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Role of Immunological Biomarkers, interleukin-40 and Interleukin-41 in Serum of Diabetic Patients with End Stage Renal Disease

A Thesis

Submitted to the Council of the College of Medicine/ University of Karbala in Partial Fulfillments of the Requirements for the Degree of Master in Medical Microbiology

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

(وَقُلْ رَبِّ زِدْنِي عِلْمًا)

صدق الله العلي العظيم

(طه :114)

Dedication

To ….

The dearest man in my life who lighted my way, to the bright star in my sky… guiding my nights for better tomorrows, my father.

To….

The kindest *woman in my life with most pure and endless love in the universe, my mother.*

To….

My companion...to the one who holds my hand in every endeavor I pursue, with her endless support...I share every dream and success with her, my dear wife

2025 ***Hussein***

Supervisors Certification

We certify that this M.Sc. thesis titled:

Role of Immunological Biomarkers, interleukin-40 and Interleukin-41 in Serum of Diabetic Patients with End Stage Renal Disease

Was prepared under our supervision in the College of Medicine/ University of Karbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology.

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Certification

We, the examiners committee, certify that we've read the M.Sc. thesis entitled: (Role of Immunological Biomarkers, interleukin-40 and Interleukin-41 in Serum of Diabetic Patients with End Stage Renal Disease).

We have examined the student (Hussein Abdul Raheem Abdul Nabi Jabr) in its contents. In our opinion, it meets the standards of a thesis for the degree of Masters in Medical Microbiology.

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Summary

Diabetic kidney disease (DKD) represents a one of severe chronic complications of diabetes mellitus and constitutes one of the primary causes of end-stage kidney disease globally. DKD also lead to changes in the hemodynamic of the immune cells components and pathophysiological inflammation which influence in the production, suppression, or downregulation of mediators.

this study aimed to evaluate IL-40 and IL-41 levels in diabetic patients generally and those with diabetic kidney disease specifically compared to healthy controls, determine the predictive value of these biomarkers, and investigate relationships between these novel indicators and traditional laboratory markers (HbA1c, creatinine, urea, ferritin). The study adopted a case-control design with 128 participants: 42 DKD patients, 43 diabetic patients without kidney disease (DM), and 43 healthy individuals. DKD and DM patients were recruited from Imam AL-Hussein Hospital in Karbala, while controls were collected from Al-Majar Al-Kabir Hospital in Maysan, during period between Non.2024 to Jan.2025. Blood samples were analyzed using ELISA technique (HUMAN German company kits) to measure IL-40 and IL-41 levels, alongside other laboratory indicators. Statistical analysis employed ANOVA, Kruskal-Wallis tests, correlation analysis, and ROC curves.

Key results revealed that IL-40 levels decreased by approximately 47.2% (15.8 ± 4.0 pg/mL) in DKD patients and 52.8% (14.1 ± 3.7 pg/mL) in DM patients, compared to healthy controls (29.9 ± 8.7 pg/mL). Similarly, IL-41 levels declined by about 82.4% (0.53 ± 0.18 pg/mL) in DKD patients and 78.7% (0.64 ± 0.11 pg/mL) in DM patients, relative to healthy individuals (3.01 ± 0.98 pg/mL), with all differences being statistically significant ($p < 0.001$). IL-41 demonstrated excellent predictive performance with an AUC of 98.4% and 95.3% accuracy in

distinguishing DKD patients from controls, whereas IL-40 showed good predictive utility with an AUC of 89.6% and 90.6% accuracy. additional biochemical changes in DKD patients included elevated urea and creatinine, decreased hemoglobin, and disturbed calcium and phosphate levels. No significant correlations were found between IL-40/IL-41 levels and traditional clinical indicators, except for a positive correlation between IL-40 and IL-41 in the DKD group ($r=0.335$, $p=0.030$).

This study confirmed a significant decrease in the levels of IL-40 and IL-41 in previously diagnosed diabetic kidney disease and diabetic patients compared to healthy individuals, suggested a potential role for these markers as complementary or predictive biological indicators. Notably, IL-41 demonstrated very high sensitivity and specificity in distinguishing known diabetic kidney disease patients.

However, study limitations included small sample size, lack of assessment of other inflammatory factors, and absence of long-term follow-up. The study recommends larger longitudinal clinical research to verify integrating these indicators into early diagnosis programs. In conclusion, this study provided initial evidence supporting the predictive potential and role of IL-40 and especially IL-41 in diabetic kidney disease of previously diagnosed patients, and due to the lack of correlation with traditional markers, it is recommended to conduct large-scale and longitudinal studies to validate these findings and explore their clinical applicability especially in the early stages of the disease to confirm their capability for early diagnosis or prediction.

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List of abbreviation

Abbreviation	Full meaning in English
ACR	Albumin-to-Creatinine Ratio
ADA	American Diabetes Association
ACE	Angiotensin-converting enzyme
AGEs	Advanced Glycation End-products
ALP	Alkaline Phosphatase
Anti ccp	Anti -cyclic citrullinated peptide antibody
ALT	Alanine Aminotransferase
AMPK	AMP-activated Protein Kinase
AMP	2-Aino-2-methyl-1-propanol
APOC-1	Apolipoprotein C1
APOE	Apolipoprotein E
AngII	Angiotensin II
ANOVA	Analysis of Variance
APCS	Amyloid P Component Serum
AST	Aspartate Aminotransferase
AUC	Area Under the Curve
B.Urea	Blood Urea
BIGE	Body index of gene expression
BCG	Bromocresol green
BSA	Bovine serum albumin
CAT	Catalase

Abbreviation	Full meaning in English
Cu^{2+}	Divalent copper ions
CBC	Complete blood count
CGM	Continuous Glucose Monitoring
CKD	Chronic Kidney Disease
C17	Chromosome 17
CLIA	Chemiluminescent Immunoassay
CCL3	Chemokine (c-c motif) ligand 3
C2C12	C2C12 myoblast cell line
COL4A3	Collagen Type IV Alpha 3 Chain
CV	Coefficient of Variation
CD	Cluster of Differentiation
CLSI	Clinical and laboratory standards institute
CRP	C-reactive protein
COPD	Chronic obstructive pulmonary disease
CVD	Cardiovascular Disease
DALYs	Disability-Adjusted Life Years
DKD	Diabetic Kidney Disease
DCCT	Diabetic control and complications trial
DAS28-ESR	Disease activity score 28-erthrocyte sedimentation rate
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

Abbreviation	Full meaning in English
DFIS	Diabetic food infections
EASD	European Association for the Study of Diabetes
ECM	Extracellular Matrix
eGFR	estimated Glomerular Filtration Rate
eNOS	Endothelial nitric oxide synthase
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
EP5-A2	Evaluation Protocol 5 – Version A2
EndMT	Endothelial-Mesenchymal Transition
ESKD	End Stage Kidney Disease
EDTA	Ethylenediaminetetraacetic acid
ESRD	End-Stage Renal Disease
FBG	Fasting blood gloucose
FPOX	Fructosyl peptide oxidase
Fe^{3+}	Ferric ions
GA	Glycated Albumin
GAD	Glutamic Acid Decarboxylase
GADA	Glutamic Acid Decarboxylase Antibodies
GDM	Gestational Diabetes Mellitus
GLDH	Glutamate Dehydrogenase
GOD-POD	Glucose Oxidase-Peroxidase
GPR91	G Protein-Coupled Receptor 91
GBD	Global burden of disease

Abbreviation	Full meaning in English
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
HB	Hemoglobin
HbA1c	Hemoglobin A1c
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
hsCRP	High sensitivity C-reactive protein
HSPG2	Heparin sulfate proteoglycan 2
H2O2	Hydrogen peroxide
IA-2	Insulinoma-Associated-2
ICA	Islet cell antibodyes
IGF-1	Insulin like growth factor
IBM	International business machines
IA-2A	Insulinoma-Associated-2 Antigen
IAA	Insulin Autoantibodies
ISE	Ion selective electrode
IDMS	Isotope Dilution Mass Spectrometry
ICE	Interleukin-1 Converting Enzyme
IQC	Internal quality control
INF-γ	Interferon gamma
IDF	International Diabetes Federation
IgM	Immunoglobulin M
IgG	Immunoglobulin G

Abbreviation	Full meaning in English
IgA	Immunoglobulin A
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IL-40	Interleukin 40
IL-41	Interleukin 41
L.F.T	Liver function test
LCD	Liquid crystal display
LLOD	Lower limit of detection
JAK-STAT	Janus Kinase - Signal Transducer and Activator of Transcription
KDLGO	Kidney disease improving global outcomes
LDH	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinases
MCP-1	Monocyte Chemotactic Protein-1
MDH	Malate Dehydrogenase
MetS	Metabolic Syndrome
MCP-1	Monocyte chemoattractant protein 1
M2	Macrophage 2
MMP-13	Matrix metalloproteinases 13
miR/miRNA	MicroRNAs
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDKD	Non diabetic kidney disease
NADH	Nicotinamide adenine dinucleotide hydrogenated
NAD	Nicotinamide adenine dinucleotide
NETosis	Neutrophil extracellular traps

Abbreviation	Full meaning in English
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NOS	Nitric Oxide Synthase
NLRP3	NOD-like receptor family pyrin domain containing 3
NIST SRM	National institute of standers and technology stander reference material
NGSP	National glycohemoglobin standardization program
NPV	Negative Predictive Value
OGTT	Oral Glucose Tolerance Test
$O2^{\cdot}$	Superoxide anion radical
$^{\cdot}OH$	Hydroxyl radical
$ONOO^{\cdot}$	Peroxynitrite anion
ORF	Open reading frame
OD	Optical density
p-NPP	p-nitrophenylphosphate
PKC	Protein Kinase C
PLT	Platelets
PAD	Peripheral arterial disease
PBS	Phosphate buffer solution
PH	Power of hydrogen
PEITA	Particle enhanced immunoturbidimetric assay
POD	Peroxidase
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
ppar δ	Peroxisome Proliferator-Activated Receptor delta
PPV	Positive Predictive Value

Abbreviation	Full meaning in English
p-NPP	P-nitrophenylphosphate
RAS/RAAS	Renin Angiotensin (Aldosterone) System
RBS	Random Blood Sugar
RBCs	Red blood cells
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RA	Rheumatoid arthritis
RF	Rheumatoid factor
R.F.T	Renal function test
S.Albumin	Serum Albumin
S.Ca	Serum Calcium
S.Cr	Serum Creatinine
S.Ferritin	Serum Ferritin
S.Na	Serum Sodium
$S.PO_4$	Serum Phosphate
S.Protein	Serum Total Protein
S.GOT	Serum Glutamate-Oxaloacetate Transaminase
S.GPT	Serum Glutamate-Pyruvate Transaminase
SNP	Single Nucleotide Polymorphism
SOX	Sarcosine oxidase
SGLT	Sodium-Glucose Transport Protein2 Inhibitors
SOD	Superoxide Dismutase
SPSS	Statistical Package for the Social Sciences

Abbreviation	Full meaning in English
STAT3	Signal transducer and activator of transcription
supAR	Soluble urokinase plasminogen Activator Receptor
T1DM	Type 1 Diabetes Mellitus
TRis	(hydroxymethyl)aminomethane
TMB	tetramethylbenzidine, TMB
T2DM	Type 2 Diabetes Mellitus
TAK-STAT	TGF-beta Activated Kinase - Signal Transducer and Activator of Transcription
TOOS	N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine
TGF-β/TGFβ1	Transforming Growth Factor Beta 1
TIBC	Total Iron Binding Capacity
TNF-α	Tumor Necrosis Factor Alpha
Tri-SNP	Triple Single Nucleotide Polymorphism
UIBC	Unsaturated Iron Binding Capacity
Urease-GLDH	Urease-Glutamate Dehydrogenase
VEGF	Vascular Endothelial Growth Factor
VOX	Vanadate oxidase method
WBC	White Blood Cells
WNT/B-catenin	Wnt/B-catenin signaling pathway
WHO	World Health Organization
ZnT8A	Zinc Transporter 8 Antibody
QC	Quality control

Chapter One
Introduction and Literature Review

1.1. Introduction:

Diabetic kidney disease(DKD) emerges as a major microvascular complication of diabetes mellitus, constituting a substantial healthcare burden through its considerable contribution to patient morbidity and mortality rates. Globally, DKD represents approximately 40% of global end-stage kidney disease prevalence, with differential impact rates of 30-50% among type 1 diabetic populations and 20-40% in type 2 diabetics. The pathophysiological progression of DKD is characterized by sustained albuminuria, decline in glomerular filtration rate, and the development of systemic complications.**(Templer *et al*., 2024;Caramori and Rossing , 2000 ; Hara, 2024)**.

Despite substantial advances in understanding DKD pathophysiology, the underlying immunological mechanisms remain inadequately characterized. Emerging evidence increasingly points to Chronic inflammation and mediators dysregulation as key drivers of disease progression, highlighting their potential as targets for early diagnosis and therapeutic intervention,**(Donate-Correa, *et al*., 2021)**.

In particular, two novel mediators, Interleukin-40 (IL-40) and Interleukin-41 (IL-41) have recently garnered attention for their immunomodulatory and metabolic functions. IL-40, identified in 2017, regulates B-cells and mucosal immunity and is linked to autoimmune diseases like rheumatoid arthritis and lupus. Recent findings demonstrate elevated IL-40 levels in type 2 diabetes, implying a possible role in diabetes-related inflammation **(Catalan-Dibene *et al*., 2017; Nussrat and Ad'hiah, 2023)**.

Interleukin-41 (IL-41) a newly discovered protein with 46% similarity to the neurotrophic factor Meteorin. Unlike Meteorin (which is brain-specific), IL-41 (Metrnl) is highly expressed in metabolic tissues and barrier organs, function as a myokine, released from the macrophage during muscle contraction, and enhances insulin sensitivity in skeletal muscle by reducing inflammation**(Shi *et al*., 2024)**.

Despite associations between IL-40/IL-41 and metabolic or autoimmune disorders, their precise involvement in diabetic kidney disease pathogenesis remains unclear. Current research has not clarified their relationship with diabetic renal inflammation, highlighting a critical need for further studies The research gap is widening in the Middle East particularly Iraq amid rapidly increasing diabetes rates and its complications. Unique genetic, environmental, and behavioral factors among Iraqis may influence cytokine profiles and disease progression, necessitating localized studies. recent Iraqi diabetes research has yielded critical insights into disease complications and inflammatory pathway.

Oleiwi, Al-Taie, **and Al-Hilali (2022)** documented a 63.6% nephropathy prevalence among 121 T2DM patients in Karbala, strongly associated with age, glycemic control, obesity and disease duration. parallel cytokine studies revealed IL-1/IL-6 dysregulation in nephropathy patients, **(Ameen *et al.,* 2020)**. IL-2/IL-17 correlations with renal markers **(Al-Rawi *et al*., 2022)** and low IL-9 predicting neuropathy **(Khalaf *et al*., 2023)**.More recently, **Muttaleb ,(2024)** established role of IL-40 in Iraqi insulin resistance. collectively, this research reveals, alarmingly high rates of diabetic complications among Iraqi patients, and the promising potential of cytokine-based biomarkers and individualized therapeutic approaches offers significant relevance for Iraq's unique patient population.

These findings underscore the urgent necessity of implementing high precision medicine strategies to address the growing diabetes burden in Iraq. Therefore, the current study aims to address this gap by examining serum levels of IL-40 and IL-41 in patients with and without diabetic kidney disease, and healthy controls. The primary hypothesis assumes the existence of clear differences in IL-40 and IL-41 levels between the study groups. The sub-hypotheses include that these cytokines may suggesting a potential role as complementary or predictive

biological indicators biomarkers for diabetic kidney disease, correlate with traditional laboratory indicators such as HbA1c, creatinine, urea, and ferritin, and may reflect distinct inflammatory pathways not detected by conventional markers.

To test these hypotheses, will measure serum IL-40 and IL-41 levels using ELISA across three distinct groups. Biochemical evaluations will include primary biomarkers (IL-40 and IL-41), conventional laboratory indicators (HbA1c, creatinine, urea, liver function tests, ferritin), hemoglobin, and electrolyte levels. we will assess both positive and negative correlations between (IL-40, IL-41) and conventional laboratory indicators. Statistical analysis will involve assessing diagnostic performance (sensitivity, specificity, predictive values), ROC curve analysis, correlation coefficients, and significance testing at a p-value threshold of <0.05. this study aims to contribute to identifying IL-40 and IL-41 a potential role as biomarkers that may serve complementary or predictive functions in the context of diabetic kidney disease, particularly in high-risk populations such as those in Iraq.

1.2. Literature Review

1.2.1. Definition of Diabetes mellitus:

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from impaired insulin secretion (as in Type 1 DM), insulin resistance (as in Type 2 DM), or a combination of both. Insulin, a hormone produced by pancreatic β-cells, plays a central role in glucose homeostasis. Dysregulation of insulin production or action leads to elevated blood glucose levels which, if left uncontrolled, contribute to the development of both microvascular complications such as retinopathy, nephropathy, and neuropathy and macrovascular complications including cardiovascular diseases **(ADA, 2023**)

Globally, DM affects more than 537 million adults, with prevalence continuing to rise, particularly in Middle Eastern countries such as Iraq. This regional burden is attributed to genetic susceptibility, sedentary lifestyles, and unhealthy dietary habits **(IDF, 2023**;**Webber ,2023).** Chronic hyperglycemia induces oxidative stress and systemic inflammation, which in turn contribute to end-organ damage. In the kidneys, this is manifested as albuminuria and a progressive decline in estimated glomerular filtration rate (eGFR); in the retina, as microaneurysms and vision impairment; and in peripheral nerves, as sensory loss and neuropathic pain **(Patil *et al*., 2023; ADA, 2023).**

1.2.2. The Global Burden and Diabetes epidemiology

Diabetes mellitus is among the fastest-growing health challenges of the twenty-first century. Over the past two decades, the global number of adults living with diabetes has more than tripled, imposing significant health and socioeconomic burdens on individuals and societies. Several factors contribute to the rising prevalence of diabetes, including demographic transitions such as population

aging, changes in dietary patterns, sedentary lifestyles, and increasing rates of overweight and obesity among both adults and children **(Sun *et al*., 2022)**.

According to the International Diabetes Federation (IDF), approximately 463 million people were living with diabetes in 2019. If current trends persist, this number is projected to rise to 700 million by 2045 **(Sun *et al*., 2022)**. more recent estimates indicate that 529 million individuals were affected by diabetes in 2021, with projections suggesting that this number will increase to 1.31 billion by 2050 **(Xu *et al*., 2024)**.This rising prevalence is expected to be accompanied by a proportional increase in diabetes-related complications, including severe morbidity, disability, reduced life expectancy, and diminished quality of life. Moreover, the disease contributes to economic losses at both individual and national levels due to decreased productivity and increased healthcare expenditures **(Sun *et al*., 2022)**.

In the Middle East, rapid socioeconomic transitions particularly in high-income oil-producing countries have led to significant lifestyle changes. These include unhealthy dietary habits, reduced physical activity, increased tobacco use, and a growing prevalence of obesity. Additionally, urbanization and longer life expectancy have further contributed to the increase in both diabetes and impaired glucose tolerance. a recent review by **Holt and Flyvbjerg,(2024)**, highlighted wide variations in diabetes prevalence across Middle Eastern countries, as well as a lack of comprehensive epidemiological data. Notably, countries such as Saudi Arabia, Kuwait, and Qatar exhibit some of the highest prevalence rates globally. Furthermore, approximately 73.4% of individuals with diabetes in Arab countries are under the age of 60, indicating a significant impact on the working-age population and exacerbating the socioeconomic burden of the disease. according to the latest statistics from the International Diabetes Federation (**IDF**) for 2024, the prevalence of diabetes among adults in Iraq (aged 20-79 years) is 13.4%. This

means that approximately 2.67 million adults in Iraq are living with diabetes. In 2021, the prevalence rate was 10.7%, indicating a significant increase in recent years.

1.2.3. Types of Diabetes:

Diabetes mellitus is primarily classified into three main categories: Type 1 diabetes, Type 2 diabetes, and gestational diabetes.

1.2.3.1. Type I diabetes

Type 1 diabetes mellitus (T1DM) is most commonly caused by immune-mediated destruction of the insulin-producing pancreatic β-cells. Although the precise trigger for this autoimmune response remains unknown, it leads to absolute insulin deficiency and a lifelong dependence on exogenous insulin therapy ,the disease is typically preceded by a variable-length preclinical phase during which circulating autoantibodies such as those targeting insulin, glutamic acid decarboxylase (GAD), and insulinoma-associated antigen 2 (IA-2) can be detected, indicating autoimmune activity against pancreatic islet cells **(Holt and Flyvbjerg ,2024)**. Clinically, Type 1 diabetes is characterized by overt hyperglycemia and the classic triad of symptoms: polyuria, polydipsia, and polyphagia **(Balaji,Duraisamy and Kumar ,2019)**.

Early histopathological studies of pancreatic tissues from individuals who died shortly after the onset of type 1 diabetes revealed significant structural alterations in the islets of Langerhans, including fibrosis, hyalinosis, atrophy, and infiltration by inflammatory cells. These findings provided early evidence of an immune-mediated process affecting the endocrine pancreas. Furthermore, impaired leukocyte migration in response to pancreatic islet antigens was also observed, supporting the role of autoimmune mechanisms in the pathogenesis of type 1 diabetes **(Holt and Flyvbjerg ,2024**). Accordingly, autoimmune responses

targeting pancreatic β-cells are now recognized as a central feature in the development of this disease. the natural history of autoimmune type 1 diabetes follows a well-defined progression characterized by the presence of islet autoantibodies, β-cell dysfunction, and the eventual onset of symptomatic hyperglycemia. This process unfolds over months to years and involves three distinct stages of disease development, each defined by immunologic and metabolic criteria. These stages, along with their immunogenetic and environmental determinants, are summarized in **Figure (1.1**.)

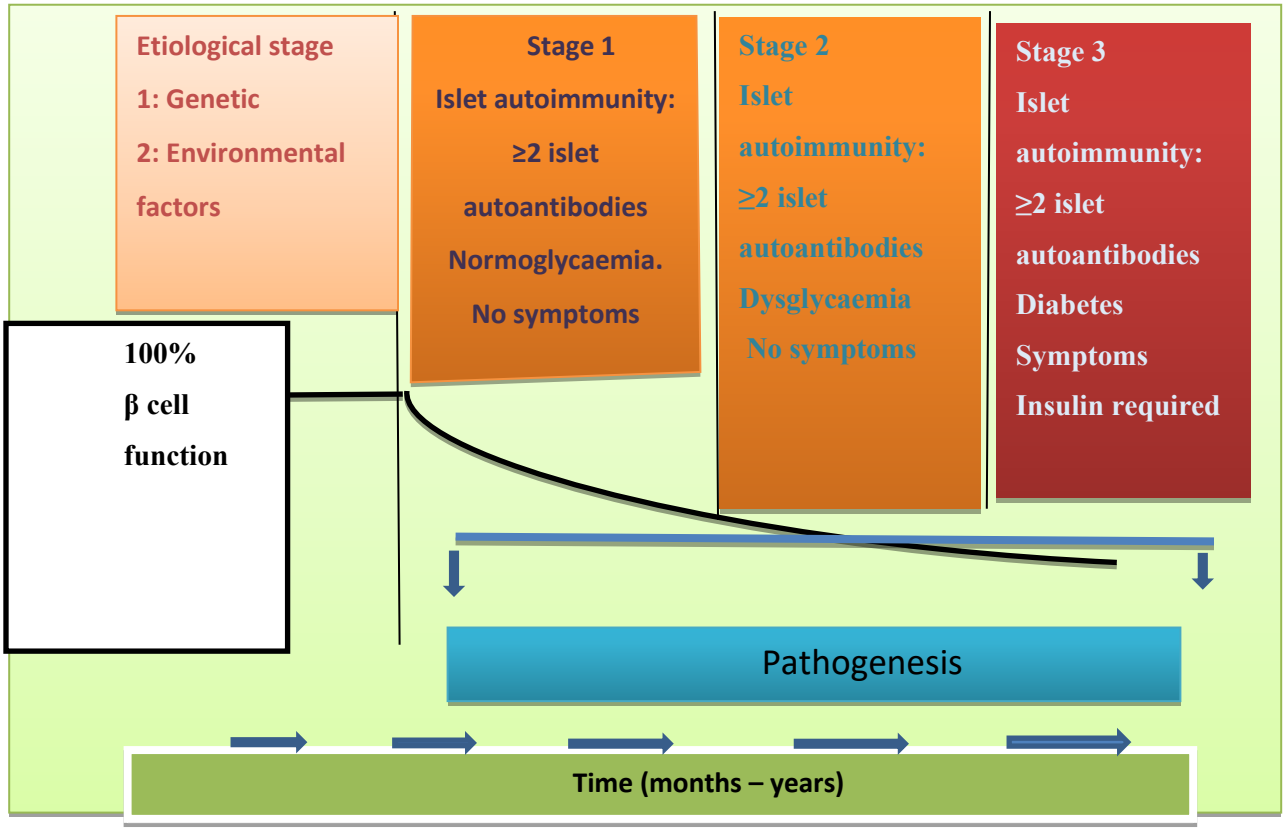

Figure 1.1: Staging of autoimmune type 1 diabetes mellitus based on immunological and metabolic criteria **(Insel *et al*., 2015).**

The figure illustrates the natural course of type 1 diabetes mellitus, beginning with an etiological phase influenced by genetic (e.g., HLA-DR3-DQ2, HLA-DR4-DQ8) and environmental triggers (e.g., viral infections). Stage 1 is characterized by the presence of ≥2 islet autoantibodies with normoglycemia and no clinical symptoms. Stage 2 includes the persistence of autoantibodies and evidence of dysglycemia, while stage 3 marks the onset of symptomatic diabetes with the need for exogenous insulin. β-cell function progressively declines across stages. The pathogenesis evolves over time, influenced by immune biomarkers such as IAA, GADA, IA-2A, and ZnT8A **(Insel *et al*., 2015).**

The pathogenesis of T1DM begins with a predisposed genetic background. The strongest genetic risk is associated with specific human leukocyte antigen (HLA) class II haplotypes, particularly HLA-DR3-DQ2 and HLA-DR4-DQ8 **(Pociot and Lernmark, 2016)**. The initial appearance of β-cell autoantibodies is often linked to these alleles: IAA (insulin autoantibodies) are strongly associated with HLA-DR4-DQ8, while GADA (glutamic acid decarboxylase antibodies) are linked to HLA-DR3-DQ2. Notably, GADA-first autoimmunity is observed predominantly in children homozygous for DR3-DQ2 **(Redondo *et al*., 2024).**

Extended haplotypes in the HLA region are inherited due to linkage disequilibrium and low recombination rates, complicating the identification of specific causal alleles. Recently, an intronic polymorphism (tri-SNP) in HLA-DRA was proposed to influence DQ and DR heterodimer expression **(Nygård *et al.*, 2021)**. Certain amino acid residues encoded by DQA1-DQB1**, such as α1, α44, α157, α196 on the α-chain, and β9, β30, β57, β70, β135 on the β-chain, have been shown to mediate either susceptibility or protection against islet autoimmunity **(Zhao *et al*., 2021).**

Environmental factors also play a crucial role in triggering the autoimmune response. Viral infections including coxsackie virus B, rotavirus, rubella,

cytomegalovirus, Epstein Barr virus, and mumps have been implicated in initiating or accelerating β-cell destruction **(Rajsfus *et al.,* 2023; Lloyd *et al*.,2022)**. These infections may contribute via molecular mimicry, bystander activation, or direct cytotoxicity.

