseases like DN (Y.-P. Han et al., 2023). ERS increases GRP78 expression, activates caspase-12 pathways, and causes

apoptosis in mouse podocytes, possibly leading to the development of DKD. Previous research by N. Ma et al. discovered greater serum GRP78 concentrations in patients with T2DM than in the healthy control group (N. Ma et al., 2021). Research indicates that in mice with DKD, there is damage to the glomeruli. In DKD mice, there was an increase in the expression of GRP78 and caspase-12, while the expression of BCL-2 was decreased (R. Zhang et al., 2023).

The GRP87 level was significantly increased in macro- and microalbuminuria compared to normoalbuminuria and controls. A similar result was reported in the literature (N. Ma et al., 2021). In addition to the statistically significant elevation displayed in stages 3 and 2 compared to stage 1 according to DKD stages, as demonstrated by our study data, GRP87 also positively correlated with LC3-II in all groups based on proteinuria, as exhibited in our findings.

BCL-2 functions as a regulator of apoptosis, providing antiapoptotic effects. The protein BCL-2 works by stopping the release of Cytochrome-C and the apoptosome from forming with Apaf1. These actions eventually end in the suppression of caspase-9 and, subsequently, caspase-3 (Q. Zhang et al., 2019).

BCL-2, a critical mediator, plays a significant role in autophagy and apoptosis counter-regulation. It interacts with beclin-1, a pro-autophagic protein, and influences cell selection between apoptosis and autophagy (Hamzawy et al., 2017; Valle et al., 2021). Beclin-1 deactivates BCL-2 by inducing phosphorylation, which leads to enhanced apoptotic cell death. (Munasinghe et al., 2016).

Furthermore, the anti-apoptotic protein BCL-2 inhibits Beclin-1-dependent autophagy. As a result, BCL-2 family members acted as autophagic pathway inhibitors (P. Zhang et al., 2020). Prior studies have documented that the phosphorylation of BCL-2 at Ser70 can prevent BCL-2 degradation, boost the stability of the BCL-2/Bax interaction to suppress apoptosis, and disrupt the BCL-2/Beclin-1 complex to encourage autophagy (X. qi Liu et al., 2022). The findings of the previous study by Guo et al. were consistent with earlier studies that demonstrated that patients with DM had higher levels of apoptotic mediators and extra cell death due to apoptosis (Guo et al., 2021).

Additionally, it was found that exposure to hyperglycemia resulted in an induction of Bax protein expression and a downregulation of BCL-2 protein expression (Y. Chen

et al., 2019). Phosphorylation of BCL-2 and other anti-apoptotic BCL-2 family members contributes to its inactivation (Y. H. Shen et al., 2018).

According to research by Naguib et al., there is a connection between the presence of DKD and the severity of albuminuria, as well as the reduced blood concentration of the autophagy biomarker Beclin-1 (K. Cui & Li, 2023). Another study investigated the effect of BCL-2 on renal damage in hyperglycemia cells and found a significant decrease in its expression. This reduction in BCL-2 levels suggested the occurrence of severe renal damage and the initiation of cell apoptosis (H. Liu & Sun, 2019)

The level of BCL-2 showed a significant increase in microalbuminuria compared to normoalbuminuria. Furthermore, our study revealed a statistically significant increase in stages 3 and 2 compared to stage 1, as defined by DKD stages. Additionally, our results indicated a positive correlation between BCL-2 and LC3-II across all groups based on proteinuria levels. Ultimately, our interpretation indicated a moderate decline in serum levels of BCL-2 with the progression of DKD. Consequently, this decline disrupts the regulation of renal autophagy. Based on our discoveries, we propose that the LC3-II autophagic biomarker serves as a promoter for antiapoptotic BCL-2.

Podocin is the essential protein located on the slit diaphragm of podocytes, which exhibits unique expression exclusive to podocytes. The pathogenesis of proteinuria, observed in both congenital nephrotic syndrome and developed glomerular disease, directly affected podocin (S. M. Zhao et al., 2019). Nephrin, located on the side of the cell and extending into the slit diaphragm, controls the structure of the cytoskeleton and influences the shape and survival of podocytes. Within the podocyte foot process's lipid rafts, podocin interacts with nephrin's cytosolic tail, which is located in the precise same spot as nephrin. (X. Cao et al., 2019).

The study by Mollet et al. showed that mice lacking the podocin gene had a variety of glomerular lesions, demonstrating that the histologic patterns of renal injury were dependent on the developmental stage. The results showed that people with diabetes had higher levels of podocin than a control group, regardless of their ACR (Mizdrak et al., 2022).