The hallmark of early T1DM is the appearance of islet autoantibodies, including IAA, GADA, IA-2A and ZnT8A. Their presence indicates a break in immune tolerance and marks the onset of Stage 1, even in the absence of glycemic abnormalities **(Holt and Flyvbjerg, 2024)**. Stage 2 is defined by the persistence of these autoantibodies in combination with dysglycemia (impaired fasting glucose or impaired glucose tolerance), and Stage 3 is marked by clinical symptoms and a definitive diagnosis of diabetes

Progression through these stages varies among individuals. The number and titer of autoantibodies are predictive of disease trajectory: higher levels of IAA and IA-2A (but not GADA) are associated with faster progression to Stage 3 ***(*Steck *et al.*, 2015)**. Risk scoring systems have been developed to estimate the time to clinical onset among individuals with multiple autoantibodies **(Beyerlein *et al*., 2019)**. While HbA1c is a specific marker for glycemic abnormality, it lacks sensitivity in early detection and should be complemented with glucose tolerance testing or continuous glucose monitoring **(Salami e*t al*., 2022; Hao et al., 2017).**

In Stage 2 pancreases, insulitis (mononuclear cell infiltrates) is detected in a minority of islets and primarily consists of CD3+CD8+ T cells **(Smeets *et al*., 2021)**, highlighting the role of cytotoxic T cells in β-cell destruction.

The HLA class II molecules, particularly those encoded by DR and DQ loci, facilitate antigen presentation to CD4+ T-helper cells, which in turn support the activation of CD8+ cytotoxic T cells and B lymphocytes. This cascade promotes

epitope spreading and the emergence of additional autoantibodies, perpetuating the autoimmune cycle **(Holt and Flyvbjerg ,2024).**

1.2.3.2. Type II diabetes.

Type 2 Diabetes Mellitus (T2DM) is one of the most prevalent chronic diseases worldwide, with few societies or ethnic groups spared from its burden. Its development is strongly associated with several risk factors, including increased age, obesity particularly central obesity excessive caloric intake, high consumption of animal fats and sugar-sweetened beverages, sedentary lifestyle, family history of diabetes, previous gestational diabetes, polycystic ovary syndrome, hypertension, dyslipidemia, and other cardiometabolic disorders **(Holt and Flyvbjerg ,2024)**.

T2DM is a complex, multifactorial disease that results from a combination of genetic predisposition and environmental influences, with obesity, aging, and unhealthy lifestyle habits being the most prominent contributing factors. Hyperglycemia, driven by both insulin resistance and pancreatic β-cell dysfunction, is the hallmark metabolic feature of T2DM **(Lima, Moreira, and Sakamoto-Hojo, 2022)**.

A central mechanism in the pathophysiology of T2DM involves the overproduction of reactive oxygen species (ROS) and the resulting oxidative stress. Under physiological conditions, ROS are generated primarily in the mitochondria during aerobic respiration **(Pácal *et al.*, 2011)**. These species include both free radicals such as superoxide ($O2^{\bullet -}$) and hydroxyl radicals (·OH) and non-radical molecules like hydrogen peroxide (H2O2) and peroxynitrite ($ONOO^-$) **(Schieber and Chandel ,2014; Zhang e*t al*., 2016).**

Normally, antioxidant defense systems counteract ROS activity. For example, superoxide dismutase (SOD) converts $O2^{\bullet-}$ into H2O2, which is then neutralized by catalase (CAT) and glutathione peroxidase (GPx). However, in the presence of iron (Fe^{2+}), H2O2 can generate the highly toxic ·OH radical via the Fenton reaction, against which the body has no natural defense **(Galaris, Barbouti, and Pantopoulos, 2019)**. As a result, cellular systems tightly regulate iron to prevent this process.

In T2DM, glucotoxicity increases mitochondrial membrane potential, leading to excessive ROS production. This disturbs the regulation of glycolysis, activates NF-κB signaling, and contributes to β-cell apoptosis **(Dinić *et al*., 2022)**. Furthermore, lipotoxicity from free fatty acids in obese individuals works synergistically with glucotoxicity to induce β-cell dysfunction and death **(Lytrivi *et al*., 2020; Cerf ,2020)**.

1.2.3.3. Gestational diabetes

Gestational diabetes mellitus (GDM) is currently the most common medical complication associated with pregnancy. It is characterized by hyperglycemia first diagnosed during pregnancy and affects approximately 15% of pregnancies worldwide, contributing to an estimated 18 million births annually **(Modzelewski *et al*., 2022)**. The global prevalence of GDM continues to rise, posing increased risks for both maternal and fetal complications **(McIntyre *et al*., 2019)**.

The exact mechanisms responsible for insulin resistance in late pregnancy remain incompletely understood. However, it is believed to result from a combination of elevated levels of placental and maternal hormones such as human placental lactogen, progesterone, estrogen, prolactin, and cortisol alongside increased free fatty acid concentrations and placental cytokines, including tumor

necrosis factor-α (TNF-α), leptin, and adipocyte fatty acid binding protein **(Fasshauer, Blüher, and Stumvoll, 2014)**.

GDM is associated with significant short and long term health risks. Mothers with GDM are at increased risk for developing type 2 diabetes (T2DM) and cardiovascular diseases, while their offspring face elevated risks of obesity and impaired glucose metabolism later in life **(Plows *et al*., 2018)**. Moreover, the growing incidence of GDM represents a substantial economic burden on healthcare systems, emphasizing the need for improved awareness, screening, and prevention strategies **(Xu *et al*., 2017).**

1.2.4. Tests for diagnosis of diabetes

The diagnosis of diabetes mellitus is based on criteria established by leading international organizations such as the International Diabetes Federation (IDF), the American Diabetes Association (ADA), the European Association for the Study of Diabetes (EASD), and the World Health Organization (WHO). These criteria include the following tests:

1: Glycated Hemoglobin (HbA1c):

HbA1c reflects the average blood glucose levels over the preceding 6 to 8 weeks and is widely considered a reliable retrospective marker of glycemic control **(Ortiz-Martínez *et al.*, 2022)**. Compared to traditional glucose testing, HbA1c offers several advantages, including convenience, stability, and reduced day-to-day variability. Despite its higher cost and limited availability in some regions, it remains a pivotal tool for both diagnosis and long-term management of diabetes **(Tiwari and Aw ,2024)**. Normal: <5.7%, Prediabetes: 5.7–6.4%, Diabetes: ≥6.5%.

tight glycemic control is associated with a significant reduction in diabetes-related complications. For instance, maintaining HbA1c below 7% results in reductions of

76% in retinopathy, 54% in nephropathy, 60% in peripheral neuropathy, and 35% in cardiovascular disease risk **(Hare, Shaw, and Zimmet, 2012)**.

2: Random blood glucose test: This test measures blood glucose without regard to food intake. A random glucose level ≥200 mg/dL (11.1 mmol/L) is indicative of diabetes, especially when accompanied by symptoms of hyperglycemia **(Genuth *et al.*, 2015 ; Gobena *et al.*, 2022).**

3: Fasting blood glucose test (FBG): Following an overnight fast, a blood sample will be obtained. It is normal to have a fasting blood sugar level of less than 100 mg/dL (5.6 mmol/L). Prediabetes is defined as a fasting blood sugar level between 100 and 125 mg/dL (5.6 and 6.9 mmol/L). It indicates diabetes if it is 126 mg/dL (7 mmol/L) or greater on two different tests **(Goyal *et al.*, 2020)**.

4: Oral glucose tolerance test (OGTT): is most frequently used method for assessing glucose tolerance, insulin sensitivity and secretion. It has been widely used both in clinical practice and in research settings for a long time. Following ADA guidelines, the test should be performed in the morning after at least 3 days of unrestricted diet and normal physical activity **(Klein *et al*., 2021)**. According to the oral glucose tolerance test (OGTT), a normal blood glucose level is less than 140 mg/dL after two hours. Prediabetes is defined as 140–199 mg/dL, while diabetes is diagnosed at 200 mg/dL or higher. **(Chanda *et al.*, 2020)**.

There are additional differentiating markers such as:

A. The immune marker glutamic acid decarboxylase (anti-GAD65) and the measurement of C-peptide levels are the most crucial differentiating tests for distinguishing between the two primary forms of diabetes. Type 1 diabetes is confirmed by a C-peptide level less than 5 μU/mL and a positive anti-GAD65 test **(Rao *et al*., 2022)**.

B. Anti-Glutamic acid decarboxylase antibodies (Anti-GADA65): 85–90% of people with a recent diagnosis of T1D have this autoimmune condition, which is increasingly being diagnosed in outpatient clinics. Based on the mode of onset, T1D is challenging to diagnose and necessitates the presence of islet cell antibodies (ICA) and/or anti-glutamic acid decarboxylase antibodies (GADA). Both a clear cell-mediated immune response to GAD and auto-antibodies to GAD (GADA), which can be used as a predictor of diabetes, are evidence that GAD is a major auto-antigen in the process that leads to type 1 diabetes. Numerous studies have demonstrated that administering the isoform GAD65 can stop the autoimmune destruction of pancreatic beta cells **(Koopman *et al*., 2019)**.

1.2.5. Complications of diabetes:

According to **Holt and Flyvbjerg, (2024)**, the complications of diabetes are broadly classified into macrovascular and microvascular complications. This classification highlights the systemic impact of chronic hyperglycemia on both large and small blood vessels, contributing to a wide range of clinical outcomes.

1.2.5.1. Macro vascular complications in diabetes including:

1.2.5.1.1. Cardiovascular complications

Cardiovascular disease (CVD) represents the leading cause of morbidity and mortality among individuals with type 2 diabetes mellitus (T2DM), accounting for over two-thirds of deaths in this population **(Shah, Isath, and Aronow, 2022)**. T2DM is currently the ninth leading cause of death worldwide, and its prevalence continues to rise. The cardiovascular complications associated with diabetes include atherosclerosis, coronary artery disease, nephropathy, stroke,

thromboembolism, and peripheral vascular disease. There is a strong and well-established association between diabetes mellitus and cardiovascular disease. Common cardiovascular risk factors such as obesity, hypertension, and dyslipidemia are frequently present in individuals with T2DM, significantly increasing their risk of adverse cardiac events **(Leon and Maddox, 2015).**

1.2.5.1.2. Diabetic foot complication.

Foot ulceration and lower limb amputation are among the most debilitating complications of diabetes, significantly contributing to patient morbidity and mortality **(Committee, 2023)**. These complications arise from a combination of peripheral neuropathy, peripheral arterial disease (PAD), and structural foot deformities. Additional contributing factors include psychosocial stressors and abnormal plantar pressure distribution, which exacerbate the risk of skin breakdown and ulcer formation **(Core *et al*., 2018)**. Diabetic foot infections (DFIs) are particularly serious, often developing in the presence of ischemia and neuropathy, which impair wound healing and increase the risk of deep tissue infection and eventual amputation **(Deng *et al*., 2023).**

1.2.5.2. Microvascular complications in diabetes including:

1.2.5.2.1. diabetic neuropathy:

Diabetic neuropathy is a common and debilitating microvascular complication of diabetes mellitus, affecting a substantial proportion of patients globally. It arises primarily due to chronic hyperglycemia-induced metabolic and vascular disturbances that lead to progressive nerve fiber damage. Clinically, diabetic neuropathy manifests with a broad spectrum of symptoms, including

peripheral pain, paresthesia, sensory loss, muscle weakness, and autonomic dysfunction. These manifestations contribute significantly to functional impairment, increased risk of foot ulceration, and a diminished quality of life in affected individuals **(Strand *et al*., 2024).**

1.2.5.2.2. Diabetic retinopathy

Diabetic retinopathy is a microvascular complication of diabetes mellitus that affects the retinal vasculature and remains one of the leading causes of vision impairment and preventable blindness globally. It develops as a result of chronic hyperglycemia-induced damage to retinal blood vessels, leading to increased vascular permeability, capillary occlusion, ischemia, and, in advanced stages, neovascularization. The condition progresses through non-proliferative and proliferative stages and may be accompanied by diabetic macular edema, further compromising visual function **(Holt and Flyvbjerg ,2024)**.

1.2.5.2.3. Diabetic nephropathy:

Diabetic nephropathy (DN) is a major microvascular complication of diabetes and a leading cause of end-stage renal disease (ESRD) worldwide, contributing substantially to the morbidity and mortality among individuals with diabetes **(Dwivedi and Sikarwar ,2024)**.

Chronic kidney disease (CKD), commonly arising from diabetes, imposes significant personal, societal, and economic burdens **(Holt and Flyvbjerg ,2024)**. risk factors for diabetic kidney disease (DKD) are generally divided into modifiable and non-modifiable categories. modifiable risk factors include hyperglycemia, hypertension, dyslipidemia, and smoking, whereas non-modifiable

ones encompass age, male sex, ethnicity, and genetic predispositions involving genes such as ACE, APOC1, APOE, HSPG2, eNOS, VEGF, TGFβ1, and PPARγ **(Mallik and Chowdhury, 2022)**.

The pathogenesis of DKD is multifactorial, involving glomerular hyperfiltration, chronic inflammation, oxidative stress, and progressive fibrosis **(Pelle *et al*., 2022)**. the term "diabetic nephropathy" refers to a histologically confirmed kidney disease typically associated with prolonged duration of diabetes **(Initiative *et al*., 2007)**. It is clinically characterized by persistent albuminuria and/or reduced estimated glomerular filtration rate (eGFR). According to the American Diabetes Association (ADA), pathological albuminuria is defined as an albumin-to-creatinine ratio (ACR) greater than 30 mg/g. however early identification and aggressive management of DKD risk factors can significantly delay progression to ESRD and improve patient outcomes.

1.2.5.2.3.1. Epidemiology and burden of diabetic nephropathy:

Chronic kidney disease (CKD) is a prevalent and severe complication of diabetes mellitus, contributing substantially to global morbidity and mortality. End-stage kidney disease (ESKD), the most advanced form of CKD, imposes a heavy economic and social burden on both individuals and healthcare systems **(Holt and Flyvbjerg, 2024)**. Diabetic kidney disease (DKD) affects nearly 50% of individuals with type 2 diabetes mellitus (T2DM) and approximately one-third of those with type 1 diabetes mellitus (T1DM) **(Sun *et al*., 2022)**.

The Global Burden of Disease Study (GBD) in (2017) ranked CKD as the 12th leading cause of death worldwide. Its prevalence ranges from 5% to 15% among adults in developed countries, with even higher rates in low- and middle-

income nations due to limited access to care and higher rates of hypertension and diabetes **(Deng *et al*., 2021)**. More than 75 million people are affected globally, and CKD now surpasses diseases like osteoarthritis and depression in terms of population impact **(Crews *et al*., 2019; Kyu *et al*., 2018)**.

In 2019, the WHO reported that diabetes related kidney diseases were responsible for nearly 2 million deaths globally, with half of those occurring in individuals under the age of 70. In the United States, diabetes is the leading cause of ESKD, accounting for 44% of patients undergoing dialysis or having received a kidney transplant in 2023. In the United Kingdom and Australia, these rates are 28% and 38%, respectively. Local studies have documented diabetic nephropathy rates ranging from 35% to 63.6% among diabetic patients in outpatient clinical settings, with particularly high prevalence in southern governorates including Karbala, where **Oleiwi *et al*., (2022)**. reported a 63.6% nephropathy rate among 121 type 2 diabetic patients attending outpatient clinics. These elevated rates are attributed to multiple factors including delayed diagnosis, suboptimal glycemic control, genetic predisposition, and limited access to specialized nephrology care, making Iraq a critical focus area for diabetic kidney disease research and intervention strategies.

1.2.5.2.3.2. Pathophysiology of diabetic kidney disease:

Diabetic kidney disease represents a complex pathophysiological process involving multiple interconnected mechanisms that ultimately lead to progressive renal dysfunction. Understanding these mechanisms is crucial for identifying potential therapeutic targets and developing novel diagnostic approaches **(Yu *et al*., 2023)**.

1.2.5.2.3.2.1. Metabolic and hemodynamic factors.

Prolonged hyperglycemia serves as the primary initiating factor in diabetic nephropathy, particularly in type 1 diabetes, where duration of hyperglycemia constitutes one of the strongest risk factors for kidney disease development **(Holt and Flyvbjerg ,2024)**. The pathophysiological cascade begins with alterations in renal hemodynamics, characterized by glomerular hyperfiltration and increased intraglomerular pressure **(Tuttle ,2016)**.

Hyperglycemia induces renal damage through multiple molecular pathways, including oxidative stress generation, activation of the renin-angiotensin aldosterone system (RAAS), upregulation of transforming growth factor beta-1 (TGF-β1), and depletion of adenosine triphosphate **(Dugbartey, 2017)**. these metabolic disturbances create a pro-inflammatory environment that perpetuates kidney damage through sustained activation of deleterious signaling pathways. Figure (1.2)

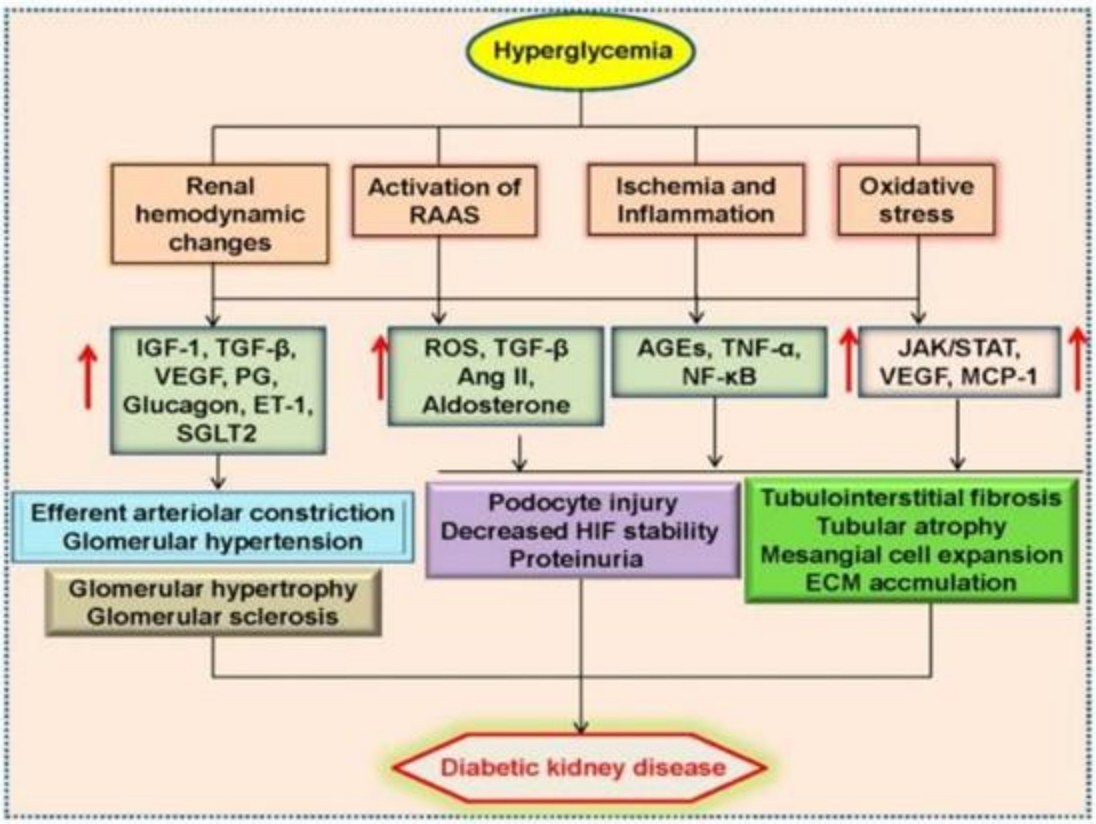

Figure 1.2: molecular and cellular pathways mechanisms in development of Diabetic Kidney Disease. **(Sun *et al*., 2019)**.

1.2.5.2.3.2.2. Structural and cellular changes.

The progression of diabetic kidney disease involves characteristic structural alterations that reflect underlying pathophysiological processes. Key morphological changes include thickening of the mesangial matrix, fusion of podocyte foot processes, progressive loss of podocytes, and expansion of the glomerular basement membrane **(Alicic *et al*., 2017)**. These structural modifications compromise filtration barrier integrity and contribute to the development of proteinuria, a hallmark of diabetic nephropathy.

Podocytes emerge as critical targets of injury during DKD progression, with extensive evidence demonstrating their central role in disease pathogenesis **(Hishikawa *et al.*, 2018)**. Podocyte dysfunction and loss result from multiple factors including oxidative stress, inflammatory mediator exposure, and activation of apoptotic pathways triggered by hyperglycemic conditions. Figure (1.2)

1.2.5.2.3.2.3. Oxidative stress and advanced glycation.

Reactive oxygen species (ROS) generation plays a pivotal role in diabetic kidney disease pathophysiology. Multiple sources contribute to ROS production, including uncoupled nitric oxide synthase (NOS), NADPH oxidase activation, and mitochondrial respiratory chain dysfunction **(LeBaron *et al.*, 2019)**. Excessive ROS accumulation triggers activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways, ultimately promoting extracellular matrix (ECM) production and progressive fibrosis **(Lee *et al*., 2003).** Advanced glycation end products (AGEs) formation represents another crucial mechanism linking hyperglycemia to kidney damage. AGEs accumulation activates inflammatory pathways and promotes oxidative stress through interaction with specific receptors, further amplifying the pathological cascade **(Potenza *et al*., 2009)**. Figure (1.2)

1.2.5.2.3.2.4. Renin-Angiotensin-Aldosterone system activation.

The renin-angiotensin-aldosterone system (RAAS) plays a central role in diabetic kidney disease progression. Elevated angiotensin II levels cause efferent arteriole constriction, disrupting autoregulation mechanisms and resulting in glomerular hypertension **(Tuttle ,2016)**. Beyond hemodynamic effects, angiotensin

II promotes renal fibrosis and tubular dysfunction through activation of pro-fibrotic pathways **(He *et al*., 2013).**

Aldosterone emerges as another key mediator in DKD pathophysiology, contributing to disease progression through enhanced macrophage infiltration and promotion of renal fibrosis **(Ritz and Tomaschitz, 2009)**. These findings highlight the importance of RAAS modulation in therapeutic approaches to diabetic nephropathy. Figure (1.2)

1.2.5.2.3.2.5. Inflammatory mechanisms and immune system involvement

Accumulating evidence demonstrates that chronic inflammation constitutes a fundamental mechanism underlying diabetic kidney disease initiation and progression **(Pérez-Morales *et al*., 2019)**. The inflammatory response involves multiple cellular and molecular components that create a self-perpetuating cycle of kidney damage.

1.2.5.2.3.2.5.1. Cellular inflammatory response.

Intrinsic renal cells including glomerular endothelial, tubular epithelial, and mesangial cells serve as both sources and targets of pro-inflammatory mediators.**(García-García *et al*., 2014)**. In response to chronic hyperglycemia, these cells increasingly express cytokines such as interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), which play significant roles in the initiation and progression of diabetic kidney disease **(Fathy *et al*., 2019)**. Additionally, chemokines like monocyte chemoattractant protein-1 (MCP-1) and acute-phase proteins such as high-sensitivity C-reactive

protein (hsCRP) have been linked to renal inflammation and damage in type 2 diabetic patients **(Fang *et al*., 2024)**. The infiltration of immune cells especially T lymphocytes and macrophages into renal tissue further amplifies this inflammatory milieu, promoting fibrotic signaling and tissue injury**(Duran-Salgado and Rubio-Guerra ,2014)**. These immune mechanisms, including NLRP3 inflammasome activation and cytokine-mediated pathways, underscore the immune-mediated nature of renal injury in DKD **(Ma *et al*., 2025)**.

1.2.5.2.3.2.5.2. Molecular inflammatory pathways

Inflammatory cytokines activate multiple intracellular signaling cascades that promote kidney damage. Key pathways include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways **(Mora and Navarro ,2006)**. These transcription factors regulate expression of numerous genes involved in inflammation, fibrosis, and cell survival.

The inflammatory milieu in diabetic kidneys involves complex interactions between pro-inflammatory and anti-inflammatory mediators. Disruption of this balance toward sustained pro-inflammatory conditions contributes to progressive kidney damage and functional decline **(Donate-Correa *et al*., 2020)**.

1.2.5.2.3.2.6. Fibrotic processes and tissue remodeling.

Renal fibrogenesis represents a complex process involving dysregulated extracellular matrix assembly, degradation, and fibroblast activation ***(Kanasaki et al.*,2014)**.

Progressive fibrosis affects both glomerular and tubulointerstitial compartments, ultimately leading to end-stage kidney disease.

Epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition (EndMT) constitute alternative mechanisms contributing to renal fibrosis **(Harris and Neilson ,2006)**. EndMT processes are particularly important in diabetic kidney fibrosis, responsible for depositing activated fibroblasts and myofibroblasts in affected tissues **(Zeisberg *et al*., 2008)**.

1.2.5.2.3.2.7. Genetic and epigenetic factors.

Genetic susceptibility significantly influences diabetic kidney disease development and progression. Genome-wide association studies have identified 16 novel loci associated with diabetic kidney disease, with the strongest signal involving a protective missense mutation in the COL4A3 gene encoding glomerular basement membrane components **(Salem *et al*., 2019)**.

Epigenetic mechanisms, including DNA methylation, chromatin modifications, and non-coding RNA regulation, modulate gene expression patterns in diabetic kidney disease **(Kato and Natarajan, 2019)**. microRNAs (miRNAs) emerge as particularly important regulators, with specific miRNAs such as miR-192 and miR-21 being upregulated and contributing to renal fibrosis, while others like miR-200 and miR-23b are downregulated **(Liu*et et al.*, 2019)**.

1.2.5.2.3.3. Prognosis of chronic kidney disease (CKD) based on eGFR and albuminuria

The KDIGO (Kidney Disease: Improving Global Outcomes) guidelines emphasize that estimated glomerular filtration rate (eGFR) and urinary albumin excretion (albuminuria) are among the most important indicators for assessing the progression of chronic kidney disease (CKD) and predicting its prognosis. A combined classification based on eGFR and albuminuria allows for a more accurate estimation of mortality risk, cardiovascular complications, and kidney function decline than relying on either parameter alone. eGFR reflects kidney function and filtration capacity, allowing determination of CKD stage, while albuminuria reflects glomerular damage and indicates disease severity and activity. The natural history of classical diabetic nephropathy illustrates the typical progression of the disease over time. In the corresponding figures, the white bar represents the normal range for each variable, allowing comparison of gradual changes in kidney function and albuminuria with normal values. In general, this natural history includes a gradual increase in urinary albumin several years after diabetes onset, a progressive decline in eGFR as the disease advances, and ultimately reaching end-stage kidney disease (ESKD) if no therapeutic interventions are applied. Patients with low eGFR and severely increased albuminuria have the highest risk of progressing to ESKD, while those with low eGFR but no significant albuminuria generally experience slower CKD progression yet remain at increased risk compared to individuals who have never had elevated albuminuria. KDIGO utilizes this information to classify CKD stages and estimate patient risk, aiding clinicians in developing individualized monitoring and treatment plans aimed at slowing kidney function decline and delaying progression to end-stage disease, as illustrated in Figures (1.3) and (1.4)

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012

GFR categories (mL/min per 1.73 m²) description and range				Persistent albuminuria categories description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	>90			
	G2	Mildly decreased	60–89			
	G3a	Mildly to moderately decreased	45–59			
	G3b	Moderately to severely decreased	30–44			
	G4	Severely decreased	15–29			
	G5	Kidney failure	<15			

1.2.5.2.3.3. Classification of Chronic Kidney Disease (CKD) and diabetes nephropathy:

For enhanced clinical decision making and therapeutic stratification, **Kitada and Koya, (2019)** proposed a refined classification framework that distinguishes chronic kidney disease in diabetic patients into three principal categories:

1. **Diabetic Kidney Disease (DKD):** Kidney impairment primarily attributable to diabetes-induced metabolic and hemodynamic disturbances. This form is directly linked to hyperglycemia-driven pathophysiological mechanisms.

2. **Non-Diabetic Kidney Disease (NDKD) with Diabetes:** Refers to chronic kidney disease (CKD) resulting from etiologies unrelated to diabetes, such as primary glomerulonephritides, hypertensive nephrosclerosis, or other intrinsic renal diseases. These conditions often develop prior to the onset of diabetes, which occurs later. This highlights the importance of differential diagnosis in diabetic patients who present with atypical renal features.
3. **Combined DKD and NDKD:** In this subgroup, both diabetes-related renal injury and an independent kidney pathology coexist, representing a diagnostic and therapeutic challenge that requires individualized management.

In parallel, the Kidney Disease Improving Global Outcomes Kidney Disease: Improving Global Outcomes (KDIGO) guidelines recommend classifying CKD using a dual-axis model based on glomerular filtration rate (GFR) and albuminuria levels **(Eknoyan *et al*., 2013)**. This approach facilitates a more precise assessment of disease severity, prognosis, and guides evidence-based treatment decisions.

Figure (1.3) the KDIGO guidelines emphasize that estimated glomerular filtration rate (eGFR) and urinary albumin excretion are key indicators for assessing CKD progression and prognosis **(Levin & Stevens, 2014)**.

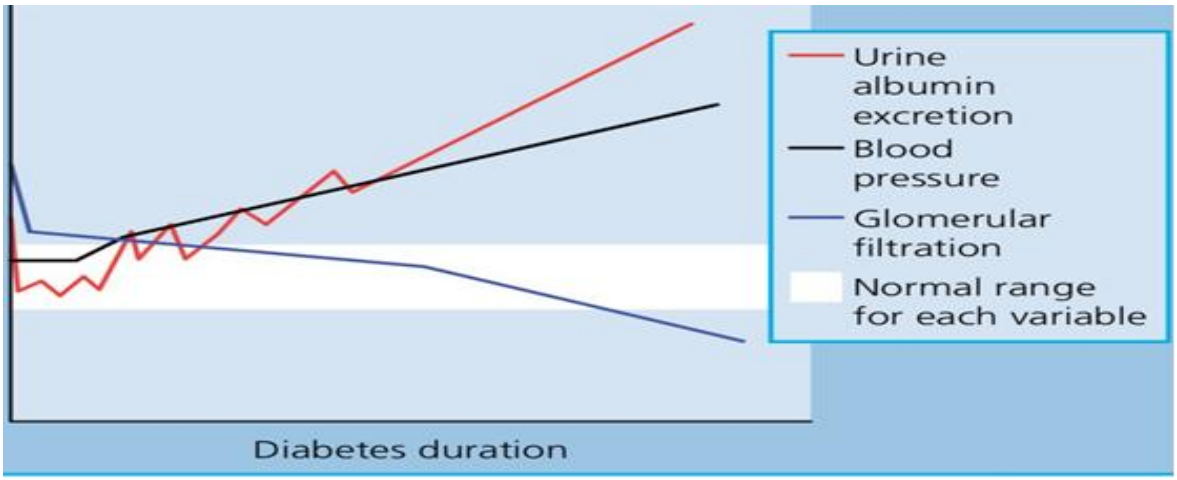

Figure (1.4) Natural history of classical diabetic nephropathy. The white bar represents the normal range for each variable **(Holt and Flyvbjerg ,2024)**.

1.2.5.2.3.4. Classification of Chronic Kidney Disease (CKD) and diabetes nephropathy:

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1.2.6. Immunological Biomarkers Associated with Diabetic Kidney Disease:

Beyond the well-established molecular pathways involved in diabetic kidney disease (DKD), recent research has increasingly focused on immunological biomarkers that may play critical roles in its pathogenesis and progression. Among these, a novel group of cytokines has garnered attention for their potential contribution to the inflammatory and immune processes underlying diabetic nephropathy, offering promising new diagnostic and therapeutic avenues **(Donate-Correa *et al*., 2021).**

1.2.6.1. Overview of Cytokines in Diabetic Kidney Disease

Low-grade chronic inflammation is a hallmark of diabetic kidney disease (DKD), wherein a variety of cytokines regulate the immune and inflammatory responses in renal tissues compromised by chronic hyperglycemia. Traditional pro-inflammatory cytokines implicated in the pathogenesis of DKD include tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-18 (IL-18) **(Donate-Correa *et al*., 2021).**

These cytokines contribute to disease progression through several mechanisms, including the recruitment of inflammatory cells to kidney tissues, increased glomerular capillary permeability, promotion of fibrosis and accumulation of glycated proteins, and modulation of gene expression in glomerular and tubular epithelial cells **(Alicic *et al*., 2017)**.

In recent years, several novel immunological biomarkers have emerged, offering deeper insights into the inflammatory landscape of diabetic kidney disease (DKD). Notable among these are IL-33, IL-37, and suPAR, which have demonstrated promising roles in early immune activation, anti-fibrotic modulation, and renal injury progression. **(Hofherr *at el*.,2024; Li Xiong *at el*.,2023; Lupușoru *at el.*,2021)** These discoveries laid the groundwork for the identification of more recently described cytokines, namely interleukin-40 (IL-40) and interleukin-41 (IL-41), which are the focus of this study due to their potentially pivotal role in the immunopathogenesis of DKD.

1.2.6.2. Interleukin-40 (IL-40)

Interleukin-40 (IL-40) represents one of the most recently discovered cytokines in the immune system, first identified by (**Catalan-Dibene *et al.* 2017)** as a novel B cell-associated cytokine encoded by the previously uncharacterized

gene C17orf99 located on chromosome 17. This small secreted protein of approximately 27 kDa belongs to the "orphan cytokines" category due to its unique structural properties that share no homology with any established cytokine families, making it a particularly intriguing addition to our understanding of immune regulation. IL-40 is exclusively present in mammalian genomes and exhibits 72% amino acid sequence similarity between humans and mice, suggesting important conserved functions across species. The protein is primarily expressed in bone marrow, fetal liver, activated B cells, and mammary glands during lactation, indicating its specialized role in mammalian-specific immune responses.

Functionally, IL-40 plays a crucial role in B cell development and homeostasis, as demonstrated by Il40−/− knockout mice which exhibit significant B cell abnormalities including reduced numbers of splenic and bone marrow B cells, decreased transitional, follicular, and marginal zone B cell subsets, and a five-fold reduction in IgA+ cells. These mice also display substantially reduced IgA levels in serum, gut, feces, and mammary gland secretions while maintaining normal IgM and IgG levels, highlighting IL-40 specific importance in IgA-mediated humoral immunity. remarkably, IL-40 deficiency leads to profound alterations in gut microbiome composition, including reduced bacterial diversity and major shifts in bacterial phylum abundances, establishing IL-40 as a critical mediator of host-microbiome interactions through its effects on mucosal immunity**(Catalan-Dibene *et al*., 2017).**

In autoimmune diseases, IL-40 has emerged as a significant player, particularly in rheumatoid arthritis (RA) where serum levels are markedly elevated compared to healthy controls and show strong correlations with disease activity (DAS28-ESR), autoantibody levels (RF-IgM and anti-CCP), and synovial fluid

leukocyte counts. The cytokine is abundantly present in RA synovial tissue, expressed by various immune cells including macrophages, T cells, B cells, and neutrophils, and demonstrates strong associations with markers of NETosis (proteinase 3 and neutrophil elastase), suggesting its involvement in neutrophil-driven inflammatory processes. Functionally, IL-40 stimulates synovial fibroblasts to produce pro-inflammatory chemokines (IL-8, MCP-1) and matrix-degrading enzymes (MMP-13), implicating it in both inflammation and joint destruction mechanisms. Importantly, B cell depletion therapy with rituximab significantly reduces IL-40 levels in RA patients, while TNF inhibitors show no such effect, indicating that IL-40 is specifically linked to B cell activation pathways rather than general inflammation. Similar patterns emerge in systemic sclerosis , where IL-40 is elevated in both serum and affected skin tissue, correlates with disease activity, gastrointestinal involvement, and fibrotic mediators including TGF-β1 and IL-8, while stimulating pro-inflammatory cytokine production in peripheral blood mononuclear cells **(Navrátilová *et al*., 2021)**.

In Sjögren's syndrome, IL-40 shows increased expression in lymphocyte-infiltrated salivary glands and is produced by various immune cell populations, further supporting its role across multiple autoimmune conditions **(Guggino *et al.,* 2023)**.

From a clinical diagnostic perspective, IL-40 demonstrates excellent diagnostic performance in ankylosing spondylitis (AUC ≥ 0.9), and remarkable accuracy in type 2 diabetes with AUC = 0.969, sensitivity 94.3%, and specificity 94.5% **(Dabbagh-Gorjani ,2024)**.

Despite these significant advances in understanding IL-40 biology, several critical knowledge gaps remain, including the identification of its specific receptor, deeper understanding of its molecular mechanisms, comprehensive clinical trials to assess therapeutic targeting potential, and development of standardized diagnostic

assays **(Dabbagh-Gorjani, 2024)**. The current evidence strongly suggests that IL-40 represents a central mediator in B cell biology and humoral immunity, a promising biomarker for diagnosis and disease monitoring across multiple conditions, a potential therapeutic target worthy of further investment, and an important link between the immune system and gut microbiome (**Catalan-Dibene *et al*., 2017; Dabbagh-Gorjani, 2024)**. Future research should focus on multi-center studies to establish reference values, development of standardized diagnostic tests, exploration of its role in other diseases including cancer and infections, and development of targeted therapies for the IL-40 pathway, as deeper understanding of this cytokine may open new avenues for treating autoimmune and inflammatory diseases while contributing to the advancement of precision medicine based on accurate biomarkers **(Dabbagh-Gorjani, 2024)**.

Although studies directly investigating IL-40 in diabetic kidney disease (DKD) are still lacking, its significant roles in B cell function, mucosal immunity, microbiome modulation, and systemic inflammation suggest potential involvement in diabetic kidney disease.

1.2.6.3. Interleukin 41 (IL-41).

Interleukin-41 (IL-41), also known as Meteorin-like (Metrnl), Meteorin-β, Subfatin, and Cometin, represents a recently discovered secretory protein that has emerged as as an identity of adipokine, exerting pleiotropic effects on glucose homeostasis, such as improving insulin sensitivity, facilitating adipose tissue browning, and increasing energy expenditure and significant player in immune regulation, inflammatory immune-related diseases, and metabolic homeostasis

(Li *et al*., 2023;Zheng *et al.*, 2016) unlike its closely related neurotrophic factor Meteorin, which shows brain-specific expression in the central nervous system, IL-41 demonstrates a broader tissue distribution, being abundantly expressed in metabolism-related organs and barrier tissues including adipose tissue, skin, mucosal barriers, skeletal muscle, and bone marrow **(Li *et al*., 2023)**.

The interleukin-41 shares 46% amino acid sequence homology with neutrophic factor Meteorin and is located on human chromosome 17q25.3, consisting of 311 amino acids encoded by 936 base pairs, notably lacking a membrane-spanning region which facilitates its secretory nature. the genomic location 17q25.3 has been identified as a susceptibility locus for cardiovascular disease, psoriasis, and atopic dermatitis, providing genomic rationale for IL-41 involvement in these pathological conditions **(Li *et al*., 2023).**

Functionally, Interleukin-41 (IL-41), also known as Meteorin-like (Metrnl), was initially studied in the context of immune regulation prior to its formal classification as an interleukin. While the name "IL-41" was first proposed by **Ushach *et al*.,** (**2015)** following a bioinformatics analysis of the Body Index of Gene Expression (BIGE) database a comprehensive repository of human gene expression profiles across multiple tissues the immunological relevance of Metrnl had already begun to emerge in earlier studies.

Their analysis revealed that Metrnl is highly expressed in activated macrophages, suggesting a potential role in immune function. Subsequent functional studies using IL-41 knockout mice demonstrated significant B-cell immune defects, including decreased serum IgG levels, impaired secretion of chemokines such as CCL3 and CCL4, and reduced interferon levels, thereby establishing its crucial role in humoral immunity **(Ushach *et al*., 2015)**.

Moreover, the immunomodulatory role of IL-41 had been partially characterized even before its formal naming, as shown in the work of (**Rao *et al*., 2014)**. Their findings demonstrated that IL-41 is associated with Type 2 immune responses, where its overexpression induces secretion of IL-4 and IL-13, subsequently enhancing M2 macrophage activation and catecholamine production. In allergic asthma models, IL-41 was shown to impair dendritic cell maturation and antigen presentation capacity, thereby dampening Type 2 inflammatory responses **(Rao *et al*., 2014; Gao *et al*., 2022)**.

The protein anti-inflammatory properties are evidenced by the fact that IL-41 knockout mice are prone to develop inflammatory lesions in multiple organs including uterus, kidneys, and liver, and show increased susceptibility to endotoxin shock, suggesting that IL-41 deficiency promotes inflammation development and that the protein may play a protective anti-inflammatory role during sepsis **(Ushach *et al*., 2015)**.

In autoimmune diseases, IL-41 shows significant involvement across multiple conditions intravenous administration of IL-41 can postpone diabetes onset in non-obese diabetic mice by decreasing islet lymphocyte infiltration and modulating immune cell responses **(Yao *et al.*, 2021)**,while in Graves' disease patients, IL-41 levels are significantly lower compared to healthy controls and positively correlate with inflammatory markers CRP and WBC count **(Gong *et al*., 2022)**.

In rheumatoid arthritis and psoriatic arthritis, IL-41 expression is significantly upregulated in synovial membranes and synovial fluid compared to osteoarthritis patients, with stromal cells identified as the dominant producers at inflammatory sites, and importantly, stromal-derived IL-41 can be further induced by pro-inflammatory cytokines IL-17A/F and TNF **(Bridgewood *et al*., 2019)**.

The protein demonstrates remarkable involvement in tissue regeneration, particularly in muscle repair where IL-41 increases dramatically (30-fold) within 24 hours after muscle injury, with macrophage-secreted IL-41 proving essential for successful muscle regeneration through direct signaling via STAT3 to promote macrophage differentiation to anti-inflammatory phenotypes and indirect promotion of muscle stem cell expansion through IGF-1 and IL-6 secretion **(Baht *et al*., 2020; Wu, Ballantyne, and others ,2017).**

In metabolic regulation, IL-41 can be strongly induced in adipose tissue upon acute cold exposure and in muscle upon exercise, leading to increased energy expenditure by stimulating browning of white adipose tissue through an eosinophil-dependent mechanism involving IL-4/IL-13 signaling cascade and M2 macrophage activation, distinguishing it from other adipokines that act directly on adipocytes **(Rao *et al*., 2014)**.

The protein shows significant therapeutic potential in insulin resistance and diabetes, where IL-41 treatment ameliorates impaired insulin response in skeletal muscle and C2C12 myoblast cells via AMPK/PPARδ-mediated signaling***(Jung et al*., 2018)**, while injection of recombinant IL-41 can reduce high glucose-induced insulin secretion and promote pancreatic β-cell function recovery by activating β-cell proliferation and inhibiting apoptosis through the WNT/β-catenin pathway in mice **(Hu, Wang, and Sun ,2021)**.

Clinical applications as a biomarker show promise across multiple diseases, with serum IL-41 levels demonstrating negative correlations with inflammatory cytokines TNF-α and IL-6 in various metabolic diseases **(Gholamrezayi *et al*., 2020)**, and positive correlations with disease activity indices like CRP and hs-CRP in polycystic ovarian syndrome, coronary artery disease, impaired glucose tolerance, Graves' disease, and type 2 diabetes mellitus (**Li *et al*., 2024).**

In cardiovascular diseases, circulating IL-41 levels are reduced in atherosclerosis and coronary artery disease patients, negatively correlating with endothelial dysfunction parameters, while lower levels in elderly patients with chronic heart failure correlate with increased cardiovascular mortality, heart failure rehospitalization, and major adverse cardiac events **(Dadmanesh *et al*., 2018)**.

Recent studies have also identified IL-41 as a novel serum marker for hepatocellular carcinoma diagnosis, particularly in alpha-fetoprotein-negative cases, suggesting its potential utility in cancer detection and prognosis **(Li *et al*., 2024)**. In inflammatory bowel diseases, IL-41 circulation concentrations are lower in both Crohn's disease and ulcerative colitis patients and inversely related to TNF-α and IL-6 levels, while experimental studies show that IL-41 injection into inflammatory bowel disease models can decrease disease activity indices and inflammatory scores through activation of STAT5/PPAR-γ signaling and promotion of adipocyte function **(Gholamrezayi *et al*., 2020; Zuo *et al*., 2019)**.

The molecular mechanisms underlying IL-41 function involve complex signaling pathways including AMPK/PPARδ, WNT/β-catenin, STAT3, and STAT5/PPAR-γ pathways, with production stimulated by various cytokines including IL-4, IL-12, IL-17α, and TNF-α, where TNF-α serves as the most potent inducer, while production can be inhibited by IFN-γ and TGF-β **(Ushach *et al*., 2015)**.

Despite significant advances in understanding IL-41 biology, several critical knowledge gaps remain including the identification of its specific receptor, deeper mechanistic understanding of its pleiotropic effects, standardization of clinical assays, and comprehensive clinical trials to assess its therapeutic targeting potential, though current evidence strongly supports IL-41 as a promising biomarker for multiple diseases and a potential therapeutic target for inflammatory, autoimmune, and metabolic disorders **(Shi *et al*., 2024).**

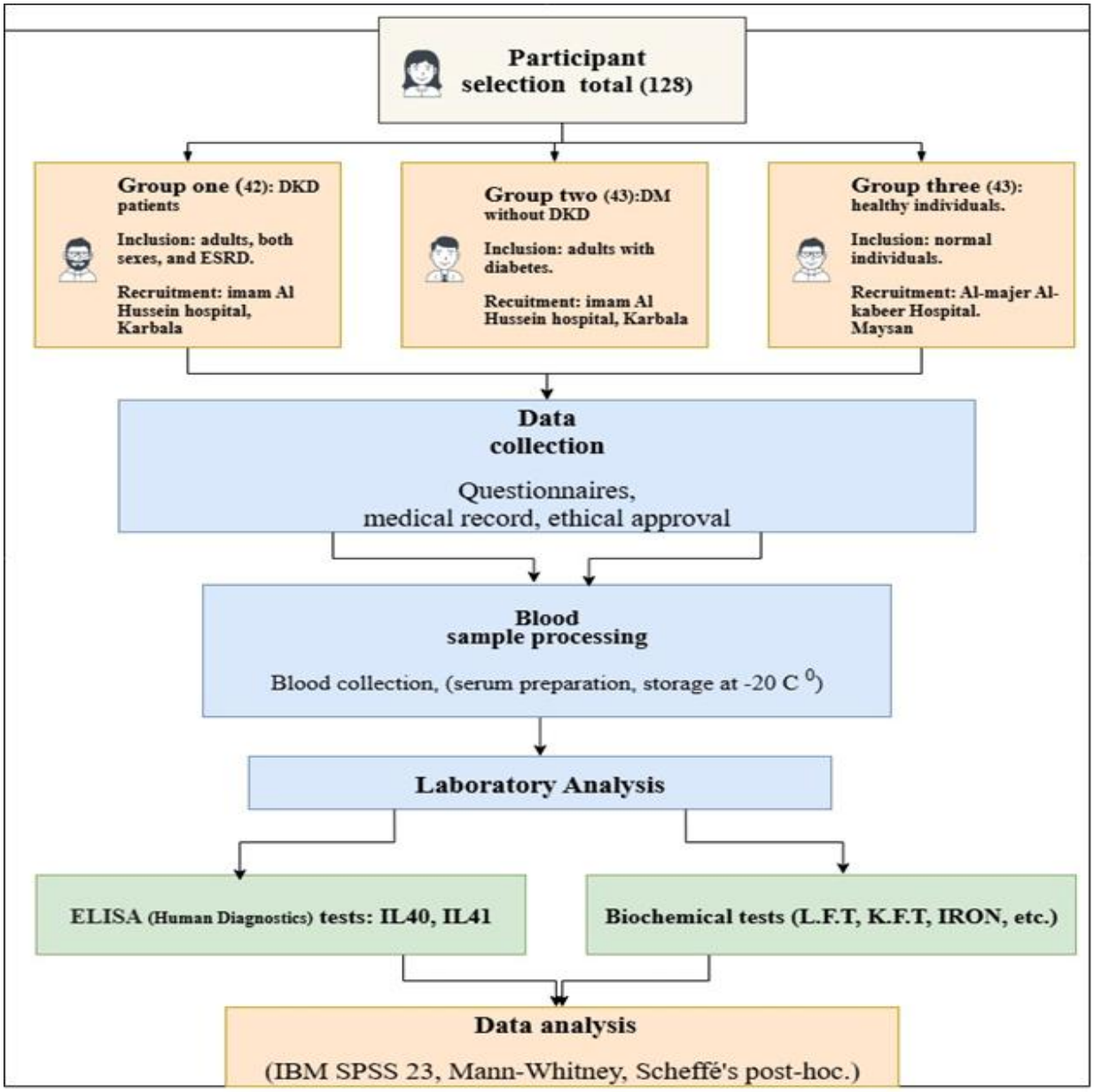
Chapter Two

Materials and Methods

2.Subject, Materials and Methods

2.1. Study Design:

This is a case-control study that was done at Habib ibn Mudhaher dialysis center in Imam AL Hussein Teaching Hospital in Karbala and Al-Majjar Al-Kabir Hospital in Maysan Province Hospital. blood samples were collected from all participants during the period between November 2024 and January 2025



2.2. Subjects Group

A total of 128 participants were enrolled in the study and classified into three groups: 42 patients diagnosed with diabetic kidney disease (DKD), 43 patients with diabetes mellitus (DM) without nephropathy, and 43 healthy individuals. Diabetic patients, including both DKD and DM groups, were recruited from Imam Al-Hussein Teaching Hospital in Karbala, while healthy controls were selected from Al-Majar Al-Kabeer Hospital in Maysan Province. For each participant, detailed demographic and clinical information was collected, including age, sex, type of antidiabetic treatment (insulin, oral agents, or a combination), and family history of diabetes. In the DKD group, the number of weekly dialysis sessions was also recorded. Biochemical investigations covered a wide range of parameters. Glycemic control was assessed using random blood sugar (RBS) and hemoglobin A1c (HbA1c). Renal function was evaluated through serum creatinine and blood urea levels. Liver function tests included aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), alkaline phosphatase (ALP), total bilirubin, and total serum protein. Mineral and electrolyte levels, including serum calcium, phosphate, and sodium, were also measured. Additionally, iron metabolism was assessed using serum ferritin, serum iron, and unsaturated iron-binding capacity (UIBC).