Additionally, Tagawa et al. have documented that the impairment of podocyte autophagy worsens proteinuria in cases of DN (Dong et al., 2019). Podocyte injury in

DN, such as a decrease in the number and density of podocytes, the disappearance of the foot processes, the detachment and destruction of the slit diaphragm, the death of podocytes, and then hypertrophy and/or dedifferentiation, which leads to podocyte death rates (C. Wang et al., 2018).

The podocyte plays a vital role in maintaining the integrity of the glomerular filtration barrier, hence inhibiting the passage of proteins into the urine (Samsu et al., 2019). Several studies have shown that hyperglycemia, insulin resistance, and inflammatory and toxic insults caused by AGEs can lead to podocytopathy, which can lead to proteinuric DN. It was shown that hyperglycemia impacts excess mitochondria and enhances the production of ROS. Stimulates the process of apoptosis in glomerular podocytes (Heyman et al., 2022).

Even though albuminuria is a hallmark of renal impairment in patients with T2DM, a significant number of DKD patients do not acquire it (Gonzalez et al., 2021). PDCN was found to be considerably lower in T2DM patients with microalbuminuria and macroalbuminuria as compared to healthy subjects. Since tubulointerstitial impairment instead of glomerular damage is highly linked with the deterioration of kidney function, PDCN can be regarded as a sign of advanced-phase DN (El-Shazly et al., 2019).

In comparison to normoalbuminuria, serum PDCN levels were significantly higher in macroalbuminuria, as indicated by the proteinuria groups. Furthermore, our results show a significant elevation in serum PDCN levels in stage 3 compared to stage 1, as observed in the DKD stages. Additionally, our study revealed a significant positive correlation between PDCN and LC3-II levels across all categories, based on the proteinuria levels analyzed.

In the scope of this research, the performance of LC3-II in the differential diagnosis of macroproteinuria T2DM patients from normoproteinuria T2DM patients was evaluated, as were the levels of podocin, FBG, and HbA1c. LC3-II was the parameter with the highest diagnostic performance. LC3-II showed adequate performance in the differential diagnosis of T2DM patients with macroproteinuria from T2DM patients with normoproteinuria, with an AUC of 0.709 (0.605–0.812) and 73.6% accuracy. Based on our findings, serum LC3-II levels might be useful as a new biomarker to detect the difference between T2DM patients with proteinuria and those in the DKD stages. To

complete this view, further studies should evaluate the levels of serum LC3-II and how they relate to other biochemical markers in individuals with type 2 diabetes who have renal impairment. Centrally, the clinician and laboratorian for diagnosis and follow-up in T2DM and DKD patients profoundly take care of FBS, HbA1c, and kidney function tests (Scr. BUN, dipstick). As mentioned earlier, following our results, we suggest the insertion of extra tests to determine UPCR, ACR, eGFR, and LC3-II biomarkers for a specific diagnosis of DKD within the initial stages and controlling T2DM.

DKD is often diagnosed clinically based on a high level of albuminuria and/or decreased eGFR without any symptoms or evidence of other fundamental kidney injury (ElSayed et al., 2023). Impaired kidney function frequently appears with normal albuminuria (ACR < 30 mg/g), particularly in elderly people. However, renal failure without albuminuria is unusual (Persson & Rossing, 2018).

The results of our study show that patients with T2DM who consistently have hyperglycemia have higher levels of advanced glycation end products (AGEs) and hyperlipidemia. These metabolic changes occurring in T2DM patients may trigger the development of chronic renal disease through various biochemical pathways. One of these pathways could involve chronic inflammation in renal tubular cells due to the decrease in hemoglobin levels, as evidenced by our research findings, which may lead to tubular necrosis. The elevation in TGF- β 1 levels in the data we examined supports this pathway.

Another pathway may involve increased apoptosis in renal tubular cells due to the decrease in anti-apoptotic BCL-2 levels observed in DKD patients, in accordance with our results. The metabolic changes leading to decreased BCL-2 levels have been shown to increase the levels of LC3-II, a key protein involved in autophagic signaling pathways known for their protective effects on renal tubular cells in DKD patients, as presented in our results. This increase in LC3-II levels, associated with disease duration and severity, may be considered a new signaling pathway for DKD, as it reduces pro-inflammatory responses in renal tubular cells by decreasing IL-1 β and TGF- β 1 levels.