2.3. Inclusion and Exclusion Criteria

2.3.1. Inclusion criteria for DKD patients, DM patients without nephropathy, and healthy controls:

Participants were selected based on predefined inclusion and exclusion criteria designed to each study group:

- **DKD group (Diabetic Kidney Disease):**

  Adults of both sexes, aged 18 to 70 years, with a confirmed diagnosis of diabetes mellitus (regardless of type) for at least six months and clinically diagnosed diabetic kidney disease (stages 5) according to Kidney Disease: Improving Global Outcomes (KDIGO) guidelines. Inclusion required with albuminuria, or eGFR <15 mL/min/1.73m², with a clinically stable condition for at least four weeks prior to enrollment.

- **Diabetic group without nephropathy:**

  Adults aged 18 to 70 years with a history of diabetes mellitus (any type) for at least six months, with normal kidney function (eGFR ≥90 mL/min/1.73m²), no proteinuria, and no clinical signs of diabetic kidney involvement. Participants were required to have stable glycemic control for at least four weeks before inclusion.

- **Healthy Control Group:**

  Healthy adults aged 18 to 70 years with no history of diabetes or impaired glucose tolerance, confirmed by a fasting blood glucose <4.6 mmol/L and not more than HbA1c <5.7%. All participants in this group had normal kidney function (eGFR ≥90 mL/min/1.73m²) and no chronic medical conditions.

2.3.2. Exclusion criteria

For all groups:

Participants were excluded if they had a current malignancy or a history of cancer within the past five years, or if they had immunological disorders other than diabetes mellitus, such as systemic lupus erythematosus or rheumatoid arthritis. Chronic inflammatory diseases not related to diabetes, or recent use of immunosuppressive therapy (within the last three months), were also excluded. Cardiovascular exclusions included acute myocardial infarction within the last six months, congestive heart failure. Respiratory exclusions included severe asthma requiring systemic steroids and advanced COPD. Patients were also excluded if they had liver disease (cirrhosis or acute hepatitis), active infection within the past two weeks, being pregnant or lactating, unable to provide informed consent.

2.4. Data Collection.

2.4.1. questionnaire design

A structured, validated questionnaire was developed in Arabic and English, included demographical history (appendices-1-p161)

2.5. Patient general data

Comprehensive demographic and clinical data were collected for all study participants. These included age, sex, type of antidiabetic therapy (insulin, oral hypoglycemic agents, or combination therapy), and family history of diabetes mellitus. For participants diagnosed with diabetic kidney disease (DKD), additional information such as the number of hemodialysis sessions per week was also documented.

Data collection was conducted through structured interviews, supplemented and cross-verified with available medical records to enhance the accuracy and reliability of the information.

2.6. Materials and equipment.

2.6.1. Equipment and instruments utilized in the study.

Table 2.1: Equipment and instruments used.

Equipment	Model	Manufacturer	Country	Calibration Status
Automated Chemistry Analyzer	MINDRAY BS-230	MINDRAY	China	Calibrated monthly
Alcohol (70%)	-	Human	China	-
Complete Blood Counter	CBC M30	MINDRAY	China	Daily QC
Cobas C111	Roche diagnostics	Roche diagnostices international	switzerland	Daily 2-point calibration
Centrifuge	High-speed, refrigerated	Kokusan	Japan	Annual certification
Cotton	-	BDH	China	-
Deep Freezer (-20°C)	Ultra-low temperature	Hettich	Korea	Daily monitoring
EDTA tube 5ml	-	ALS	China	-
ELISA Reader/Washer	Human Diagnostics	Human	Germany	Weekly maintenance
Eppendorf tube 0.5 ml	-	ALS	China	-

Equipment	Model	Manufacturer	Country	Calibration Status
ELISA printer	Human Diagnostics	Human	Germany	-
Gloves	-	BDH	China	-
Gel Tubes 6 ml	-	ALS	China	-
Microplate Incubator	Temperature controlled	Memmert	Germany	Monthly verification
Micropipettes	10-1000 µL range	SLAMED	Germany	6-monthly calibration
Pipette tip	-	ALS	China	6-monthly calibration
Refrigerator (2-8°C)	Pharmaceutical grade	Panasonic	Korea	Continuous monitoring
Syringe 5 ml	-	Bio	Egypt	-
Timer	-	Human	Germany	-

2.6.2. Commercial Kits

Table 2.2: Commercial Kits and Reagents

Parameter	Kit Name	Manufacturer	Catalog No.	Lot No.	Expiry Date
ALT	ALT Kit (IFCC Method)	MINDRAY	110-9101/R1	MR240305	Jul. 2026
AST	AST Kit (IFCC Method)	MINDRAY	110-1213/R1	MR240306	Aug. 2026
ALP	Alkaline Phosphatase Kit (IFCC)	MINDRAY	110-1415/R1	MR240307	Sep. 2026
B.Urea	Urea Kit (Urease-GLDH)	MINDRAY	110-5678/R1	MR240304	Jun. 2026
Creatinine	CREA Kit (SOX Method)	MINDRAY	110-1234/R1	MR240303	May. 2026
Glucose	Glucose Kit (GOD-POD)	MINDRAY	110-2419/R1	MR240301	Mar. 2026

HbA1c	HbA1c Kit (Enzymatic)	MINDRAY	110-3527/R1	MR240302	Apr. 2026
Hb	CBC Complete Blood Count Kit	MINDRAY	CBC-M30/R1	MR240315	
IL-40	Human Protein IL-40 ELISA Kit	BT LAB	E4654Hu	BTL240101	Jan. 2026
IL-41	Human Meteorin-like Protein ELISA	BT LAB	E3941Hu	BTL240102	Feb. 2026
IRON	Serum Iron Kit (Ferrozine)	MINDRAY	110-2425/R1	MR240313	Mar. 2026
S.Albumin	Albumin BCG Kit	Roche Diagnostics	05467005	RO-645343	May. 2026
S.Ca+2	Serum Calcium Kit (Arsenazo III)	MINDRAY	110-2021/R1	MR240310	Dec. 2026
S.Na+2	Sodium ISE Electrode Kit	MINDRAY	ISE-Na+/R1	MR240311	Jan. 2026
S.PO_4	Serum Phosphate Kit (Molybdate UV)	MINDRAY	110-2829/R1	MR240316	May. 2026
S.Ferritin	Serum Ferritin Kit (CLIA)	MINDRAY	110-2223/R1	MR240312	Feb. 2026
T.S.P	Total Serum protein Kit (BCG)	MINDRAY	110-1819/R1	MR240309	Nov. 2026
UIBC	UIBC Kit (Unsaturated Iron Binding)	Copas c111	110-2627/R1	MR240314	Apr. 2026

Table 2.3: Reagents and quantity (IL 40) ELISA kits.

Components	Quantity (96T)	Quantity(48T)
Standard Solution (192ng/L)	0.5ml x1	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1	12 * 4 well strips x1
Standard Diluent	3ml x1	3ml x1
Streptavidin-HRP	6ml x1	3ml x1
Stop Solution	6ml x1	3ml x1
Substrate Solution A	6ml x1	3ml x1
Substrate Solution B	6ml x1	3ml x1
Wash Buffer Concentrate	20ml x1	20ml x1
Biotinylated Human C17orf99 Antibody	1ml x1	1ml x1
User Instruction	1	1
Plate Sealer	2 pics	2 pics
Zipper bag	1 pic	1 pic

Table 2.4: Reagents of human meteorin like protein ELISA kit

Components	Quantity (96T)	Quantity(48T)
Standard Solution (192ng/L)	0.5ml x1	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1	12 * 4 well strips x1
Standard Diluent	3ml x1	3ml x1
Streptavidin-HRP	6ml x1	3ml x1
Stop Solution	6ml x1	3ml x1
Substrate Solution A	6ml x1	3ml x1
Substrate Solution B	6ml x1	3ml x1
Wash Buffer Concentrate	20ml x1	20ml x1
Biotinylated Human METRNL Antibody	1ml x1	1ml x1
User Instruction	1	1
Plate Sealer	2 pics	2 pics
Zipper bag	1 pic	1 pic

2.7. Sample collection procedures.

2.7.1. Biological sampling.

2.7.1.1. Blood sample collection.

Venipuncture was the preferred method for blood sampling, as it provides reliable access to venous blood with minimal discomfort to the patient. A **5** mL syringe was used, with the barrel size selected based on the required sample volume. Larger syringes were avoided to prevent vein collapse due to excessive vacuum pressure.

All necessary equipment and materials were prepared in advance and placed within safe and easy reach on a rack. Care was taken to arrange all items clearly and systematically to ensure a smooth and accurate execution of the procedure.

2.7.2. Laboratory methods

2.7.2.1. Serum preparation

blood sample was divided into two parts:

- The first portion was added to a serum gel tube. The tube was gently inverted several times to activate clotting. The blood was allowed to clot at room temperature for 20 to 30 minutes, followed by centrifugation for 10 minutes to separate the serum from the clot. The serum was then transferred to Eppendorf tube. This process was completed within one hour of sample collection to ensure optimal results.
- The second portion was added to a tube containing an EDTA K3 anticoagulant, intended for tests requiring whole, non-clotted blood.

2.8. Biochemical Analysis

2.8.1. Glycemic control assessment

2.8.1.1. Estimation of random blood sugar (RBS)

Blood glucose concentration was measured in serum samples using the Glucose Oxidase–Peroxidase (GOD-POD) method with the Mindray Glucose Assay Kit **(Catalog No. 110-2419/R1; Mindray, 2023)**. The glucose oxidase method is considered the gold standard enzymatic approach for glucose determination due to its high specificity and accuracy **(Tietz, 2017)**.

This enzymatic colorimetric method involves the oxidation of glucose by the GOD enzyme, producing hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminoantipyrine and p-hydroxybenzoate sodium in the presence of peroxidase (POD) to form a colored quinoneimine complex, which was measured spectrophotometrically at an absorbance of 510 nm.

Analysis was performed using a Mindray BS-230 automated chemistry analyzer under the following conditions **(Mindray, 2023)**:

- **Sample preparation**: Serum was separated by centrifugation at 3000 rpm for 10 minutes within 30 minutes of blood collection.
- **Storage conditions**: Samples remained stable for up to 8 hours at 15–25°C or 72 hours at 2–8°C.
- **Quality control**: Three-level internal quality control samples were analyzed with each batch to ensure accuracy.

Results were reported in mg/dL, using the established adult reference range of 70–110 mg/dL **(Tietz, 2017)**.

2.8.1.2. Estimation of Glycated hemoglobin (HbA1c)

The concentration of HbA1c was determined using the Mindray enzymatic assay kit **(Catalog No. 110-3527/R1)** on a Mindray BS-230 automated chemistry analyzer **(Mindray, 2023)**. Measurement of hemoglobin A1c is essential for assessing long-term glycemic control in diabetic patients **(Tietz, 2017)**.

Whole blood samples collected in EDTA-K_3 were centrifuged at 2,000 × g for 10 minutes. On-board lysis was then performed by mixing 10 µL of whole blood with 200 µL of pretreatment reagent, followed by incubation at room temperature for 4 minutes.

The analysis was carried out in non-twin test mode using the following parameters:

- **Primary wavelength:** 505 nm
- **Sample volume:** 12 µL
- **Reagent addition:** 180 µL of R1 (containing proteinase and DA67 chromogen), followed by 60 µL of R2 (containing peroxidase and fructosyl peptide oxidase [FPOX])
- **Incubation:** Two separate 5-minute incubations at 37°C

The assay demonstrated a coefficient of variation (CV) ≤3.5%, in compliance with CLSI EP05-A3 standards, and showed linearity in the range of 3–16% (NGSP) or 20.2–42.06 mmol/mol (IFCC) **(Mindray, 2023).**

Results were reported in both national glycohemoglobin standardization programe (NGSP) (%) and International Federation of Clinical Chemistry (IFCC) (mmol/mol) units. The conversion between units was performed using the standardized

equation:

NGSP (%) = 0.0915 × IFCC (mmol/mol) + 2.15 (NGSP, 2023).

The reference range for healthy adults was 4.0–6.0% (NGSP/DCCT standard) **(Tietz, 2017**).

2.8.2. Renal Function Parameters

2.8.2.1. Estimation of Serum Creatinine

Serum creatinine concentration was measured using the enzymatic sarcosine oxidase (SOX) method with the Mindray Creatinine Kit (Catalog No. 110-1234/R1) on a Mindray BS-230 automated chemistry analyzer **(Mindray, 2023)**. Creatinine is a key biomarker for evaluating kidney function and plays an essential role in the diagnosis and monitoring of renal disorders **(Tietz, 2017)**. The enzymatic method offers higher specificity, particularly in samples containing interfering substances **(Tietz, 2017).**

This method involves a four-step enzymatic reaction:

1. Creatinine is hydrolyzed to creatine by creatininase.
2. Creatine is converted to sarcosine and urea by creatinase.
3. Sarcosine is oxidized by sarcosine oxidase to generate hydrogen peroxide (H_2O_2).
4. H_2O_2 reacts with 4-aminoantipyrine and (TOOS) in the presence of peroxidase to form a quinoneimine chromogen, measured photometrically at 546 nm.

Serum samples were centrifuged at 3,000 × g for 10 minutes within 30 minutes of collection. The processed samples remained stable for up to 7 days at 2–8°C or 3 months at –20°C **(Mindray, 2023).**

The assay utilized ready-to-use reagents:

- Reagent 1 (R1): Containing creatininase, creatinase, sarcosine oxidase, and (TOOS).
- Reagent 2 (R2): Containing peroxidase and 4-aminoantipyrine.

The automated procedure consisted of mixing 5 µL of sample with 150 µL of R1, followed by incubation at 37°C for 5 minutes, addition of 50 µL of R2, and a second incubation for 5 minutes before measuring the change in absorbance (ΔA) **(Mindray, 2023).**

The method showed excellent precision with a coefficient of variation (CV) ≤2.48% and conformed to CLSI EP05-A3 guidelines. Linearity was established over a range of 10–7,000 µmol/L .

The adult reference ranges were 0.64–1.0 mg/dL for males and 0.46–0.8 mg/dL for females **(Tietz, 2017)**. Quality assurance included two-point calibration using the Mindray Multi Sera Calibrator and daily quality control using two levels (normal and abnormal), following CLSI EP15-A3 guidelines **(CLSI, 2014; Mindray, 2023).** Traceability to international standards, including NIST SRM 927 and isotope dilution mass spectrometry (IDMS), ensured measurement accuracy and reliability **(IDMS Working Group, 2006;NIST, 2023).**

2.8.2.2. Estimation of Blood Urea

Serum urea concentration was measured using the Mindray Urea Kit (Catalog No. 110-5678/R1) based on the enzymatic Urease-GLDH method, performed on the Mindray BS-230 automated chemistry analyzer **(Mindray, 2023)**. The urease-glutamate dehydrogenase (GLDH) method is a widely accepted enzymatic approach for determining urea levels in clinical diagnostics **(Tietz, 2017)**.

This UV-kinetic method involves the enzymatic hydrolysis of urea by urease to yield ammonia and carbon dioxide. The produced ammonia reacts with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase to form glutamate and NAD^+. The decrease in NADH concentration, measured as a reduction in absorbance at 340 nm, is directly proportional to the urea concentration in the sample **(Mindray, 2023)**.

The assay used two reagents:

- **Reagent 1 (R1):** TRIS buffer, α-ketoglutarate, and urease.
- **Reagent 2 (R2):** NADH, GLDH, and stabilizers.

Serum samples were centrifuged within 30 minutes of collection at 3,000 × g for 10 minutes. Samples were stored at 2–8°C and remained stable for up to 24 hours. The analyzer automatically calculated urea concentration based on the kinetic rate of absorbance change, with results expressed in mg/dL.

The assay had a linear measurement range of 0.6–40 mmol/L. The established adult reference range was 2.5–7.1 mmol/L (15–43 mg/dL) **(Tietz,**

2017). Quality control samples at two levels were analyzed with each batch to ensure precision and accuracy **(Mindray, 2023)**.

2.8.2.3. Estimation of serum albumin.

Serum albumin concentration was measured using the Bromocresol Green (BCG) dye-binding method on the Mindray BS-230 photometric analyzer **(Mindray, 2023)**. This colorimetric assay is widely used for albumin determination due to its high specificity and reliability **(Tietz, 2017)**.

The method is based on the specific binding of BCG dye to albumin in an acidic environment (pH 4.2–4.66), forming a blue-green complex. The intensity of the complex is directly proportional to the albumin concentration and is measured photometrically at 630 nm.

The reaction mixture consisted of 1,000 µL of reagent and 5 µL of serum sample, incubated for 1–3 minutes at room temperature. The ready-to-use reagents contained BCG, succinic acid buffer, sodium hydroxide, surfactants, and stabilizers (Mindray, 2023).

Calibration was performed using certified albumin standards traceable to NIST SRM 927 and WHO reference standards, with a two-point linear calibration conducted every 28 days or when reagent lots were changed **(NIST, 2023; Mindray, 2023)**.

The assay demonstrated a linear measurement range of 0.4–8.0 g/dL. It showed high analytical precision, with within-run coefficient of variation (CV) between 1.2–1.5% and between-run CV between 1.2–1.3% **(Mindray, 2023)**. The established adult reference range was 3.4–4.8 g/dL **(Tietz, 2017)**.

Quality control procedures included analysis of two levels of controls (normal and abnormal) with each batch. Reagents were stored at 2–8°C and remained stable for up to 12 months after opening and for up to 2 months onboard the analyzer

2.8.3. Estimation of hemoglobin

Cell counts were evaluated and analyzed using the CBC BC-30 automated hematology analyzer, a high-precision instrument capable of measuring 20 hematological parameters. The CBC BC-30 operates on the electronic impedance (resistance) detection method to count and differentiate leukocytes (WBCs), erythrocytes (RBCs), and platelets.

The system utilizes three hydraulic subsystems to process WBCs, RBCs, hemoglobin, and platelets. Results are displayed on a liquid crystal display (LCD) alongside histograms and are printed on thermal paper for record-keeping.

Quality Control, (QC) protocols were followed for the CBC BC-30 analyzer, including weekly, and monthly maintenance and calibration (using a three-part method) to ensure consistent accuracy. Additionally, a delta check was performed by reanalyzing a previous day's blood sample before each use to verify result reliability.

2.8.4 Liver function tests

2.8.4.1. Estimation of alanine aminotransferase (ALT)

Serum alanine aminotransferase (ALT) activity was measured using a UV kinetic enzymatic method based on the International Federation of Clinical Chemistry (IFCC) reference procedure without pyridoxal phosphate activation, performed on the Mindray BS-230 automated chemistry analyzer **(Mindray, 2023;**

IFCC, 2002). ALT is a key hepatic enzyme, and its measurement is essential for assessing liver function **(Tietz, 2017)**.

The method involves the enzymatic transfer of an amino group from L-alanine to α-ketoglutarate by ALT, forming L-glutamate and pyruvate. Pyruvate is subsequently reduced to lactate by lactate dehydrogenase (LDH), coupled with the oxidation of NADH to NAD^+. The decrease in absorbance at 340 nm due to NADH oxidation is directly proportional to ALT enzymatic activity.

The analysis employed two reagents:

- Reagent 1 (R1): TRIS buffer, L-alanine, LDH, and NADH.
- Reagent 2 (R2): α-ketoglutarate.

Serum samples were centrifuged at 3,000 × g for 10 minutes within 30 minutes of collection and analyzed immediately. Samples were stable for up to 3 days at 2–8°C. Hemolyzed specimens were excluded due to the risk of falsely elevated ALT activity **(Mindray, 2023)**.

The analyzer automatically calculated ALT activity in U/L based on the rate of absorbance change per minute (ΔA/min). The method demonstrated linearity up to 1100 U/L **(Mindray, 2023)**.

Quality control procedures included the use of two-level control materials (normal and abnormal) with each analytical batch. The established reference ranges for ALT activity were:

- Adult males: 7–56 U/L , Adult females: 7–45 U/L **(Tietz, 2017)**

2.8.4.2. Estimation of aspartate aminotransferase (AST)

Serum aspartate aminotransferase (AST) activity was determined using the International Federation of Clinical Chemistry (IFCC) standardized UV kinetic method on the Mindray BS-230 automated chemistry analyzer. AST is a crucial biomarker for hepatocellular injury and myocardial damage assessment **(Tietz, 2017)**. In this enzymatic reaction, AST catalyzes the transfer of an amino group from L-aspartate to α-ketoglutarate, producing L-glutamate and oxaloacetate. The oxaloacetate is subsequently reduced to malate by malate dehydrogenase (MDH), accompanied by oxidation of NADH to NAD^+. The rate of NADH absorbance decrease at 340 nm is directly proportional to AST activity **(IFCC, 2002)**. The assay employed two reagents: R1 containing TRIS buffer, L-aspartate, NADH, MDH, and LDH; and R2 containing α-ketoglutarate and pyridoxal-5-phosphate (P-5-P) as a coenzyme activator **(Mindray, 2023)**. Serum samples were separated within 30 minutes of collection and remained stable for up to 3 days at 2–8°C. **(Mindray, 2023)**. The analyzer calculated enzyme activity in U/L based on the absorbance change per minute (ΔA/min). The assay showed a linear range up to 1500 U/L **(Mindray, 2023)**. Quality control materials were included in every analytical run. The established reference intervals were: adult males: 8–40 U/L; adult females: 5–32 U/L **(Tietz, 2017)**.

2.8.4.3. Estimation of alkaline phosphatase (ALP)

Serum alkaline phosphatase (ALP) activity was measured using the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) modified method on the Mindray BS-230 automated chemistry analyzer. ALP is a key enzyme for assessing hepatobiliary disorders and bone diseases, making it essential for clinical diagnosis and monitoring **(Tietz, 2017)**. In this kinetic colorimetric assay, ALP catalyzes the hydrolysis of p-nitrophenylphosphate (p-NPP) to p-nitrophenol and inorganic phosphate in the presence of magnesium ions. The rate of p-nitrophenol formation, quantified by the increase in absorbance at 405 nm, is directly proportional to ALP activity in the sample **(IFCC, 2011)**.

The method utilized two reagents: R1 containing AMP buffer (435 mmol/L), magnesium acetate (2.5 mmol/L), and zinc sulfate (1.2 mmol/L); and R2 containing p-NPP (60 mmol/L) **(Mindray, 2023)**. Serum samples were separated by centrifugation within 30 minutes of collection and analyzed following manufacturer guidelines. The procedure involved pre-incubation with Reagent 1 at 37°C, followed by addition of Reagent 2 and monitoring absorbance changes over 3 minutes. The analyzer calculated ALP activity in U/L based on the rate of absorbance change per minute (ΔA/min). The method demonstrated linearity up to 1500 U/L. Quality control samples were included in each analytical run to ensure result validity **(Mindray, 2023)**.

The established reference interval for adults (≥18 years) was 30–120 U/L for both males and females **(Tietz, 2017)**.