Moreover, it was found that LC3-II is associated with clinical and laboratory data in DKD and is considered a key protein that may contribute to podocyte damage through various signaling pathways in patients with T2DM. Serum LC3-II levels, shown in our results to have sufficient diagnostic performance for DKD, could be considered a useful laboratory parameter for monitoring the development of renal damage in patients with T2DM.



The conventional biomarker approach has certain limitations that need to be acknowledged and addressed

The research investigation had a limited number of participants and was conducted at a single center, which may not accurately represent the characteristics of DKD patients from multiple centers. Furthermore, this clinical study provides evidence of the evolution of serum autophagic biomarkers such as TGF- β 1, IL-1 β , CYS-C, GRP78, BCL-2, LC3-II, and PDCN. To address the study's limitations, future research should include biopsy specimens and assess these biomarkers alongside urine samples and gene analysis. Additionally, the literature lacks sufficient information on current topics such as autophagy, apoptosis, ERS, podocyte injury, inflammation, and necrosis biomarkers across various types of specimens. This gap hinders precise diagnosis and leaves the progression from DM to DKD poorly understood.

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7 SUFFICES

Suffice-1

INFORMED VOLUNTEER CONSENT FORM PATIENTS GROUP

Statement of the Researcher/Physician

We are planning to conduct scientific research to determine biochemical parameters that will help diagnose patients with diabetes-related kidney disease (diabetic nephropathy). The name of the planned research is "Evaluation of the relationship between autophagy and renal functions in diabetic nephropathy patients".

We invite you to participate in this study, which focuses on patients diagnosed with diabetic nephropathy. Participants will be followed up for as long as their medical condition meets the criteria of the patient sample for this study. **The patient sample consists of patients with T2DM, have no cancer, liver disease, T1DM, pregnancy, and infectious diseases.** However, it is essential to clarify that participation in this research is entirely voluntary. You must make your own decision to participate in this scientific study of your own free will. No one can suggest or pressure you into making this decision.

Before you make your decision, we would like to inform you about the scientific research in question and the procedures that will be undertaken if you agree to participate. If, after reading and understanding this information, you wish to take part in this scientific research, please sign the form.

Information about scientific study

The reason for your invitation to participate in this research is that you fall outside the age range of 35–85. Furthermore, inclusion in the study requires that you do not have any other kidney diseases aside from diabetes and that you have not experienced any infections or engaged in drug use that could affect your kidneys in the last 15 days. Another criterion for participation is the absence of a history of heart or liver failure or cancer or type 1 diabetes mellitus, and pregnancy. This research is conducted in collaboration with the Department of Biochemistry and the Department of Internal Medicine. The tests to be conducted in this study include novel tests aimed at elucidating conditions causing kidney damage due to diabetes, as well as tests utilized in monitoring diabetes and kidney disease. We will examine these tests in diabetic patients, individuals with diabetes-related kidney disease, and healthy individuals to assess potential differences among them. Through this, we aim to evaluate the impact of this new method of measuring kidney damage in diabetic patients and the potential utility of these tests in diagnosis and follow-up care.

1. Autophagic Markers
2. Urea, creatinine
3. Cystatin-C
4. Complete urine tests (TIT)
5. Glucose, Insulin

6. Albumin
7. Urine Albumin
8. Urine creatinine
9. Urine Glucose
10. Urine Total Protein
11. HbA1c

Within the scope of this study, we will collect two tubes of blood and a urine sample from you for the purpose of conducting the aforementioned tests.

Situations that need to be known within the scope of the study and rules that researchers and volunteers must follow

If you choose to participate in the research:

1. You will not be required to pay any fees.
2. You will not receive any additional payment for your participation in the study.
3. Your confidentiality will be strictly maintained, with your information kept solely between you and the physician and treated with great care and respect.
4. Your personal information will be safeguarded meticulously when the research results are utilized for educational and scientific purposes.
5. The researchers bear full responsibility for any health-related or other adversities that may arise during the study.
6. You have the option to withdraw from the research at any point, voluntarily. However, it is essential to inform the researchers before doing so.
7. Declining to participate in the study will not affect your treatment or clinical follow-up. Your disease will continue to be treated with the same level of care and attention as always.

Declaration of Participant (Volunteer) / Patient

Assoc. Dr. Köksal Deveci has communicated that research will be conducted in collaboration with the Gazi Osmanpaşa University Faculty of Medicine, specifically involving the Department of Biochemistry and the Department of Internal Medicine. I have been informed about this research endeavor. Subsequently, I was invited to participate in this research as a "participant."

If I choose to participate in this research, it has been clearly stated that the confidentiality of my information, which is to be kept strictly between myself and the physician, will be handled with the utmost care and respect throughout the research process. Furthermore, it has been assured that my personal information will be diligently safeguarded when the research results are utilized for educational and scientific purposes.

I am not liable for any financial expenses related to the research. It has been explicitly stated that I will not be charged any fees, nor will I receive any payments for my participation.

I've been informed that I have the right to withdraw from the research at any point during its execution without providing a reason. However, I understand that it would be considerate to inform the researchers in advance of my decision to withdraw to avoid placing them in a challenging position. Furthermore, I acknowledge that the researcher may opt to exclude me from the research, provided it does not pose any risk to my medical condition.

The responsibility for any adverse health conditions that may arise during the research process, whether directly or indirectly, lies with the researchers. I will not bear any financial burden associated with such occurrences.

When encountering a health-related issue in my research, I can reach out to Associate Professor Dr. Köksal Deveci at any time of the day for advice at 5057570305.

I am not obligated to participate in this research, and I may choose not to do so. I have not experienced any coercive behavior regarding my involvement in the research. Additionally, I understand that declining to participate will not adversely affect my medical care or my relationship with the physician.

I have thoroughly understood all the explanations provided to me. Following a period of reflection, I have chosen to participate in this research project as a "participant" (volunteer) of my own free will. I gladly accept the invitation in this capacity and volunteer my participation with great pleasure.

Participant (Volunteer) Name and Surname:

Address:

Telephone:

Signature:

Interview Witness

Name and surname:

Address:

Telephone:

Signature:

Researcher Interviewing Participant (Volunteer)

Name, Surname, and Title:

Address:

Telephone:

Signature:

(A copy of this form, with all pages signed, will be provided to the participant.)

Suffice-2

INFORMED VOLUNTEER CONSENT FORM CONTROL GROUP

Statement of the Researcher/Physician

We are planning to conduct scientific research to determine biochemical parameters that will help diagnose patients with diabetes-related kidney disease (diabetic nephropathy). The name of the planned research is "Evaluation of the relationship between autophagy and renal functions in diabetic nephropathy patients".

We invite you to participate in this study, which focuses on patients diagnosed with diabetic nephropathy. Participants will be followed up for as long as their medical condition meets the criteria of the control sample for this study. **The control sample consists of individuals who are considered healthy, have no chronic systemic or metabolic diseases, and have not been diagnosed with diabetic nephropathy.** However, it is essential to clarify that participation in this research is entirely voluntary. You must make your own decision to participate in this scientific study of your own free will. No one can suggest or pressure you into making this decision.

Before you make your decision, we would like to inform you about the scientific research in question and the procedures that will be undertaken if you agree to participate. If, after reading and understanding this information, you wish to take part in this scientific research, please sign the form.

Information about scientific study

The reason for your invitation to participate in this research is that you fall outside the age range of 35–85 and do not have kidney disease caused by diabetes (diabetic nephropathy). Furthermore, inclusion in the study requires that you do not have any other kidney diseases aside from those related to diabetes and that you have not experienced any infections or engaged in drug use that could affect your kidneys in the last 15 days. Another criterion for participation is the absence of a history of heart or liver failure or cancer or type 1 diabetes mellitus, and pregnancy. This research is conducted in collaboration with the Department of Biochemistry and the Department of Internal Medicine. The tests to be conducted in this study include novel tests aimed at elucidating conditions causing kidney damage due to diabetes, as well as tests utilized in monitoring diabetes and kidney disease. We will examine these tests in diabetic patients, individuals with diabetes-related kidney disease, and healthy individuals to assess potential differences among them. Through this, we aim to evaluate the impact of this new method of measuring kidney damage in diabetic patients and the potential utility of these tests in diagnosis and follow-up care.

1. Autophagic Markers
2. Urea, creatinine
3. Cystatin-C
4. Complete urine tests (TIT)
5. Glucose, Insulin
6. Albumin

7. Urine Albumin
8. Urine creatinine
9. Urine Glucose
10. Urine Total Protein
11. HbA1c

Within the scope of this study, we will collect two tubes of blood and a urine sample from you for the purpose of conducting the aforementioned tests.

Situations that need to be known within the scope of the study and rules that researchers and volunteers must follow

If you choose to participate in the research:

8. You will not be required to pay any fees.
9. You will not receive any additional payment for your participation in the study.
10. Your confidentiality will be strictly maintained, with your information kept solely between you and the physician and treated with great care and respect.
11. Your personal information will be safeguarded meticulously when the research results are utilized for educational and scientific purposes.
12. The researchers bear full responsibility for any health-related or other adversities that may arise during the study.
13. You have the option to withdraw from the research at any point, voluntarily. However, it is essential to inform the researchers before doing so.
14. Declining to participate in the study will not affect your treatment or clinical follow-up. Your disease will continue to be treated with the same level of care and attention as always.