2.8.4.4. Estimation of total serum protein

Serum total protein concentration was measured using the Biuret method, a colorimetric assay performed on the Mindray BS-230 automated chemistry analyzer. The Biuret method is a classical and reliable technique based on the reaction of divalent copper ions (Cu^{2+}) with the peptide bonds of proteins in an alkaline medium, forming a purple-colored biuret complex **(Tietz, 2017)**. The intensity of this complex, measured photometrically at 540 nm, is directly proportional to the total protein concentration in the sample.

The assay utilized two ready-to-use reagents:

- **Reagent 1 (R1):** Alkaline biuret reagent containing copper sulfate and sodium potassium tartrate.
- **Reagent 2 (R2):** Assay activator solution.

The procedure involved sequential reagent mixing, incubation at 37°C, and photometric absorbance measurement under kinetic principles (Mindray, 2023).

Calibration was carried out using the Mindray Human Multi-Calibrator, traceable to international reference materials, and quality control was ensured through the use of two levels of assayed control materials in each analytical batch **(Mindray, 2023)**. The method exhibited the following analytical performance:

- **Linearity:** 2–12 g/dL (20–120 g/L)
- **Detection limit:** 0.5 g/dL (5 g/L)

The **reference range** for total serum protein in healthy adults was:

- **6.4–8.3 g/dL (64–83 g/L)** (Tietz, 2017).

Method validation followed the CLSI EP5-A2 guidelines (CLSI, 2014). Accurate pH and temperature control were essential to maintain the specificity and consistency of the reaction. The Biuret reaction complies with Beer's law up to protein concentrations of at least **10 g/dL**, further confirming its precision and applicability in clinical biochemistry **(Tietz, 2017).**

2.8.5. Electrolytes profile

2.8.5.1. Estimation of serum calcium

Serum calcium concentration was quantitatively determined using the Arsenazo III method, a colorimetric in vitro assay performed on the Mindray BS-230 automated chemistry analyzer. Calcium assessment is clinically significant for evaluating parathyroid gland function, bone metabolism, and various metabolic and renal disorders **(Tietz, 2017)**.

This method is based on the formation of a colored complex between calcium ions and Arsenazo III [2,2′-[1,8-Dihydroxy-3,6-disulfonaphthylene-2,7-bisazo]-bisbenzenearsonic acid] under mildly acidic conditions (pH ~6.5). The resulting purple complex exhibits maximum absorbance at ~650 nm, and its intensity is directly proportional to the calcium concentration in the sample.

The assay was performed using the Calcium Kit (Arsenazo III Method) supplied by the manufacturer **(Mindray, 2023)**. The working reagent consisted of:

- Buffered solution containing ≥0.2 mmol/L Arsenazo III
- Surfactants and stabilizers

All reagents and consumables were certified calcium-free to prevent contamination. Serum samples were obtained via centrifugation within 30 minutes of collection and stored at 2–8°C for up to 7 days before analysis. **(Mindray, 2023)**.

The automated procedure involved mixing the serum sample with the working reagent at 37°C, followed by photometric measurement at 650 nm. Calcium concentrations were calculated automatically by the analyzer using a standard calibration curve and reported in mg/dL.

The method showed high specificity and reproducibility under controlled conditions. The established adult reference range for total serum calcium was:
equivalent to 8.8–10.6 mg/dL) **(Tietz, 2017)**.
Strict adherence to the manufacturer's instructions and laboratory quality assurance protocols ensured accurate and reliable results.

2.8.5.2. Estimation of serum phosphate

Serum phosphate concentration was measured using the photometric endpoint method. assay performed on the Mindray BS-230 automated chemistry analyzer. Phosphate measurement is essential for assessing bone metabolism, kidney function, and mineral disorders **(Tietz, 2017)**. in this reaction, inorganic phosphate reacts in an acidic medium with ammonium molybdate to form a molybdophosphate complex, which absorbs light at 340/656 nm. The intensity of the color produced is directly proportional to the phosphate concentration. The reaction was carried out using 158 µL of total reaction volume, and serum was preferred as the specimen type **(Mindray, 2023)**. The assay demonstrated excellent precision (CV < 2%) within the therapeutic range and was linear from 0.10 to 6.46 mmol/L **(Mindray, 2023)**. The established adult reference range for serum phosphate was 0.81–1.45 mmol/L (2.5–4.5 mg/dL) **(Tietz, 2017)**. Calibration was

performed biweekly using linear regression, and quality control procedures included routine analysis of both normal and pathological control materials **(Mindray, 2023)**.

2.8.5.3. Estimation serum sodium

Serum sodium concentration was quantitatively determined using the Ion-Selective Electrode (ISE) method on the cobas C111 automated chemistry analyzer **(Roche Diagnostics)**. The ISE technique is the gold standard method for electrolyte measurement, providing high specificity and accuracy for sodium determination **(Tietz, 2017)**. This technique utilizes a sodium-selective membrane that generates an electrical potential corresponding to the activity of sodium ions in the sample. This potential is measured and converted into sodium concentration through calibration with standard solutions **(Roche Diagnostics, 2023)**. Non-hemolyzed serum samples were used, and the analysis was carried out according to the manufacturer's instructions **(Roche Diagnostics, 2023)**. The results were reported in millimoles per liter (mmol/L). The established reference range for serum sodium was 135–145 mmol/L **(Tietz, 2017)**. Routine quality control procedures were applied with each batch of samples using normal and pathological control materials to ensure analytical accuracy and precision **(Roche Diagnostics, 2023)**.materials to ensure analytical accuracy and precision.

2.8.6. Iron metabolism assessment

2.8.6.1. Estimation of serum ferritin

Serum ferritin concentration was measured using a particle-enhanced immunoturbidimetric assay (PEITA) performed on the Mindray BS-230 analyzer. Ferritin is the primary iron storage protein and serves as a key biomarker for assessing iron status and diagnosing iron deficiency or iron overload disorders **(Tietz, 2017)**. This method relies on the agglutination of latex particles coated with anti-human ferritin antibodies when they react with ferritin antigens in the sample, producing turbidity proportional to ferritin concentration. Absorbance was measured at 570 nm. The assay used 5 µL of serum or heparinized plasma per test, with samples stable for 7 days at room temperature or 2–8°C, and up to one year at –20°C (single freeze-thaw cycle) **(Mindray, 2023)**. The reagents included Tris buffer (R1), antibody-coated latex particles (R2), and WHO-traceable calibrators (WHO Standard 94/572) **(Mindray, 2023)**. Calibration was done every 28 days or with reagent lot changes using a multi-point method **(Mindray, 2023)**. The assay was linear from 10 to 1000 ng/mL, with a detection limit of 10 ng/mL. Samples above this range were diluted 1:10 with saline **(Mindray, 2023)**. The established reference intervals were 30–400 ng/mL for males and 15–150 ng/mL for females **(Tietz, 2017)**. Quality control involved two-level internal controls with each batch **(Mindray, 2023)**.

2.8.6.2. Estimation of serum iron

Serum iron concentration was determined using a second-generation colorimetric assay on the cobas C111 automated chemistry analyzer (Roche Diagnostics). Measurement of serum iron is clinically significant test result are used for the diagnosis of iron deficiency anemia, iron overload syndromes, and for monitoring iron status in various systemic and metabolic disorders **(Tietz, 2017).**

The assay involves the liberation of ferric ions (Fe^{3+}) from their binding with transferrin through the action of citric acid and thiourea. Subsequently, Fe^{3+} is reduced to ferrous ions (Fe^{2+}) using sodium ascorbate, and Fe^{2+} then reacts with FerroZine to form a stable purple-colored complex. The intensity of the resulting color, measured photometrically at 552 nm, is directly proportional to the serum iron concentration **(Roche Diagnostics, 2023)**.

The test was performed using 8.5 µL of serum, with a total reaction volume of 160 µL. The method demonstrated linearity within the range of 1.0–179 µmol/L (5.9–1000 µg/dL) and a detection limit of 1.0 µmol/L. The assay exhibited high precision with a coefficient of variation (CV) between 1.5% and 2.6% **(Roche Diagnostics, 2023)**.

The reference range for serum iron in adults was established as 5.83–34.5 µmol/L (33–193 µg/dL) **(Tietz, 2017)**. The method was validated to be free from significant analytical interference by hemolysis, lipemia, icterus, within clinically relevant concentrations **(Roche Diagnostics, 2023)**.

Calibration was carried out every 28 days using linear regression, with deionized water as the zero calibrator. To ensure accuracy and analytical reliability, quality

control procedures incorporated normal and pathological human serum-based controls with each analytical batch.

2.8.6.3. Estimation of unsaturated iron-binding capacity (UIBC) and total iron-binding capacity (TIBC)

The UIBC (Unsaturated Iron-Binding Capacity) assay is a colorimetric endpoint method based on an inverse measurement principle performed on the cobas C111 automated chemistry analyzer. UIBC measurement is essential for evaluating iron metabolism disorders and assessing iron-binding capacity of transferrin **(Tietz, 2017)**. In this assay, excess ferrous ions (Fe^{2+}) are added to the serum sample. Under alkaline conditions, Fe^{2+} oxidizes to Fe^{3+} and binds to unoccupied transferrin. Any unbound Fe^{2+} then reacts with FerroZine to form a colored complex. The absorbance measured at 546 nm is inversely proportional to the UIBC value. Reagents included ferrous chloride, TRIS buffer, and FerroZine **(Roche Diagnostics, 2023)**. Serum (20 µL) were used, with serum preferred due to better accuracy. **(Roche Diagnostics, 2023)**. Calibration was performed using a two-point method (deionized water and iron standard) and repeated daily or with reagent lot changes. The established UIBC reference range for adults is 112–347 µg/dL (20.0–62.1 µmol/L) **(Tietz, 2017)**. TIBC (Total Iron-Binding Capacity) was calculated using the formula: TIBC = Serum Iron + UIBC, with an adult reference range of 250–400 µg/dL (44.8–71.6 µmol/L) **(Tietz, 2017)**. Quality controls were analyzed at the start and end of each analytical shift **(Roche Diagnostics, 2023)**.

2.8.7. Instrument calibration and operation

All automated analyzers (Mindray BS-230, cobas C111, CBC) underwent routine self-checks and calibration procedures as per the manufacturers' instructions prior to use. Calibration was performed using traceable reference standards at the frequencies recommended by the instrument manuals or upon reagent lot changes.

Quality assurance measures:

Internal quality control (IQC) was implemented by analyzing at least two levels (normal and pathological) of assayed control materials at the start of each analytical run. Control results were reviewed and validated before proceeding with patient sample testing.

Sample analysis:

Patient samples were carefully loaded into designated analyzer positions, and tests were conducted using predefined assay protocols stored in the instrument software. The automated systems monitored reaction parameters including temperature, timing, and absorbance readings in real time.

Result reporting:

Results were automatically generated by the analyzer software and subsequently manually reviewed and verified by qualified laboratory personnel to ensure validity and accuracy before being documented for statistical analysis.

2.9. immunological markers

2.9.1. Determination the level of interleukin-40 protein ELISA kit (Cat. No E4654Hu)

2.9.1.1. Analytical performance characteristics

Assay Validation Parameters: the analytical performance of the ELISA kit was evaluated according to established guidelines for bioanalytical method validation.

Precision Assessment:

• Intra-assay precision was determined by analyzing three samples of known concentrations in triplicate within a single assay run (2.5%). The coefficient of variation (CV) was calculated using the formula: CV (%) = (SD/mean) × 100.

• Inter-assay precision was assessed by testing the same three samples across multiple independent assay runs (3.3%).

• Acceptance criteria: Intra-assay CV < 8%; Inter-assay CV < 10% Analytical Range and Sensitivity:

• Standard curve range: 1.5 - 96 ng/mL

• Lower limit of detection (LLOD): 0.65 ng/mL

• The assay demonstrated linear response across the specified concentration range

2.9.1.2. Principle of sandwich ELISA technique:

The concentration of C17orf99 (IL-40) in serum samples was quantified using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cat. No. E4654Hu, BT LAB, china) according to the manufacturer's instructions. this highly specific immunoassay employs the principle of sandwich ELISA technology, which relies on high-affinity antigen–antibody interactions combined with enzymatic signal

amplification for precise quantitative analysis of target proteins in biological samples.

The sandwich ELISA technique utilizes two distinct antibodies that recognize different epitopes on the same target antigen, creating a molecular "sandwich" configuration that ensures high specificity and sensitivity. The 96-well ELISA microplate was pre-coated with a monoclonal anti-human C17orf99 capture antibody, which specifically binds to the N-terminal domain of the target protein present in serum samples. This immobilized capture antibody serves as the foundation of the sandwich complex, providing stable antigen binding throughout the assay procedure.

A biotinylated detection antibody, recognizing a different epitope on C17orf99, was then added to form a sandwich complex. this was followed by the addition of streptavidin-conjugated horseradish peroxidase (HRP), which binds to the biotinylated antibody.

After incubation, unbound materials were removed by multiple wash steps. A chromogenic substrate solution (typically tetramethylbenzidine, TMB) was added. The HRP catalyzed a reaction producing a blue color, which turned yellow upon the addition of an acidic stop solution.

The absorbance was measured by ELISA Reader at 450 nm, and the color intensity was directly proportional to the concentration of C17orf99 in the sample.

Quantification was achieved by constructing a standard curve from known concentrations of recombinant C17orf99. the concentration of C17orf99 in the unknown samples was interpolated from this curve using curve-fitting software.

2.9.1.3. Reagent preparation

Objective: Standards for IL-40 ELISA assay were prepared using enhanced serial dilution methods with improved quality control measures.

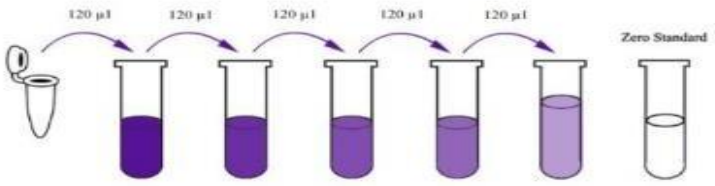
Pre-protocol preparation: All reagents were removed from refrigeration 30 minutes before use to equilibrate to room temperature (20-25°C). all solutions were inspected for precipitates, contamination, or unusual appearance. Labeled Eppendorf tube were prepared for each standard concentration. test was ambient at temperature was recorded and adequate lighting was ensured while direct sunlight exposure was avoided.

Standard stock preparation: The IL-40 standard was reconstituted by combining 120 µl of the provided standard (192 ng/ml) with 120 µl of standard diluent in a sterile Eppendorf tube. The mixture was gently mixed by pipetting up and down 3-4 times, with foam formation being avoided. this yielded 240 µl of stock standard at 96 ng/ml concentration. The reconstituted standard was allowed to equilibrate for 15 minutes at room temperature with gentle agitation applied every 5 minutes. This incubation period ensured complete dissolution and homogenization of the standard.

Serial dilution protocol: A serial dilution method was employed to prepare a range of IL-40 standard concentrations. Initially, the stock standard solution at a concentration of 96 ng/ml was prepared by reconstituting the lyophilized IL-40 standard: 120 µl of the original 192 ng/ml standard was mixed with 120 µl of standard diluent in a sterile Eppendorf tube, resulting in 240 µl of the stock solution. The mixture was gently pipetted up and down several times without generating foam and was incubated at room temperature for 15 minutes with gentle mixing every 5 minutes to ensure complete dissolution and homogeneity. From this stock, a set of working standards was prepared using a 1:2 serial dilution protocol.

Standard 6 (72 ng/ml) was prepared by mixing 150 µl of the stock solution with 50 µl of standard diluent to yield a total volume of 200 µl. Standard 5 (48 ng/ml) was prepared by mixing 100 µl of the stock solution with 100 µl of diluent. standard 4 (24 ng/ml) was obtained by diluting 100 µl of Standard 5 with 100 µl of diluent, followed by Standard 3 (12 ng/ml), standard 2 (6 ng/ml), and Standard 1 (3 ng/ml), each prepared by mixing 100 µl of the preceding standard with 100 µl of diluent. A blank (0 ng/ml) was also included, consisting solely of 200 µl of standard diluent. All standards were prepared in triplicate to ensure reliability and enable the generation of a precise standard curve for the quantification of IL-40 levels in serum samples.

Wash buffer preparation: (PBS, BSA) The 25x wash buffer concentrate was examined for crystal formation. When crystals were present, the solution was warmed gently to 37°C and mixed until completely dissolved. Using a graduated cylinder, 20 ml of the concentrate was accurately measured and added to a clean 500 ml container. 480 ml of deionized or distilled water was added to achieve a final volume of 500 ml (1x wash buffer). The solution was mixed thoroughly for 2-3 minutes by gentle swirling. The pH was verified to be within the acceptable range of 7.2-7.4 using calibrated pH strips. The prepared wash buffer was clear and free of particulates.



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
192ng/ml	96ng/ml	48ng/ml	24ng/ml	12ng/ml	6ng/ml

Figure (2.1): Concentration of standards of IL40

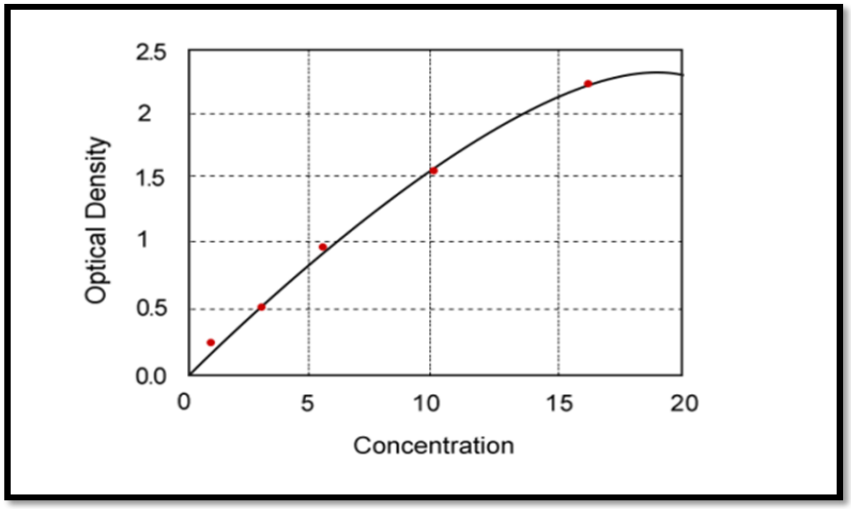


Figure (2.2): The standard curve of IL-40 concentration

2.9.1.4. Assay procedure:

The quantification of serum C17orf99 (IL-40) was performed using a sandwich ELISA kit according to the manufacturer's instructions. the assay was conducted at room temperature unless otherwise specified. the procedure consisted of the following steps:

1. **Reagent preparation**

   All reagents, standards, and serum samples were brought to room temperature prior to use. Standards and working solutions were prepared as per kit instructions.

2. **Plate preparation**

   The required number of ELISA strips was determined and placed into the assay frame. unused strips were sealed and stored at 2–8°C for future use.

3. **Standard and sample addition**

   • Standards: 50 µL of standard solution (pre-mixed with biotinylated antibody) was added to designated standard wells.

   • Samples: 40 µL of serum sample was added to each sample well, followed by 10 µL of biotinylated anti-C17orf99 antibody with gentle mixing.

   • Subsequently, 50 µL of streptavidin-HRP conjugate was added to both sample and standard wells (excluding blank control wells).

   • The plate was gently mixed to ensure homogeneous distribution, sealed with a plate sealer, and incubated at 37°C for 60 minutes.

Washing step

After incubation, the plate sealer was removed, and wells were washed five times with wash buffer. Each wash involved soaking with 300 µL of buffer for 30 seconds to 1 minute. Plates were blotted dry on absorbent material after washing.

4. **Substrate reaction**
   - 50 µL of Substrate Solution A (TMB) was added to each well, followed by 50 µL of Substrate Solution B (H_20_2).
   - The plate was covered with a new sealer and incubated in the dark at 37°C for 10 minutes to allow color development.
5. **Reaction termination**
   - 50 µL of Stop Solution (H_2S0_4) was added to each well, resulting in an immediate color change from blue to yellow.
6. **Absorbance measurement**
   - The optical density (OD) of each well was measured at 450 nm using a microplate reader within 10 minutes of adding the stop solution.

2.9.1.5. Calculation of results

Quantification of serum C17orf99 (IL-40) concentrations was achieved by generating a standard curve. The average optical density (OD) values for each standard concentration were plotted on the **Y-axis**, while the corresponding concentrations were plotted on the **X-axis**. A best-fit curve was constructed using regression analysis.

To ensure accuracy and reproducibility, data analysis was performed using computer-based curve-fitting software, which allows for the generation of a

standard curve through non-linear or linear regression, depending on the standard distribution. Sample concentrations were automatically interpolated from this curve based on their corresponding OD values.

All measurements were performed in duplicate, and the mean OD was used for calculations. Only standard curves with an acceptable coefficient of determination ($R^2 \geq 0.98$) were considered valid for result interpretation.

2.9.2. Determination the level of interleukin-41 protein (Cat.NoE3941Hu)

2.9.2.1. Analytical performance characteristics

The Human Meteorin-like protein (METRNL) or IL-41 ELISA Kit (Cat.No E3941Hu) demonstrated
the following validated analytical parameters:

Technical specifications:

- Standard curve range: 0.05 - 15 ng/mL
- Analytical sensitivity (LLOD): 0.023 ng/mL
- Sample volume required: 40 µL per test

Precision assessment:

- Intra-assay precision was determined by analyzing three quality control samples of known concentrations in triplicate within a single assay plate
- Inter-assay precision was evaluated by testing the same three samples across multiple independent assay runs
- Precision calculation: CV (%) = (SD/mean) × 100

Acceptance criteria:

- Intra-assay precision: CV < 8%
- Inter-assay precision: CV < 10%

These analytical parameters ensure reliable quantitative measurement of serum METRNL concentrations within the physiological range.

2.9.2.2. Principle of sandwich ELISA technique:

Serum METRNL (Meteorin-like protein / IL-41) concentration was measured using a sandwich-based Enzyme-Linked Immunosorbent Assay (ELISA). The assay utilizes a 96-well microplate pre-coated with a monoclonal antibody specific to human METRNL. When the serum sample is added, METRNL present in the sample binds to the immobilized antibodies.

Subsequently, a biotinylated anti-human METRNL detection antibody is added, which binds specifically to the captured METRNL. This complex is then recognized by streptavidin conjugated to horseradish peroxidase (HRP), forming an enzyme-labeled immune complex.

After an incubation period, unbound components are removed through multiple washing steps. A chromogenic substrate solution is then added, initiating a colorimetric reaction. The intensity of the developed color is directly proportional to the concentration of METRNL in the sample. The enzymatic reaction is terminated by adding an acidic stop solution, resulting in a color change from blue to yellow. The absorbance is measured at 450 nm using a microplate reader.

The standard curve was generated using known concentrations of METRNL ranging from 0.05 to 15 ng/mL. The concentration of METRNL in the test samples was determined by comparing their absorbance values to the standard curve using appropriate curve-fitting software.

2.9.2.3. Reagent preparation

Objective: Standards for IL-41 ELISA assay were prepared using enhanced serial dilution methods with improved quality control measures to ensure accurate quantitative analysis.

Pre-protocol preparation: All reagents were removed from refrigeration 30 minutes before use to equilibrate to room temperature (20-25°C). All solutions were inspected for precipitates, contamination, or unusual appearance. Labeled eppendorf tube were prepared for each standard concentration. Ambient temperature was recorded and adequate lighting was ensured while direct sunlight exposure was avoided.

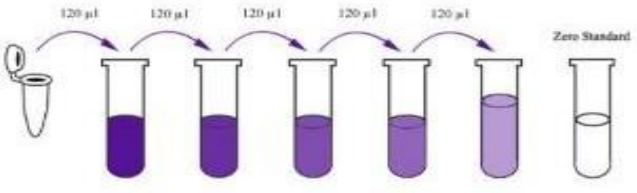
Materials and reagents used: The following materials were utilized: IL-41 Standard (16 ng/ml, 120 µl), Standard Diluent, Wash Buffer Concentrate (25x), Deionized/Distilled Water, Micropipettes (10-200 µl, 200-1000 µl), Sterile eppendorf tube (1.5 ml), and Graduated cylinder (500 ml).

Standard stock preparation: The IL-41 standard was reconstituted by combining 120 µl of the provided standard (16 ng/ml) with 120 µl of standard diluent in a sterile eppendorf tube. The mixture was gently mixed by pipetting up and down 3-4 times, avoiding foam formation. This yielded 240 µl of stock standard at 8 ng/ml concentration. The reconstituted standard was allowed to equilibrate for 15 minutes at room temperature with gentle agitation applied every 5 minutes. This incubation period ensured complete dissolution and homogenization of the standard.

Serial dilution protocol: The following standard concentrations were prepared using a systematic 1:2 serial dilution approach, with duplicates being prepared for precision. Standard 5 (8 ng/ml) was the stock solution prepared above. Standard 4

(4 ng/ml) was prepared by mixing 100 µl of Standard 5 with 100 µl standard diluent to yield 200 µl. Standard 3 (2 ng/ml) was prepared by mixing 100 µl of Standard 4 with 100 µl standard diluent to yield 200 µl. Standard 2 (1 ng/ml) was prepared by mixing 100 µl of Standard 3 with 100 µl standard diluent to yield 200 µl. Standard 1 (0.5 ng/ml) was prepared by mixing 100 µl of Standard 2 with 100 µl standard diluent to yield 200 µl. The Blank/Zero Standard (0 ng/ml) consisted of 200 µl of standard diluent only.

Wash buffer preparation: (PBS, BSA) The 25x wash buffer concentrate was examined for crystal formation. When crystals were present, the solution was warmed gently to 37°C and mixed until completely dissolved. Using a graduated cylinder, 20 ml of the concentrate was accurately measured and added to a clean 500 ml container. 480 ml of deionized or distilled water was added to achieve a final volume of 500 ml (1x wash buffer). The solution was mixed thoroughly for 2-3 minutes by gentle swirling. The pH was verified to be within the acceptable range of 7.2-7.4 using calibrated pH strips. The prepared wash buffer was clear and free of particulates.



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
16ng/ml	8ng/ml	4ng/ml	2ng/ml	1ng/ml	0.5ng/ml

Figure (2.3): Concentration of standards of IL41

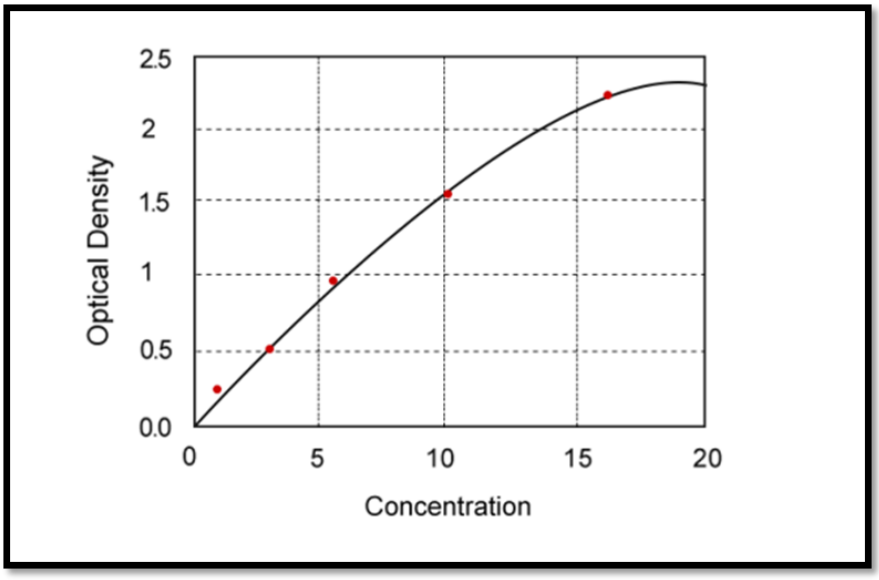


Figure (2.4): The standard curve of IL-41 concentration

2.9.2.4. Assay procedure

The determination of serum METRNL concentration was performed using a standardized sandwich ELISA protocol according to the manufacturer's instructions. The procedure involved the following steps:

1. **Reagent preparation**:
   All reagents, standard solutions, and samples were prepared as instructed in the kit manual. Prior to the assay, all components were brought to room temperature (~22–25°C) to ensure optimal reaction conditions.
2. **Microplate setup**:
   The required number of 96-well microplate strips was selected based on the number of samples and standards. unused strips were immediately returned to storage at 2–8°C in a sealed pouch with desiccant to maintain stability.
3. **Standard and sample loading**:
   - **Standards**: 50 μL of METRNL standard solution was added to the designated wells.
     Note: The standard already contains the biotinylated detection antibody; therefore, no additional antibody was added to these wells.
   - **Samples**: 40 μL of each serum sample was added to the corresponding wells, followed by 10 μL of anti-METRNL antibody solution.
   - 50 μL of streptavidin-HRP conjugate was then added to all standard and sample wells (excluding blank wells).
     The plate was gently mixed and covered with a sealing film.
4. **Incubation**:
   The microplate was incubated for 60 minutes at 37°C to allow for immunocomplex formation.

5. **Washing**:

   After incubation, the plate was uncovered and washed five times using the provided wash buffer. Each wash cycle involved soaking the wells with 300 µL of wash buffer for 30 seconds to 1 minute. For manual washing, wells were emptied and blotted on absorbent material. For automated washing, aspiration or decanting was followed by buffer addition and mixing.

6. **Substrate reaction**:
   - 50 µL of Substrate Solution A(TMB) and 50 µL of Substrate Solution B (H_2O_2) were added sequentially to each well.
   - The plate was resealed and incubated for 10 minutes at 37°C in the dark to allow color development.

7. **Reaction termination**:

   A volume of 50 µL Stop Solution (H_2SO_4) was added to each well, resulting in an immediate color change from blue to yellow.

8. **Optical density measurement**:

   The absorbance (OD) of each well was read within 10 minutes after adding the stop solution, using a microplate reader set to 450 nm.

2.9.2.5. Calculation of results

The concentration of METRNL in serum samples was determined by constructing a standard curve. The procedure involved plotting the mean optical density (OD) values for each standard on the Y-axis against their corresponding concentrations (ng/mL) on the X-axis.

A best-fit curve was generated using non-linear regression analysis, which is recommended for ELISA data due to the sigmoidal nature of antigen-antibody

binding responses. The standard curve was constructed using computer-based curve-fitting software, which allowed interpolation of unknown sample concentrations directly from the OD values.

All sample OD readings were within the linear range of the assay.

2.10. Assay validation and quality control.

2.10.1. Assay validation

The ELISA assay was validated according to ICH Q2(R1) guidelines (Guideline, 2005) to ensure analytical method reliability and reproducibility. Precision was comprehensively assessed by analyzing three serum samples in six replicates for intra-assay variability (CV% ≤ 10%) and over three consecutive days for inter-assay variability (CV% ≤ 15%), with all values falling within acceptable limits (<15%). Accuracy was rigorously determined through spike-and-recovery studies (n=5) conducted at three concentration levels across the assay's dynamic range, demonstrating mean recovery rates within the acceptable range of 85-115%.

Method linearity was confirmed through serial dilutions spanning the assay's specified concentration range, achieving correlation coefficients of $R^2 \geq 0.995$. The assay demonstrated appropriate sensitivity with limits of detection calculated as three times the standard deviation of blank measurements (3×SD of blank). Specificity testing revealed no significant cross-reactivity (<5%) with analogous proteins, confirming the method's high selectivity for the target analytic. These comprehensive validation parameters collectively ensured the analytical method met international standards for bioanalytical procedures.

2.10.2. Quality Control Measures

Duplicates of each standard concentration were prepared to ensure statistical reliability. Fresh pipette tips were used for each solution to prevent cross-contamination. Preparation times were recorded for each step to maintain consistency. All solutions were visually inspected for clarity and homogeneity. Volumes were measured with precision (±1 µl accuracy). The standard curve demonstrated linearity with $R^2 \geq 0.995$, coefficient of variation (CV) ≤ 5% between duplicates, and recovery of 90-110% for intermediate standards.

2.11. Statistical Analysis

The statistical analysis was performed with IBM SPSS Statistics version 23. The results from the analysis were subsequently summarized adopting descriptive statistics. Likewise, the Mean and Standard Deviation have been determined. A probability criterion of $p < 0.05$ was utilized to evaluate the statistical significance of the experimental findings. Moreover, the Shapiro-Wilk test has been employed to determine the normality of the data, and the Levene test has been conducted to examine the homogeneity of variance. Additionally, the Mann-Whitney Test and Independent T-Test were utilized to determine statistical differences between two distinct groups. The analysis of variance (ANOVA) was employed to do multiple comparisons among the groups. The Scheffé's post-hoc test was utilized at a significance threshold of $p < 0.05$ to do multiple comparisons among groups. Asterisks represent data which has a *P* value below 0.05.

2.12. Ethical Approval

The study protocol was approved by the Scientific Research Ethics Committee at the College of Medicine in Kerbala, the ethical committees in Kerbala and Al-Majar Al-Kabir Hospital in Maysan Governorate. Verbal consent was

obtained from patients' relatives before sample collection, with all necessary health and preventive measures followed during the sampling process.

Chapter Three

Results

3.1. Demographic and clinical characteristics of study group.

The sex distribution among the three groups showed a remarkable balance. In the Control Group, males constituted 48.8% and females 51.2%. Similarly, in the DKD Group, males and females (50% each). In the DM Group, males accounted for 51.2% and females 48.8%. Overall, the total sex distribution was nearly equal, with males comprising 50% and females 50% of the total study participants. The age groups of the participants were distributed as follows: (30) were between 19-30 years old, (63) between 31-40 years old, and (35) were over 40 years old. The overall mean age of the study participants was 36.05 ± 7.95 years. When considering the groups individually, the mean age in the Control Group was 35.16 ± 6.78 years, in the DKD Group it was 36.69 ± 8.79 years, and in the DM Group it was 36.30 ± 8.28 years.

The types of treatment varied between the DKD and DM patient groups. In the DKD Group, (12) of patients received insulin therapy, (17) received oral therapy, and (13) received mixed therapy (insulin and oral). In the DM Group, (17) received insulin therapy, (15) received oral therapy, and (11) received mixed therapy Overall, among all participants, (29) received insulin, (32) received oral medication, and (24) received mixed therapy. The Control Group did not receive any treatment, as they represented healthy individuals.

Data showed that (61) of DKD patients and 74.4% of DM patients had a family history of the disease, in contrast, the Control Group had no family history of the disease Overall, 47.7% of all participants had a family history of the disease.

Regarding the number of dialysis sessions, neither the Control Group nor the DM Group received any dialysis sessions (100.0% for each). However, in the DKD Group, (18) of patients underwent one to two sessions per week, while (24) underwent three to four sessions per week Overall, (86) of all participants had no dialysis sessions, (18) had 1-2 sessions/week, and (24) had 3-4 sessions/week.

Table 3.1: Demographic and basic characteristics of study group

Parameters	Level	Control Group	DKD Group	DM Group	Total
Sex	Male	21 48.8%	21 50.0%	22 51.2%	64 50.0%
	Female	22 51.2%	21 50.0%	21 48.8%	64 50.0%
Type of treatment					
	Insulin	0 0.0%	12 28.6%	17 39.5%	29 22.7%
	Oral	0 0.0%	17 40.5%	15 34.9%	32 25.0%
	Mix	0 0.0%	13 31.0%	11 25.6%	24 18.8%
Family history to disease	No	0 0.0%	13 31.0%	11 25.6%	24 18.8%
	Yes	0 0.0%	29 69.0%	32 74.4%	61 47.7%
Dialysis Sessions					
	None	43 100.0%	0 0.0%	43 100.0%	86 67.2%
	1-2 Sessions/Week	0 0.0%	18 42.9%	0 0.0%	18 14.1%
	3-4 Sessions/Week	0 0.0%	24 57.1%	0 0.0%	24 18.8%
Age Group	19 - 30	11 25.6%	10 23.8%	9 20.9%	30 23.4%
	31 -40	22 51.2%	19 45.2%	22 51.2%	63 49.2%
	> 40	10 23.3%	13 31.0%	12 27.9%	35 27.3%
Age Mean		35.16 ± 6.78	36.69 ± 8.79	36.30 ± 8.28	36.05 ± 7.95

3.2. Biochemical parameters.

Table presented a comparison of key biochemical parameters among the three groups. ANOVA and Kruskal-Wallis tests were used for the statistical analysis.

The results showed highly significant statistical differences ($p = 0.0009$) in HbA1c levels among the groups.The mean HbA1c in the Control Group was significantly lower compared to the DKD Group and the DM Group, 4.728 ± 0.368, 12.235 ± 2.108, and 12.481 ± 1.946, respectively.

There were highly significant statistical differences ($p = 0.0002$) in RBS levels among the groups, with the Control, DKD, and DM Groups recording mean values of 109.023 ± 10.498, 233.248 ± 72.932, and 333.326 ± 68.545, respectively.

Statistically significant differences ($p = 0.0006$) were observed in hemoglobin levels. The Control Group had the highest mean hemoglobin level (12.743 ± 1.411), while the DKD Group recorded the lowest mean (9.007 ± 1.466), and the DM Group was in between (10.698 ± 1.980).

The differences in serum creatinine levels were highly statistically significant ($p = 0.0004$). The DKD Group recorded the highest mean (6.994 ± 1.386). In contrast, creatinine levels in the Control Group (0.500 ± 0.202) and the DM Group (0.765 ± 0.187) were within or close to the normal range.

Blood urea levels showed highly significant statistical differences ($p = 0.0009$). The DKD Group had the highest mean urea (134.079 ± 27.634), while the Control Group (26.605 ± 6.776) and the DM Group (29.209 ± 5.680) were within normal levels.

There were highly significant statistical differences (p = 0.0001) in serum albumin levels. The DKD Group recorded the lowest mean (3.429 ± 0.424), while the Control Group (3.826 ± 0.274) and the DM Group (3.981 ± 0.411) were higher.

Statistically significant differences (p = 0.0007) were observed in serum calcium levels. The DKD Group had the lowest mean calcium (8.541 ± 0.697), while the Control Group (8.928 ± 0.310) and the DM Group (8.863 ± 0.369) were higher.

Serum phosphate levels showed highly significant statistical differences (p = 0.0003). The DKD Group recorded the highest mean (5.055 ± 1.379), followed by the DM Group (4.079 ± 0.353), while the Control Group had the lowest (3.228 ± 0.474)

Table 3.2: Biochemical and hematological parameters

Parameters	Control Group		DKD Group		DM Group		p. value
	Mean	Std. D	Mean	Std. D	Mean	Std. D	
HBA1C %	4.728[a]	0.368	12.235[b]	2.108	12.481[b]	1.946	0.0009
RBS (mg/dL)	109.023[a]	10.498	233.248[b]	72.932	333.326[c]	68.545	0.0002
Hemoglobin (g/dL)	12.743[a]	1.411	9.007[b]	1.466	10.698[c]	1.980	0.0006
S.Creatinine (mg/dL)	0.500[a]	0.202	6.994[b]	1.386	0.765[a]	0.187	0.0004
B.Urea (mg/dL)	26.605[a]	6.776	134.079[b]	27.634	29.209[a]	5.680	0.0009
S.Albumin (g/dL)	3.826[b]	0.274	3.429[a]	0.424	3.981[b]	0.411	0.0001
S.Ca (mg/dL)	8.928[b]	0.310	8.541[a]	0.697	8.863[b]	0.369	0.0007
S.PO4 (mg/dL)	3.228[a]	0.474	5.055[b]	1.379	4.079[c]	0.353	0.0003

Note: DKD = Diabetic Kidney Disease; DM = Diabetes Mellitus; HbA1c = Glycated Hemoglobin; RBS = Random Blood Sugar; S. = Serum; B. = Blood. Data presented as mean ± standard deviation. Different superscript letters (a,b,c) indicate statistically significant differences between groups (p<0.05).

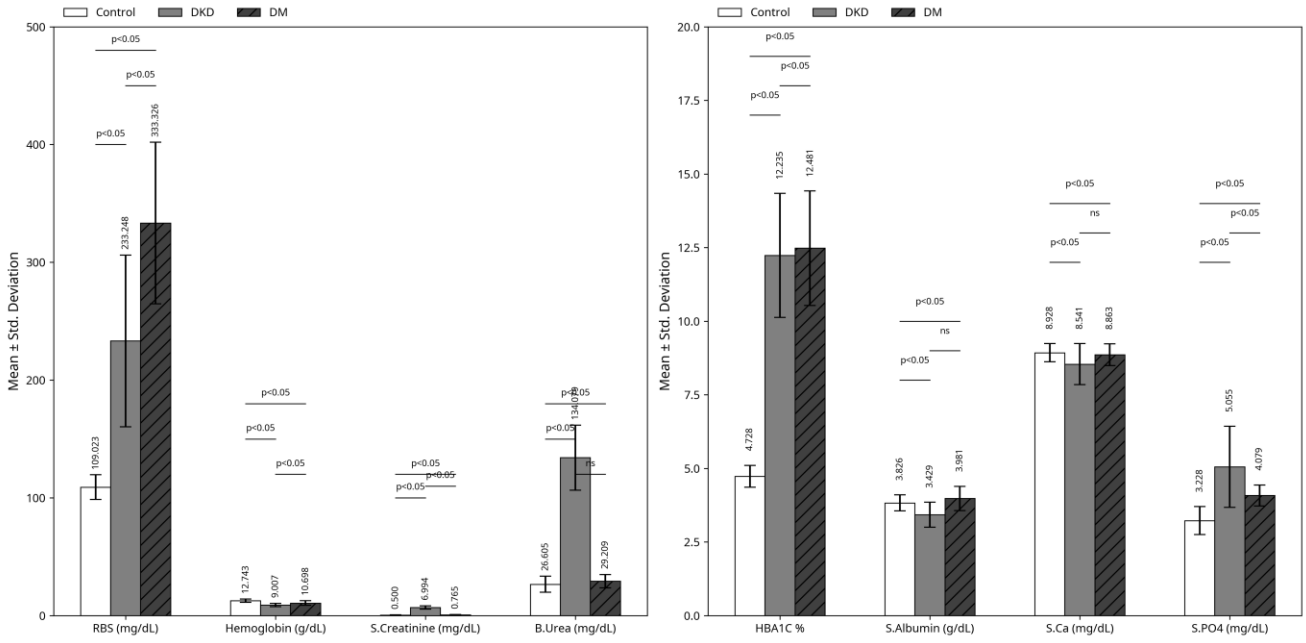


Figure (3.1) the study result compared between the of biochemical and hematological parameters among the three groups.

3.3. Inflammatory markers and iron metabolism parameters

Table 3.3 showed a comparison of key biochemical parameters among the three groups. ANOVA and Kruskal-Wallis tests were used for the statistical analysis.

Interleukin-40 (IL-40) and Interleukin-41 (IL-41) levels were significantly reduced in both the DKD and DM groups compared to the control group. Serum ferritin levels were significantly elevated in the DKD group relative to the DM and control groups, while unsaturated iron-binding capacity (UIBC) was notably lower in the DM group than in the DKD and control groups. Additionally, serum iron concentrations were significantly decreased in both DKD and DM groups compared to the control group. The mean values for IL-40, IL-41, ferritin, UIBC, and serum iron were 15.791 ± 4.001, 14.114 ± 3.686, and 29.859 ± 8.742 pg/mL; 0.525 ±

0.180, 0.641 ± 0.110, and 3.013 ± 0.983 pg/mL; 315.586 ± 97.687, 122.079 ± 40.268, and 78.209 ± 22.323 ng/mL; 162.395 ± 11.632, 247.781 ± 81.081, and 228.163 ± 34.868 µg/dL; and 82.833 ± 30.662, 85.023 ± 9.580, and 104.349 ± 14.832 µg/dL in the DKD, DM, and control groups, respectively. All differences were statistically significant ($p < 0.005$).

Table (3.3): Inflammatory markers and iron metabolism parameters

Parameters	Control Group		DKD Group		DM Group		p. value
	Mean	Std. D	Mean	Std. D	Mean	Std. D	
IL-40 (pg/mL)	29.859[a]	8.742	15.791[b]	4.001	14.114[b]	3.686	0.0002
IL-41 (pg/mL)	3.013[b]	0.983	0.525[a]	0.180	0.641[a]	0.110	0.0004
S.ferritin (ng/mL)	78.209[b]	22.323	315.586[a]	97.687	122.079[a]	40.268	0.0006
UIBC (µg/dL)	228.163[b]	34.868	247.781[b]	81.081	162.395[a]	11.632	0.0004
Iron (µg/dL)	104.349[b]	14.832	82.833[a]	30.662	85.023[a]	9.580	0.0023

Note: UIBC = Unsaturated Iron Binding Capacity.

3.4. Correlation analysis of IL-40 and IL-41 with clinical parameters in the DKD group

Table (3.4) presented the correlation analysis between interleukin levels (IL-40 and IL-41) and various clinical parameters within the diabetic kidney disease (DKD) group, utilizing Pearson and Spearman's correlation tests as appropriate. No significant correlations were observed between HbA1c levels and any of the measured variables, including serum creatinine ($r = 0.207$, $p = 0.189$), blood urea ($r = -0.087$, $p = 0.586$), serum ferritin ($r = -0.004$, $p = 0.980$), IL-40 ($r = 0.092$, $p = 0.561$), or IL-41 ($r = 0.061$, $p = 0.699$). Similarly, serum creatinine did not correlate significantly with blood urea ($r = 0.014$, $p = 0.930$), serum ferritin ($r = -0.272$, $p = 0.081$), IL-40 ($r = 0.000$, $p = 0.999$), or IL-41 ($r = 0.029$, $p = 0.856$). Blood urea levels exhibited weak and non-significant correlations with serum ferritin ($r =$

0.131, p = 0.407), IL-40 (r = 0.027, p = 0.865), and IL-41 (r = 0.024, p = 0.882). Additionally, serum ferritin showed no signigicant association with IL-40 (r = -0.078, p = 0.626) or IL-41 (r = 0.073, p = 0.646). The only statistically significant correlation identified was between IL-40 and IL-41 levels (r = 0.335, p = 0.030).

Table (3.4): Correlation analysis of IL-40 and IL-41 with clinical parameters in DKD group

Parameter	Value	S.Creatinine (mg/dL)	B.Urea (mg/dL)	S.ferritin (ng/mL)	IL-40 (pg/mL)	IL-41 (pg/mL)
HBA1C %	r. value	.207	-.087	-.004	.092	.061
	p. value	.189	.586	.980	.561	.699
S.Creatinine (mg/dL)	r. value	1	.014	-.272	.000	.029
	p. value		.930	.081	.999	.856
B.Urea (mg/dL)	r. value		1	.131	.027	.024
	p. value			.407	.865	.882
S.ferritin (ng/mL)	r. value			1	-.078	.073
	p. value				.626	.646
IL-40 (pg/mL)	r. value				1	.335*
	p. value					.030

Note: * Statistical significance (p < 0.05); r = Pearson correlation coefficient

3.5. Subgroup analysis of IL-40 and IL-41 levels based on treatment type in DKD patients.

The levels of IL-40 and IL-41 in diabetic kidney disease (DKD) patients were analyzed according to the type of treatment received (insulin therapy, oral hypoglycemic agents, or a combination of both). Comparisons among the groups showed no statistically significant differences in any of the measured parameters (p > 0.05).

The mean IL-40 level was (13.830 ± 4.035 pg/mL) in the insulin group, (15.283 ± 3.855 pg/mL) in the oral treatment group, and (18.266 ± 6.022 pg/mL) in the mixed treatment group (p = 0.372).

For IL-41, the levels were (0.523 ± 0.170) pg/mL in the insulin group, (0.519 ±

0.165) pg/mL in the oral group, and (0.536 ± 0.160) pg/mL in the mixed treatment group (p = 0.998).

Table (3.5): Subgroup analysis of IL-40 and IL-41 levels based on treatment type in DKD patients

Parameters	Insulin Treatment		Oral Treatment		Mix Treatment		p. value
	Mean	Std. D	Mean	Std. D	Mean	Std. D	
IL-40(pg/mL)	13.830	4.035	15.283	3.855	18.266	6.022	0.3720
IL-41(pg/mL)	0.523	0.170	0.519	0.165	0.536	0.160	0.9980

3.6. Liver function parameters across study groups.

The table presented a comparative analysis of liver function parameters between the diabetic kidney disease (DKD) group and the diabetes mellitus (DM) group, revealing statistically significant differences in several key biomarkers, as assessed using the Independent T-Test and the Mann-Whitney U test.

Serum glutamate-oxaloacetate transaminase (S.GOT/AST) levels were slightly higher in the DKD group (15.512 ± 6.275 U/L) compared to the DM group (13.328 ± 2.604 U/L); however, this difference did not reach statistical significance (p = 0.109). Conversely, serum glutamate-pyruvate transaminase (S.GPT/ALT) levels were significantly elevated in DKD patients (16.079 ± 6.117 U/L) compared to the DM group (11.540 ± 2.194 U/L, p = 0.003).

A marked increase in alkaline phosphatase (ALP) levels was observed in the DKD group (402.652 ± 122.589 U/L) compared to the DM group (39.186 ± 8.653 U/L, p = 0.0006).

serum total protein levels were significantly reduced in DKD patients (5.517 ± 0.689 g/dL) compared to the DM group (7.107 ± 0.789 g/dL, p = 0.0001).

Table 3.6: Liver function parameters across study groups

Parameters	DKD Group		DM Group		Reference Range	p. value
	Mean	Std. D	Mean	Std. D		
S.GOT(AST)	15.512	6.275	13.328	2.604	8-40 U/L Male 5-32 U/L Female	0.1090
S.GPT(ALT)	16.079	6.117	11.540	2.194	7-56 U/L Male 7-45 U/L Female	0.0030
ALP (U/L)	402.652	122.589	39.186	8.653	30-120 U/L	0.0006
S. total protein (g/dL)	5.517	0.689	7.107	0.789	6.4-8.3 g/dl	0.0001

3.7. Detailed analysis of mineral and electrolyte profile

The table presented a comparative analysis of the mineral and electrolyte profile between patients with Diabetic Kidney Disease (DKD) and those with Diabetes Mellitus (DM), revealed several statistically significant differences in various parameters. Statistical analysis was performed using the Independent T-test and the Mann-Whitney U test, as appropriate based on data type and distribution.

Serum sodium ($S.Na^{+2}$) levels were significantly lower in DKD patients (133.481 ± 3.658 mEq/L) compared to DM patients (136.744 ± 1.136 mEq/L) ($p = 0.0001$). Similarly, serum calcium ($S.Ca^{++}$) levels were significantly reduced in the DKD group (8.541 ± 0.697 mg/dL) relative to the DM group (8.863 ± 0.369 mg/dL) ($p = 0.010$). In contrast, serum phosphate ($S.PO_4$) levels were markedly higher in DKD patients (5.055 ± 1.379 mg/dL) than in DM patients (4.079 ± 0.353 mg/dL) ($p = 0.0005$).