Declaration of Participant (Volunteer) / Patient

Assoc. Dr. Köksal Deveci has communicated that research will be conducted in collaboration with the Gazi Osmanpaşa University Faculty of Medicine, specifically involving the Department of Biochemistry and the Department of Internal Medicine. I have been informed about this research endeavor. Subsequently, I was invited to participate in this research as a "participant."

If I choose to participate in this research, it has been clearly stated that the confidentiality of my information, which is to be kept strictly between myself and the physician, will be handled with the utmost care and respect throughout the research process. Furthermore, it has been assured that my personal information will be diligently safeguarded when the research results are utilized for educational and scientific purposes.

I am not liable for any financial expenses related to the research. It has been explicitly stated that I will not be charged any fees, nor will I receive any payments for my participation.

I've been informed that I have the right to withdraw from the research at any point during its execution without providing a reason. However, I understand that it would be

considerate to inform the researchers in advance of my decision to withdraw to avoid placing them in a challenging position. Furthermore, I acknowledge that the researcher may opt to exclude me from the research, provided it does not pose any risk to my medical condition.

The responsibility for any adverse health conditions that may arise during the research process, whether directly or indirectly, lies with the researchers. I will not bear any financial burden associated with such occurrences.

When encountering a health-related issue in my research, I can reach out to Associate Professor Dr. Köksal Deveci at any time of the day for advice at 5057570305.

I am not obligated to participate in this research, and I may choose not to do so. I have not experienced any coercive behavior regarding my involvement in the research. Additionally, I understand that declining to participate will not adversely affect my medical care or my relationship with the physician.

I have thoroughly understood all the explanations provided to me. Following a period of reflection, I have chosen to participate in this research project as a "participant" (volunteer) of my own free will. I gladly accept the invitation in this capacity and volunteer my participation with great pleasure.

Participant (Volunteer) Name and Surname:

Address:

Telephone:

Signature:

Interview Witness

Name and surname:

Address:

Telephone:

Signature:

Researcher Interviewing Participant (Volunteer)

Name, Surname, and Title:

Address:

Telephone:

Signature:

(A copy of this form, with all pages signed, will be provided to the participant.)

Permission of the Ethics Committee

Lisansüstü Eğitim Enstitüsü Yürütme Kurulu
ENSTİTÜ YÖNETİM KURULU KARARLARI

TOPLANTI TARİHİ	TOPLANTI SAYISI	KARAR NO
09/09/2022	37	2022-37/ 51

Tokat Gaziosmanpaşa Üniversitesi Lisansüstü Eğitim Enstitüsü Enstitü Yönetim Kurulu, Enstitü Müdürü Vekili Doç. Dr. Hüseyin Baha ÖZTUNÇ başkanlığında toplanarak aşağıdaki kararları almıştır.

KARAR NO 2022-37/51: Tıbbi Biyokimya Anabilim Dalı Başkanlığı'nın 08/09/2022 tarih ve 202062 sayılı yazısı okundu.

Buna göre Tıbbi Biyokimya Anabilim Dalı 209080651 numaralı doktora öğrencisi Shahab Ahmed Salih GEZH tarafından Doç. Dr. Köksal DEVECİ danışmanlığında hazırlanmış olan tez önerisi doktora tez önerisi savunma sınavında başarılı olduğu anlaşıldığından, Tokat Gaziosmanpaşa Üniversitesi Lisansüstü Eğitim ve Öğretim Yönetmeliğinin 20. maddesi gereğince; tez başlığının "Diabetik Nefropati Hastalarında Otofajinin Renal Fonksiyonlar İle İlişkisinin Değerlendirilmesi" olarak kabulüne ve 09/09/2022 (2021-2022 BAHAR YARIYILI) tarihi itibarıyla tez dönemine geçmesine oy birliğiyle karar verildi.

Doç. Dr. Hüseyin Baha ÖZTUNÇ
Enstitü Müdürü V.
(İmza)

Doç. Dr. Fatih YAZICI
Müdür Yardımcısı
(Görevli/İznil)

Doç. Dr. Hüseyin Baha ÖZTUNÇ
Müdür Yardımcısı
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Prof. Dr. Çetin ÇEKİÇ
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Prof. Dr. İlhan EROĞLU
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