Serum iron levels did not show a significant difference between the DKD group (82.833 ± 30.662 µg/dL) and the DM group (85.023 ± 9.580 µg/dL) ($p = 0.784$). However, significant variations were observed in unsaturated iron-binding capacity

(UIBC) and serum ferritin levels. UIBC was significantly elevated in DKD patients (247.781 ± 81.081 µg/dL) compared to those with DM (162.395 ± 11.632 µg/dL) (p = 0.0003). Additionally, serum ferritin levels were markedly higher in the DKD group (315.586 ± 97.687 ng/mL) than in the DM group (122.079 ± 40.268 ng/mL) (p = 0.0001).

Table 3.7: Detailed analysis of mineral and electrolyte profile

Parameters	DKD		DM		Reference Range	p. value
	Mean	Std. D	Mean	Std. D		
S.Na^{+2} (mEq/L)	133.481	3.658	136.744	1.136	135-145 mmol/L	0.0001
S.Ca^{++} (mg/dL)	8.541	0.697	8.863	0.369	8.8-10.6 mg/dl	0.0100
S.PO4 (mg/dL)	5.055	1.379	4.079	0.353	2.5-4.5 mg/dl	0.0005
Iron (µg/dL)	82.833	30.662	85.023	9.580	33-193 ug/dl	0.7840
UIBC (µg/dL)	247.781	81.081	162.395	11.632	112-347 ug/dl	0.0003
S.ferritin (ng/mL)	315.586	97.687	122.079	40.268	30-400 ng/ml (M) 15-150 ng/ml (F)	0.0001

Note: M=male; F=female

3.8. Comparison of IL-40 and IL-41 levels based on family history in DKD patients

Table (3.8) presented a comparative analysis of interleukin-40 (IL-40) and interleukin-41 (IL-41) levels, along with age and unsaturated iron-binding capacity (UIBC), based on the presence or absence of a family history of diabetes in patients with Diabetic Kidney Disease (DKD). Independent T-tests and Mann-Whitney U tests were employed to assess the differences between the two groups.

The mean IL-40 level was slightly higher in patients with a family history (15.240 ± 5.503 pg/mL) compared to those without a family history (14.188 ± 4.540 pg/mL); however, this difference did not reach statistical significance (p = 0.4870).

In contrast, IL-41 levels were significantly elevated in the group with a family history (0.670 ± 0.220 pg/mL) compared to those without (0.366 ± 0.102 pg/mL), and this difference was statistically significant (p = 0.0066).

The average age of patients with a family history (36.967 ± 8.832 years) did not differ significantly from those without a family history (35.292 ± 7.590 years) (p = 0.4159).

UIBC levels were significantly higher in patients with a positive family history (213.541 ± 79.597 µg/dL) compared to those without (181.825 ± 37.686 µg/dL) (p = 0.0150).

Table 3.8: Comparison of IL-40 and IL-41 levels based on family history in DKD patients

Parameters	Family History Present		Family History Absent		p. value
	Mean	Std. Deviation	Mean	Std. Deviation	
IL-40 (pg/mL)	15.240	5.503	14.188	4.540	0.4870
IL-41 (pg/mL)	0.670	0.220	0.366	0.102	0.0066
Age	36.967	8.832	35.292	7.590	0.4159
UIBC (µg/dL)	213.541	79.597	181.825	37.686	0.0150

Note: UIBC= unsaturated iron binding capacity

3.9. Comparison of clinical parameters based on number of dialysis sessions in DKD group.

Table 3.9 presented a comparative analysis of selected clinical parameters among patients with Diabetic Kidney Disease (DKD), stratified by the number of dialysis sessions per week (1–2 sessions vs. 3–4 sessions). Independent T-tests and Mann-Whitney U tests.

The mean IL-40 level was higher in patients who underwent 3–4 dialysis sessions per week (17.168 ± 9.332 pg/mL) compared to those who received 1–2 sessions (13.956 ± 4.544 pg/mL); however, this difference was not statistically significant (p = 0.2020). Similarly, IL-41 levels were elevated in the 3–4 session group (0.652 ± 0.122 pg/mL) compared to the 1–2 session group (0.357 ± 0.120 pg/mL), with a non-significant p-value of 0.1210.

Serum creatinine levels appeared comparable between the two groups, with means of 7.057 ± 0.744 mg/dL in the 1–2 session group and 6.946 ± 1.736 mg/dL in the 3–4 session group (p = 0.7800). Blood urea levels also showed no significant difference between the groups (135.817 ± 23.496 mg/dL vs. 132.775 ± 30.807 mg/dL; p = 0.7290). Hemoglobin concentrations were similar across both groups (9.067 ± 1.576 g/dL vs. 8.963 ± 1.411 g/dL; p = 0.8230). Lastly, serum ferritin levels were moderately higher in the 3–4 session group (330.925 ± 110.322 ng/mL) than in the 1–2 session group (295.133 ± 99.386 ng/mL), though this difference also lacked statistical significance (p = 0.6010).

Table 3.9: Comparison of clinical parameters based on number of dialysis sessions in DKD group

Parameters	1-2 Sessions/Week		3-4 Sessions/Week		p. value
	Mean	Std. Deviation	Mean	Std. Deviation	
IL-40 (pg/mL)	13.956	4.544	17.168	9.332	0.2020
IL-41 (pg/mL)	0.357	0.120	0.652	0.122	0.1210
S.Creatinine (mg/dL)	7.057	0.744	6.946	1.736	0.7800
B.Urea (mg/dL)	135.817	23.496	132.775	30.807	0.7290
Hemoglobin (g/dL)	9.067	1.576	8.963	1.411	0.8230
S.ferritin (ng/mL)	295.133	99.386	330.925	110.322	0.6010

3.10. predictive performance of IL-40 and IL-41 in distinguishing between study groups.

3.10.1 predictive performance of IL-40 and IL-41 in 41 in distinguishing between diabetic kidney disease patients and healthy controls.

The Receiver Operating Characteristic (ROC) curve analysis was employed to evaluate the diagnostic performance of IL-40 and IL-41 in distinguishing Diabetic Kidney Disease (DKD) patients from healthy controls, as presented in Table (3.10.1) and Figure (3.2).

IL-40 demonstrated good diagnostic performance, with an Area Under the Curve (AUC) of 89.646%, a standard error of 0.039, and a statistically significant p-value of 0.003. The optimal cutoff value was determined to be 17.960 pg/mL. At this threshold, IL-40 achieved a sensitivity of 83.333% and a specificity of 97.674%, with an overall diagnostic accuracy of 90.588%. The positive predictive value (PPV) was 97.222%, while the negative predictive value (NPV) reached 85.714%. The 95% confidence interval ranged from 0.820 to 0.973.

In comparison, IL-41 showed superior diagnostic accuracy, with an AUC of 98.394%, a standard error of 0.011, and a highly significant p-value of 0.001. The optimal cutoff value was identified as 1.850 pg/mL. At this threshold, IL-41 achieved a sensitivity of 95.238%, specificity of 95.349%, and an overall accuracy of 95.294%. Both the PPV and NPV were 95.238% and 95.349%, respectively. The 95% confidence interval for the AUC ranged from 0.963 to 1.000.

Table (3.10.1): predictive performance of IL-40 and IL-41 in 41 in distinguishing between diabetic kidney disease patients and healthy controls.

Metrics		DKD vs Control	
		IL-40	IL-41
Std. Error		0.039	0.011
Asymptotic Sig.		0.003	0.001
Asymptotic 95% Confidence Interval	Lower Bound	0.820	0.963
	Upper Bound	0.973	1.000
Cutoff Point		17.960	1.850
Area Under Curve (AUC)		89.646%	98.394%
Sensitivity		83.333%	95.238%
Specificity		97.674%	95.349%
Accuracy		90.588%	95.294%
Positive Predictive Value		97.222%	95.238%
Negative Predictive Value		85.714%	95.349%

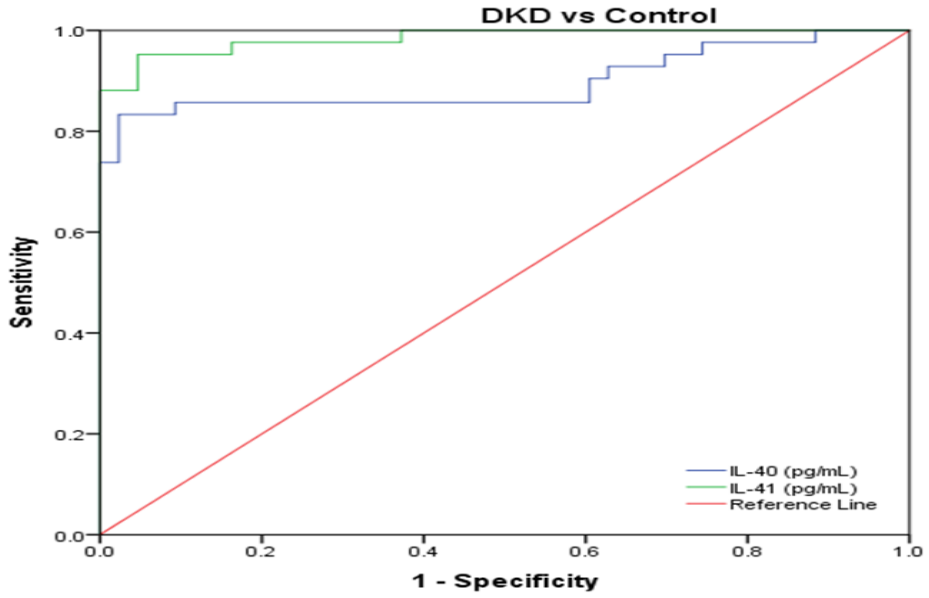


Figure (3.2): Receiver Operating Characteristic (ROC) curves for IL-40 and IL-41 in distinguishing between diabetic kidney disease patients and healthy controls.

3.10.2 predictive performance of IL-40 and IL-41 in 41 in distinguishing between diabetic mellitus patients and healthy controls.

Table 3.10.2. and figure (3.3) summarized the diagnostic performance of interleukin-40 (IL-40) and interleukin-41 (IL-41) in distinguishing patients with Diabetes Mellitus (DM) from healthy controls, using Receiver Operating Characteristic (ROC) curve analysis.

IL-40 demonstrated a high diagnostic capability, with an area under the curve (AUC) of 97.783%. The optimal cutoff value was determined to be 17.953 pg/mL. At this threshold, IL-40 achieved a sensitivity of 90.698%, specificity of 97.674%, and overall diagnostic accuracy of 94.186%. Both the positive predictive value (PPV) and negative predictive value (NPV) were 94.186%. The standard error was 0.012, with a statistically significant p-value of 0.002. The 95% confidence interval for the AUC ranged from 0.954 to 1.000. IL-41 displayed even stronger diagnostic performance, with an AUC of 99.567% and a cutoff value of 1.807 pg/mL. It yielded a sensitivity of 97.674%, specificity of 95.349%, and diagnostic accuracy of 96.512%. The PPV was 95.455%, while the NPV reached 97.619%. The standard error was 0.004, and the asymptotic significance was 0.003. The AUC 95% confidence interval ranged from 0.988 to 1.000.

Table 3.10.2: predictive performance of IL-40 and IL-41 in in distinguishing between diabetes mellitus patients and healthy controls

Metrics		DM vs Control	
		IL-40	IL-41
Std. Error		0.012	0.004
Asymptotic Sig.		0.002	0.003
Asymptotic 95% Confidence Interval	Lower Bound	0.954	0.988
	Upper Bound	1.000	1.000
Cutoff Point		17.953	1.807
Area Under Curve (AUC)		97.783%	99.567%
Sensitivity		90.698%	97.674%
Specificity		97.674%	95.349%
Accuracy		94.186%	96.512%
Positive Predictive Value		94.186%	95.455%
Negative Predictive Value		94.186%	97.619%

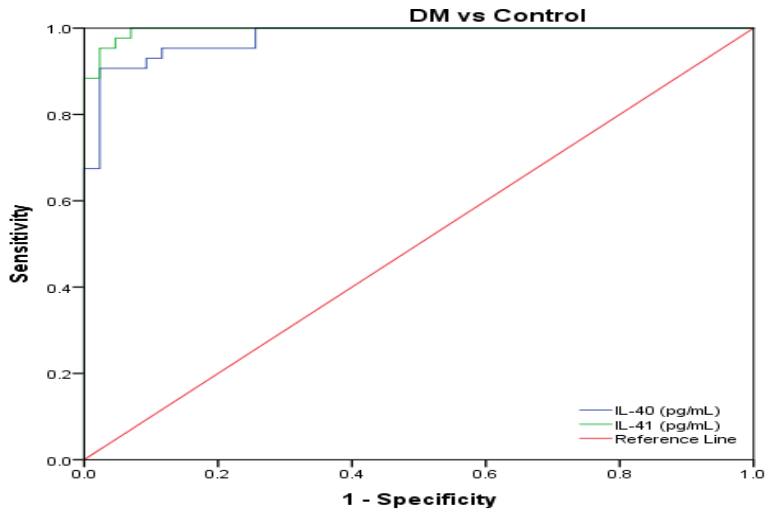


Figure (3.3): Receiver Operating Characteristic (ROC) curves for IL-40 and IL-41 in distinguishing between diabetes mellitus patients and healthy controls.

3.10.3. predictive performance of IL-40 and IL-41 in 41 in distinguishing between diabetic kidney patients and diabetes mellitus patients.

Table 3.10.3. and figure (3.4) presented the diagnostic performance of interleukin-40 (IL-40) and interleukin-41 (IL-41) in differentiating Diabetic Kidney Disease (DKD) from Diabetes Mellitus (DM) using Receiver Operating Characteristic (ROC) curve analysis.

IL-40 demonstrated limited discriminative power between DKD and DM groups, with an area under the curve (AUC) of 52.658%. The optimal cutoff value was identified as 14.604 pg/mL. At this threshold, IL-40 achieved a sensitivity of 66.667%, specificity of 48.837%, and diagnostic accuracy of only 42.353%. The positive predictive value (PPV) and negative predictive value (NPV) were 44.000% and 40.000%, respectively. The standard error was 0.064,

the p-value of 0.673 indicated no statistically significant diagnostic discrimination. IL-41 showed slightly better diagnostic potential with an AUC of 62.652% and a cutoff value of 0.183 pg/mL. It yielded a sensitivity of 42.857%, specificity of 86.047%, and overall accuracy of 35.294%. The PPV and NPV were 25.000% and 39.344%, respectively. The standard error was 0.062, and the result reached statistical significance with a p-value of 0.045. However Statistical significance was determined at the 0.05 (*) and 0.01 (**) levels.

Table 3.10.3: predictive performance of IL-40 and IL-41 in distinguishing between diabetes kidney disease patients and diabetes mellitus patients.

Metrics		DKD vs. DM	
		IL-40	IL-41
Std. Error		0.064	0.062
Asymptotic Sig.		0.673	0.045
Asymptotic 95% Confidence Interval	Lower Bound	0.401	0.505
	Upper Bound	0.652	0.748
Cutoff Point		14.604	0.183
Area Under Curve (AUC)		52.658%	62.652%
Sensitivity		66.667%	42.857%
Specificity		48.837%	86.047%
Accuracy		42.353%	35.294%
Positive Predictive Value		44.000%	25.000%
Negative Predictive Value		40.000%	39.344%

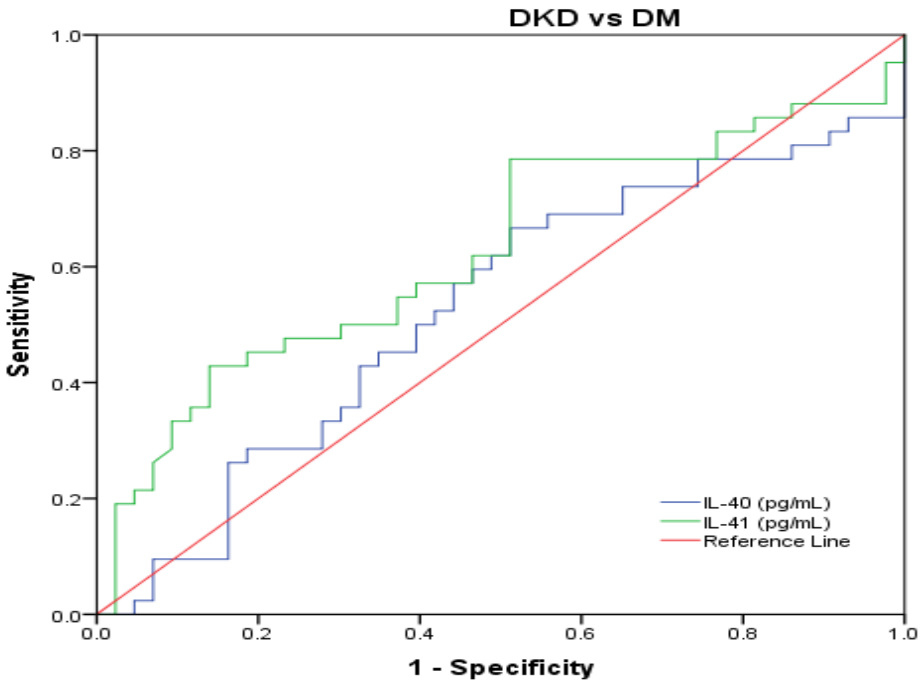


Figure (3.4): Receiver Operating Characteristic (ROC) Curves for IL-40 and IL-41 in distinguishing between diabetic kidney disease and diabetes mellitus groups

Chapter Four

Discussion

4. Discussion

4.1. Demographic and clinical characteristics of study groups.

The analysis of demographic and basic clinical characteristics revealed several important observations that enhance the validity and interpretability of the study's findings. the study results demonstrated a balanced sex distribution across all three groups with equal representation of males and females, while ages were concentrated in the thirties age group with high prevalence of family history of disease in both patient groups, and all diabetic kidney disease patients required dialysis with varying intensity distributions, while drug therapy patterns varied between the two diabetes groups. These findings are consistent with the study by **Giandalia *et al*., (2021)** which confirmed that diabetic nephropathy affects both sex at similar rates in advanced stages of the disease, and the studied age group aligns with the **Johansen *et al*., (2024)** reported which indicated that the average age of diabetic nephropathy onset ranges between (35-45) years in developing communities. The high prevalence of family history is consistent with the findings of **Seaquist *et al*., (1989)** who found that having a family history of diabetes increases the risk of kidney complications by 2.5-fold, supporting the hypothesis of genetic predisposition to diabetic nephropathy established by **Mohammedi, Marre, and Alhenc-Gelas, (2024)**, and the fact that all DKD patients require dialysis is consistent with **Klein *et al*., (2024)** who indicated that diabetic patients represent 44% of new dialysis patients.

Regarding treatment patterns, the findings align with the study on ***Betônico et al*., (2016)**, which confirmed that insulin has been traditionally considered the safe choice for treating diabetic patients with kidney injury, as recent guidelines

published by **Liu *et al*., (2021)** in diabetic kidney disease patients. This pattern is particularly relevant for new dialysis patients, as initiation of dialysis often coincides with more advanced renal dysfunction and metabolic instability, necessitating careful glycemic control. The transition to dialysis introduces additional challenges such as altered insulin clearance, increased risk of hypoglycemia, and fluid-electrolyte shifts, which further reinforce the preference for insulin therapy in this population.

Rossing *et al*., (2022) recommendations align with previous study which emphasize the necessity of adjusting medication doses with declining kidney function and the need for combination therapy in advanced cases.

Nevertheless, the current findings differ from several other studies in important aspects. Regarding sex distribution, they differ from the study by **Maric-Bilkan ,(2020)** which indicated relative protection for females in diabetic nephropathy with higher prevalence among males at 60-65%, and they varied from **Boenink *et al*., (2022)** study which mentioned that the average age of diabetic dialysis patients in Europe is 62.3 years, approximately 25 years older than the average age of our cohort, and the family history prevalence differs from **Chen *et al*., (2018)** study in Asia which revealed lower prevalence of family history (45-52%) among diabetic nephropathy patients, while the high proportion of intensive dialysis sessions differs from the ERA-EDTA Registry, (2019) the study indicated that only 23% of diabetic nephropathy patients require intensive dialysis compared to the higher proportion in this study.

Current guidelines recommend the use of SGLT2 inhibitors in combination with metformin as first-line therapy for diabetic patients with chronic kidney disease, owing to their proven benefits in slowing CKD progression, reducing cardiovascular risk, and modestly improving glycemic control (**Iyer *et al*.,2022)**.

However, their use depends on renal function, with most recommendations supporting initiation in patients with an estimated GFR above 30 mL/min/1.73m². In more advanced stages of CKD or in patients undergoing dialysis, insulin therapy remains the preferred approach due to altered drug clearance and metabolic instability. This may explain why the studied treatment patterns showed greater reliance on insulin and oral therapies rather than widespread use of SGLT2 inhibitors **(Betts *et al*., (2024)**.

These differences can be explained through several factors including genetic and ethnic variation among different populations which may affect disease susceptibility and progression patterns as indicated by research published by **Krolewski *et al*., (1988)**, differences in healthcare systems regarding early diagnostic criteria, treatment protocols, and dialysis referral criteria, and varying environmental factors and dietary patterns across different geographical regions, the similarities can be explained through the shared biological nature of diabetic nephropathy across different populations, particularly in advanced stages where pathological mechanisms overcome individual and genetic differences. The balance in demographic characteristics ensures reduction of statistical bias in comparing results and validity of conclusions related to inflammatory markers, age homogeneity reduces the impact of age-related factors on immune response, high family history indicates activation of genetic pathways associated with chronic inflammation as confirmed by the research of **Kim *et al*., (2023)**, and advanced disease severity justifies the expected elevation in interleukin levels, providing an ideal model for studying these markers.

The current and previous study support the necessity of early screening for individuals with family history in the 30-40 age group and focus on preventive approaches before reaching advanced stages, emphasize the importance of

considering disease severity when interpreting inflammatory markers in clinical practice, and open avenues for future research directions including genetic analysis of patients with strong family history to identify genetic markers associated with the disease, longitudinal studies to track disease progression from early stages to dialysis requirement, and multi-center comparisons to understand the impact of geographical and ethnic factors on disease characteristics.

4.2. Analysis of biochemical parameters across study groups.

The biochemical analysis revealed clear differences in metabolic and renal profiles among the study groups. Patients with diabetic kidney disease (DKD) exhibited the most severe abnormalities, including impaired glycemic control, deteriorated renal function, and significant disturbances in mineral metabolism. In contrast, diabetes mellitus (DM) patients showed moderate changes, mainly related to glucose regulation.

Both diabetic groups had significantly elevated glycated hemoglobin (HbA1c) levels compared to the control group, Random blood sugar levels increased progressively from the control group to the DM group and were highest in the DKD group.

Hemoglobin levels were significantly reduced in DKD patients compared to both controls and DM patients. Renal function markers, including serum creatinine and blood urea, were markedly elevated in the DKD group, Furthermore, mineral metabolism disturbances were evident in DKD patients, characterized by increased serum phosphate and decreased serum calcium and albumin levels.

The glycated hemoglobin levels align with **ElSayed *et al*., (2022)** guidelines which indicate that HbA1c levels above 7% represent suboptimal glycemic control and increased risk for diabetic complications.

Random blood sugar levels showed high elevation from controls through diabetic kidney disease to diabetes mellitus groups, indicating varying degrees of glycemic dysregulation. The severe hyperglycemia observed in our diabetic groups is comparable to findings reported by the **Prospective Diabetes Study (UKPDS,1998)**(Prospective, 1998) which demonstrated similar glucose elevations in patients with established diabetic complications.

Hemoglobin levels were significantly reduced in diabetic kidney disease patients, reflecting anemia commonly associated with chronic kidney disease. Our findings demonstrate substantially more severe anemia than reported in numerous international studies, with our diabetic kidney disease group recording a mean hemoglobin of 9.0 g/dL, lower than that recorded by **Ljungberg *et al*., (2019)** which found average hemoglobin levels of 10.2 g/dL in diabetic dialysis patients. This also differs from **Cooper *et al*., (2019)** recommendations which define anemia as hemoglobin levels below 11 g/dL in women and 12 g/dL in men, suggesting either more advanced disease stage, different management protocols, or inadequate treatment resources in our population.

One of the major mechanisms underlying this anemia is the inadequate production of erythropoietin (EPO) by the damaged kidneys. In diabetic kidney disease, interstitial fibrosis and tubular atrophy impair the function of peritubular fibroblasts, which are the primary source of EPO synthesis. This reduced EPO production leads to insufficient stimulation of erythropoiesis in the bone marrow, thereby exacerbating anemia. Moreover, anemia in DKD may be further aggravated

by factors such as chronic inflammation, iron deficiency, and uremic toxins, but impaired EPO secretion remains the central driver **(Wagner *et al.*, 2011)**.

While the KDIGO (Kidney Disease: Improving Global Outcomes, 2020) guidelines do not specify a fixed blood urea level as a sole criterion for initiating dialysis, urea levels exceeding 50 mg/dL are often considered indicative of advanced renal impairment, especially when accompanied by clinical symptoms. In our cohort, blood urea levels reached a mean of 134.079 mg/dL, significantly surpassing this threshold. This may reflect delays in therapeutic intervention, differences in local clinical practices, or limited access to specialized nephrology services.

with substantially elevated serum creatinine levels compared to both control and diabetes mellitus groups. This discrepancy may reflect the exceptionally elevated serum creatinine levels in among the diabetic kidney disease group in this study, reaching 6.994 mg/dL, significantly higher than those reported by **(Johansen *et al.*, 2021)** who revealed that the average creatinine levels of 4.2 mg/dL at dialysis initiation. This suggests that our patients represent a substantially more advanced disease stage, completely different dialysis initiation criteria, or significant delays in diagnosis and referral to specialized care, far exceeding the **Levey *et al.*, (2020)** criteria for chronic kidney disease staging.

Serum albumin concentrations were reduced in the diabetic kidney disease group to 3.429 g/dL, representing a less severe reduction than anticipated given the advanced renal dysfunction indicated by other parameters. This may suggest differences in protein loss patterns or laboratory measurement methods compared to what **Levey *et al.*, (2020)** described in their comprehensive review of chronic kidney disease complications, this may reflect different pathophysiological mechanisms

in our population or a less advanced disease stage than expected in terms of its impact on serum albumin levels.

Serum calcium concentrations were reduced in the diabetic kidney disease group to 8.541 mg/dL, representing a more pronounced reduction than typically observed compared to studies implementing **(Moschella ,2016)** .This suggests either poor management of mineral metabolism disorders, a more advanced disease stage, or inadequate therapeutic interventions to correct these imbalances.

Serum phosphate levels were elevated in the diabetic kidney disease group to 5.055 mg/dL, clearly exceeding target levels recommended by **Ketteler *et al.*,(2017)** which advocate maintaining phosphate levels within normal range. This reflects either non-adherence to dietary restrictions, inadequate availability or utilization of phosphate binders, or delayed implementation of specialized treatment protocols.

From a pathophysiological perspective, these stark differences demonstrate accelerated progression of diabetic complications as described by **Brownlee ,(2005)** in the unifying hypothesis of diabetic complications, where uncontrolled chronic hyperglycemia accelerated Advanced Glycation End Products (AGEs) formation, oxidative stress, and inflammatory pathway activation more intensively than typically observed in medically advanced societies.

The biochemical profile differences can be attribute to reflecting the complex interplay between vitamin D deficiency, calcium–phosphate imbalance, and hypoalbuminemia in the setting of advanced renal dysfunction. Serum albumin levels were moderately reduced, which may have contributed to lower measured calcium values, since a significant proportion of circulating calcium is protein-bound. When corrected for albumin, serum calcium values appeared closer to normal, suggesting that part of the apparent hypocalcemia was attributable to hypoalbuminemia rather than a true deficit in ionized calcium. Nevertheless,

impaired renal conversion of vitamin D to its active form, together with urinary losses of vitamin D–binding protein in proteinuria, likely contributed to reduced intestinal calcium absorption and secondary hyperparathyroidism, reinforcing the presence of a genuine calcium deficit. At the same time, phosphate levels were clearly elevated, reflecting phosphate retention as renal clearance declined. Hyperphosphatemia not only suppresses renal 1α-hydroxylase activity and reduces active vitamin D synthesis, but also stimulates fibroblast growth factor 23 (FGF-23) and parathyroid hormone (PTH), further disturbing calcium–phosphate homeostasis and promoting the development of chronic kidney disease mineral and bone disorder (CKD-MBD). Collectively, these biochemical alterations underscore the central role of vitamin D deficiency, hypoalbuminemia, and phosphate retention in driving mineral metabolism disturbances in DKD, leading to secondary hyperparathyroidism, disordered bone turnover, and increased risk of vascular calcification.

4.3. Concurrent disruptions in cytokines (IL-40 and IL-41) and iron metabolism in diabetes mellitus and diabetic kidney disease.

The results revealed significant alterations in study markers and iron metabolism parameters across the three groups. IL-40 and IL-41 levels were markedly reduced in both the diabetes and diabetic kidney disease groups compared to the controls. In contrast, serum ferritin levels were significantly elevated, particularly in the diabetic kidney disease group. Meanwhile, unsaturated iron-binding capacity (UIBC) and serum iron levels exhibited variable patterns among the groups, with serum iron levels notably decreased in both patient groups compared to the controls.

This results revealed a significant decrease in IL-40 and IL-41 levels in patients with diabetic kidney disease (DKD) compared to healthy individuals. A similar reduction in these cytokines was also observed in patients with diabetes mellitus (DM), suggesting that this decline may be an early feature of immune dysregulation in diabetes, which becomes more pronounced with the progression of renal complications. These findings imply a potential dysregulation of IL-40 and IL-41 within the inflammatory response associated with both diabetes and DKD. although IL-40 has traditionally been linked to B-cell-mediated immune responses, its role in chronic kidney disorders remains poorly understood. Likewise, IL-41, known for its anti-inflammatory properties, may exert a protective effect during the early stages of DKD; however, its reduction in advanced stages may indicate an exhausted or impaired regulatory immune response.**(Donate-Correa *et al*., 2021; Hassan *et al*., 2024)**

The findings are consistent with the study of **Wu *et al*., (2020),** which demonstrated a significant association between elevated serum ferritin levels and the progression of chronic kidney disease in diabetic patients. These results are further supported by the research of **Kang *et al*., (2016)**, which confirmed a positive correlation between ferritin concentrations and the severity of kidney disease.

The complex patterns observed in unsaturated iron-binding capacity (UIBC) are consistent with the study **Upadhye, Patidar, and Yadav ,(2024)**, which emphasized the multifaceted disturbances in iron metabolism resulting from the interaction between chronic inflammation and inflammatory cytokines

The observed decrease in serum iron levels aligns with the concept of "anemia of inflammation," as described in the study of **Mokgalaboni and Phoswa ,(2022)** which highlighted the role of chronic low-grade inflammation in linking type 2 diabetes with functional iron deficiency. This pattern is further supported by

findings from **Mehdi and Toto, (2009)**,which demonstrated reduced serum iron levels in DKD patients as part of the broader inflammatory response.

Our results differ significantly from the study by **Nussrat and Ad'hiah (2023b)** , which demonstrated a marked increase in IL-40 levels in diabetic patients compared to controls, with significantly higher levels recorded in patients. This stark discrepancy may reflect differences in the disease stages examined, as the aforementioned study may have included early stages of the disease, whereas our group represents more advanced stages with depletion of protective markers.

The current findings highlight an advanced pattern of iron metabolism dysregulation that has not been sufficiently documented in previous scientific literature. The simultaneous reduction in IL-40 and IL-41 levels, along with elevated serum ferritin and decreased serum iron, suggests a phenomenon that may be described as a mineral-inflammatory storm, in which the body loses its ability to regulate iron metabolism effectively due to chronic inflammation.

Our findings differ from expected patterns in the scientific literature, as the diabetes group showed a decrease in UIBC, while it increased in the diabetic kidney disease group an unusual pattern that deviates from the typical expectations of iron disorders in different stages of the disease. The severity of iron disorders in our study differs from the standard recommendations of **Bajaj *et al*., (2016)** , which suggest milder patterns of iron abnormalities at similar disease stages. In contrast, the changes in this study were more pronounced.

This current finding differ from some studies such as by Liu et al., (2023) which showed less clear patterns of serum iron reduction, whereas the reduction in our study was more severe possibly reflecting more advanced stages of inflammation or differences in geographic and ethnic contexts.

These differences can be attributed to several factors, including variation in disease stages, as our cohort may represent more advanced stages compared to those typically included in international studies. Genetic and ethnic factors may also influence immune responses and iron metabolism in distinct ways. Additionally, local environmental and dietary conditions may affect the levels of inflammatory markers and the availability of dietary iron. Differences in therapeutic interventions may also impact inflammatory and iron metabolic pathways differently. Lastly, variations in the severity of systemic inflammation, possibly due to environmental factors or chronic infections, may influence the patterns of iron metabolism responses.

The complex patterns observed in unsaturated iron-binding capacity (UIBC) may reflect divergent adaptive responses between the two patient groups. In individuals with diabetes without renal complications, the decreased UIBC may represent a protective mechanism aimed at reducing iron transport capacity as a response to oxidative stress. Conversely, the elevated UIBC in patients with diabetic kidney disease may be interpreted as a delayed compensatory response to the severe deficiency in bioavailable iron.

4.4. Correlation analysis of novel cytokines IL-40 and IL-41 with metabolic and renal parameters in study groups.

The correlation analysis in the DKD group revealed no statistically significant associations between IL-40 or IL-41 and conventional clinical parameters such as serum creatinine, blood urea, ferritin, or HbA1c. This suggests an absence of clear links between these interleukins and traditional markers of kidney function or glycemic control in this sample. However, a moderate and statistically significant correlation was observed between IL-40 and IL-41.

These findings are in agreement with previous studies, such as **Zheng *et al*.,(2021)**, which reported overlapping inflammatory pathways among various cytokines, supporting the observed relationship between IL-40 and IL-41. Similarly, **Niewczas *et al*., (2019)** reported that the correlations between certain cytokines and kidney function markers become more apparent in the very advanced or end-stage of kidney disease, which is consistent with our findings in ESRD patients.

Broader studies on inflammation in CKD have also emphasized that cytokine associations may vary depending on disease stage, aligning with the current absence of correlations. Conversely, other studies have reported differing results; For instance, **DeFronzo *et al*.,(2015)** demonstrated significant links between inflammatory cytokines and long-term glycemic control (HbA1c), while other research highlighted direct relationships between cytokines and deteriorating kidney function, including elevated creatinine, urea, and ferritin levels. These discrepancies could be due to differences in disease stage, as this study focused on advanced phases, where inflammatory responses may be saturated and less reflective in clinical parameters. Genetic, ethnic, dietary, and environmental differences, as well as variability in laboratory measurement techniques especially given that IL-40 and IL-41 are relatively novel markers may also contribute to the lack of consistency. Furthermore, treatment regimens could modulate the inflammatory and iron metabolic profiles, influencing biomarker behavior. From my perspective, the lack of correlation between IL-40 and IL-41 with standard clinical markers does not negate their potential role in DKD progression. Rather, it may reflect an involvement at earlier stages or through indirect mechanisms not easily captured by routine lab indicators. The observed association between IL-40 and IL-41 points to a possible shared immunoregulatory role.

4.5. Treatment independent regulation of novel cytokines IL-40 and IL-41 in diabetic kidney disease.

The subgroup analysis of IL-40 and IL-41 levels based on treatment type in DKD patients revealed no statistically significant differences among patients receiving insulin, oral, or mixed treatment. Despite an observable trend toward higher IL-40 levels in the mixed treatment group, this variation did not reach statistical significance. Similarly, IL-41 levels were nearly identical across the groups. These findings align with previous research suggesting that not all inflammatory cytokines are significantly influenced by treatment type, particularly newer cytokines like IL-40 and IL-41 whose regulatory pathways may differ from well characterized cytokines such as IL-6 and TNF-α. Some studies have highlighted that insulin and certain oral antidiabetic drugs possess anti-inflammatory effects that can modulate cytokine production **(Liu *et al*., 2021; Jung and Moon, 2021),** but these effects may not extend to all cytokines. In contrast, research has suggested that treatments like GLP-1 receptor agonists and SGLT2 inhibitors can reduce levels of inflammatory markers in diabetic patients**(Ren *et al*., 2025;Rykova *et al*., 2025)**. The discrepancy may stem from differences in study design, sample size, variability in treatment duration, and patient characteristics. Moreover, the inherent variability in cytokine levels and the relatively recent discovery of IL-40 and IL-41 may limit the current ability to detect treatment specific changes. From my perspective, the lack of significant variation does not rule out the potential involvement of IL-40 and IL-41 in the pathogenesis of DKD; rather, it underscores the need for more targeted studies exploring their longitudinal response to therapy, their early stage dynamics, and their role in shaping the inflammatory milieu in diabetic nephropathy. These cytokines may act through subtle or delayed mechanisms not captured in cross sectional comparisons.

4.6. Distinctive alterations in hepatic function parameters between diabetic kidney disease and uncomplicated diabetes.

The analysis of liver function parameters revealed significant differences between the DKD and DM groups. The DKD group showed elevated ALT and ALP levels, along with reduced total protein levels. AST levels, however, did not differ significantly between the groups. These alterations suggest hepatic involvement and systemic metabolic stress in DKD patients, possibly reflecting shared inflammatory and oxidative mechanisms between the liver and kidneys.

These findings are consistent with **Adiga and Malawadi (2016)** as noted elevated liver enzymes have been associated with kidney complications in diabetic patients, while increased ALP levels have been linked to biliary dysfunction and metabolic derangements in chronic conditions **(Rafaqat *et al*., 2023)**.The significant reduction in total protein is well documented in DKD, often resulting from glomerular damage and proteinuria, as reviewed by **(Cravedi and Remuzzi ,2013)**.

Conversely, some studies diverge from these results. For example, research by **Wen *et al.*,(2022)** suggested that ALT levels may not consistently differ between diabetic patients with and without nephropathy, attributing ALT changes more to hepatic factors than kidney involvement. **Chen et al., (2017)** reported no substantial changes in ALP in DKD stages, questioning its sensitivity as a marker. Regarding total protein, (**Moresco *et al*., 2013;Cravedi and Remuzzi 2013)** indicated that not all DKD cases show significant hypoproteinemia, particularly in the absence of overt proteinuria.

These discrepancies may be due to differences in disease activity, sample size, demographic composition, comorbid liver conditions, or medication use. Variations in laboratory methodologies and diagnostic criteria may also contribute,

along with whether or not DKD was clearly distinguished from general diabetes in prior studies.

From our perspective, the observed liver function changes in DKD patients may not necessarily indicate primary liver pathology, but rather reflect systemic inflammation, oxidative stress, and protein loss secondary to renal dysfunction. Monitoring liver function alongside renal parameters could enhance early detection and provide a more comprehensive view of disease progression in diabetic patients.

4.7. Comparative analysis of mineral, electrolyte, and iron metabolism profiles between diabetic kidney disease and uncomplicated diabetes.

The comparison of mineral and electrolyte profiles between the DKD and DM groups revealed several statistically significant differences. Sodium and calcium levels were reduced in the DKD group, while phosphate, UIBC, and ferritin levels were notably elevated. Interestingly, serum iron levels showed no significant difference between the two groups. These findings reflect the complex alterations in electrolyte balance and iron metabolism associated with diabetic kidney disease progression.

These results are supported by previous studies. **Liamis *et al*.,(2014)** reported that sodium imbalances are frequently observed in diabetic patients with renal impairment, often due to tubular dysfunction and hormonal dysregulation. Similarly, **Kavuparambil *et al*., (2021)** demonstrated that declining eGFR is associated with hypocalcemia and hyperphosphatemia, supporting the observed changes in calcium and phosphate. The rise in ferritin levels in the DKD group aligns with findings from **Liu *et al*., (2020)**, whose meta-analysis confirmed the role of iron overload and oxidative stress in DKD progression. Furthermore, **Kapoor**

and Sharma, (2015) highlighted that elevated UIBC may represent a compensatory response to chronic inflammation and disordered iron regulation in kidney disease.

On the other hand, some studies have yielded conflicting results. For instance, several earlier reports by **Shanahan *et al*.,(2011)** found that phosphate and calcium levels did not significantly differ in early-stage DKD, suggesting that these imbalances may only appear in later stages. Additionally, other researchers have argued that UIBC may not be a consistent marker in all types of renal dysfunction due to variations in inflammatory status and iron therapy **(Aljuraisy and Jasim, 2024)**. Regarding ferritin, while often elevated, some studies such as that by **Robles *et al*., (2018)** suggest it may fluctuate depending on infection, inflammation, and comorbidities, limiting its specificity.

From our perspective, the observed disturbances in mineral metabolism and iron regulation in DKD patients are indicative of systemic complications beyond glycemic control. These biomarkers not only reflect renal deterioration but may also play an active role in disease progression. Therefore, integrating routine monitoring of electrolytes and iron-related markers in clinical practice could enhance early detection of complications and guide timely interventions.

4.8. Familial predisposition in diabetic kidney disease.

The analysis presented reveals statistically significant differences in cytokine levels and metabolic indicators among Diabetic Kidney Disease (DKD) patients based on the presence of a family history of diabetes. Notably, IL-41 levels were significantly higher in patients with a positive family history, suggesting a potential genetic influence on inflammatory regulation. This finding aligns with the study by **Shi *et al*., (2024)**, which highlighted IL-41 (also known as Metrnl) as a novel cytokine involved in immune modulation and kidney disease development.

Additionally, UIBC levels were significantly elevated in patients with a family history, consistent with **Fayed *et al.*, (2013)**, who demonstrated altered iron metabolism indicators in diabetic nephropathy. In contrast, IL-40 levels did not show statistically significant differences between groups, despite previous studies such as **Nussrat and Ad'hiah, (2023b)** suggesting IL-40 may be a promising biomarker in diabetes, indicating its effect may not be directly linked to genetic predisposition in the context of DKD. Age also did not differ significantly between the groups, ruling it out as a confounding factor. However, these findings regarding UIBC diverge from other studies such as**(Ford *et al*., 2009; Deori and Bhuyan ,2016; Ueda and Takasawa, 2018)**, which emphasized that UIBC levels are more influenced by active inflammation, liver function, and nutritional status than by family history or genetic background. These discrepancies may be attributed to differences in activity of disease stage, study design, sample size, and the severity of systemic inflammation in the study population. From my perspective, the observed elevation in IL-41 and UIBC among patients with a family history reflects early immunological and metabolic alterations influenced by hereditary factors. These biomarkers may hold prognostic value for assessing disease risk and progression in genetically predisposed individuals.

4.9. Hemodialysis frequency and inflammatory cytokines (IL-40/IL-41) in diabetic kidney disease.

The subgroup analysis presented revealed no statistically significant differences in IL-40, IL-41, or other clinical parameters between DKD patients undergoing 1–2 versus 3–4 dialysis sessions per week. While IL-40 and IL-41 levels appeared numerically higher in the more frequent dialysis group, the differences did not reach significance, suggesting that dialysis frequency alone may

not markedly influence these cytokines in the short term. These findings are in line with **Nowak and Chonchol ,(2018)**, who reported that dialysis frequency may impact inflammatory markers but requires prolonged observation for detectable effects. Similarly, **Li *et al*., (2023)** described IL-41 (Metrnl) as a context sensitive cytokine influenced by metabolic and inflammatory states but not necessarily modulated by dialysis intensity. The non-significant changes in traditional renal markers such as serum creatinine, urea, and hemoglobin are also consistent with studies showing that increased dialysis frequency without extended treatment duration has limited impact on these parameters **(Nowak and Chonchol , 2018)**. In contrast, some studies, such as those by **Wang *et al*.,(2020)**, suggest that Metrnl levels decrease with worsening kidney function, implying that changes in cytokine levels may only become apparent with advancing disease or longer dialysis exposure. Additionally, **Masajtis-Zagajewska and and Nowicki ,(2018)** noted dialysis-induced shifts in iron metabolism, which might explain the observed (though non-significant) trend of increased ferritin levels in patients receiving more dialysis. Discrepancies may be attributed to variations in dialysis duration, modality, patient comorbidities, and inflammation burden. From our perspective, the absence of significant differences underscores the complexity of inflammatory and metabolic regulation in DKD and suggests that cytokines like IL-40 and IL-41 may not respond acutely to dialysis frequency alone.

4.10.1. predictive performance of IL-40 and IL-41 in differentiating diabetic kidney disease from healthy controls.

The results of the ROC curve analysis demonstrated that both Interleukin-40 (IL-40) and Interleukin-41 (IL-41) possess a high diagnostic capability in distinguishing patients with diabetic kidney disease (DKD) from healthy

individuals. However, IL-41 clearly outperformed IL-40, showing near-perfect performance in terms of area under the curve (AUC), sensitivity, specificity, and overall accuracy. Moreover, IL-41 exhibited a notable balance between positive and negative predictive values, making it more suitable for clinical use especially given its lower cutoff value, which facilitates its application in early screening. These findings align with those reported by **Niewczas *et al*., (2019)**, who demonstrated a strong association between inflammatory cytokines and DKD progression, thereby supporting the value of IL-41 as an inflammation-based diagnostic biomarker. The results are also consistent with **Mandrekar,(2010)** classification, which considers AUC values above 0.9 to indicate excellent diagnostic performance. Furthermore, the findings support the clinical importance of predictive values, as emphasized by **Bossuyt *et al*., (2015)**, which was clearly reflected in IL-41 balanced diagnostic performance.

On the other hand, some earlier studies did not identify IL-41 as an effective diagnostic marker for DKD, possibly due to limited exploration of this cytokine in renal disease contexts or due to methodological differences such as sample size and disease stage. For instance, studies like **Navarro-González *et al*., (2011)**, focused on conventional inflammatory markers such as TNF-α and IL-6 and did not highlight a role for non-traditional cytokines like IL-41 likely due to the limited sensitivity of detection tools at that time or differences in study populations (e.g., comorbidities, age, ethnicity). As for IL-40, there are currently no published studies directly addressing its role in DKD, most likely because it is a relatively recently identified cytokine and has yet to be integrated into predictive clinical models or large-scale epidemiological studies. However, the lack of literature should not be interpreted as an indicator of irrelevance, but rather as a call for more focused research to elucidate its mechanisms and role in chronic renal inflammation.

Notably, the current study ROC findings further validate the diagnostic efficacy of these cytokines, especially IL-41. The observed decrease in IL-40 and IL-41 levels may not reflect an absence of inflammation but rather suggest an exhausted immune response or excessive local consumption of cytokines in chronically inflamed renal tissue. The pathophysiology of DKD, marked by sustained hyperglycemia-induced microvascular damage, chronic inflammation, oxidative stress, and fibrosis, may alter cytokine dynamics either by reducing their systemic availability or impairing their synthesis by immune cells. As such, fluctuations in IL-40 and IL-41 could represent a deeper immunological dysregulation characteristic of DKD progression.

In conclusion, IL-41 emerges as a highly promising and effective diagnostic biomarker for the early prediction of DKD, offering superior accuracy and clinical applicability. IL-40, while demonstrating relatively good performance, still requires further investigation through large-scale, multi-stage studies to fully elucidate its diagnostic value and inflammatory role in diabetic kidney disease.

4.10.2. predictive performance of IL-40 and IL-41 in differentiating diabetic patients from healthy controls.

The ROC curve analysis demonstrated that both IL-40 and IL-41 have excellent diagnostic capabilities in distinguishing patients with diabetes mellitus from healthy individuals, with IL-41 showing a slight advantage in several diagnostic metrics such as sensitivity, accuracy, and negative predictive value. However, a notable finding in this study is that both cytokines were significantly decreased in diabetic patients compared to healthy controls, which is an unconventional pattern, as most inflammatory cytokines typically increase in chronic metabolic diseases like diabetes.

This decrease may reflect either excessive local consumption of these cytokines at the sites of inflammation or an immune exhaustion state where prolonged chronic inflammation suppresses their systemic production. Despite the reduced serum levels, both IL-40 and IL-41 maintained high discriminative power, indicating that lower concentrations themselves may serve as distinctive markers of disease presence and progression. This underlines the importance of recognizing not only elevated but also suppressed biomarkers in chronic disease contexts.

These findings partially align with the study by **Nussrat and Ad'hiah, (2023a)**, which identified IL-40 as a promising diagnostic marker in diabetes, though their results showed elevated levels of IL-40. This discrepancy could be attributed to differences in study design, patient demographics, disease stages, or assay techniques. Regarding IL-41, previous studies, such as **Zhang *et al*., (2023)**, highlighted its potential protective role through enhancing insulin sensitivity and regulating lipid metabolism via PPAR-δ pathways. The decreased levels observed in our diabetic cohort may thus represent a loss of this anti-inflammatory, metabolic regulatory function, particularly in advanced disease stages.

From a biological perspective, IL-40 is considered a pro-inflammatory cytokine, whereas IL-41 has been associated with anti-inflammatory responses. The simultaneous decrease of both may indicate a breakdown in immune homeostasis and cytokine balance, contributing to the chronic low-grade inflammation characteristic of diabetes mellitus. Therefore, monitoring both cytokines may offer valuable insight into the underlying immune dysregulation in diabetic patients.

In conclusion, the observed decrease in IL-40 and IL-41 despite their high predictive accuracy suggests a unique immunological signature in diabetes. Their combined assessment could enhance early prediction of complications and monitoring of disease progression.

4.10.3. predictive performance of IL-40 and IL-41 in differentiating diabetic kidney disease from diabetic patients.

The findings demonstrated the diagnostic potential of IL-40 and IL-41 in distinguishing patients with diabetic kidney disease (DKD) from those with diabetes mellitus (DM) without renal complications. Although both biomarkers showed a certain level of discriminative ability, IL-41 clearly exhibited superior predictable reliability, as reflected by its higher area under the curve (AUC), statistically significant p-value, and more favorable confidence interval range. These parameters suggest that IL-41 has greater potential as a confirmatory biomarker for identifying kidney involvement in patients already diagnosed with diabetes.

IL-40 showed higher sensitivity than IL-41, indicating a better ability to detect true cases of DKD. However, this was offset by its lower specificity, which limited its utility in accurately excluding patients without DKD. Conversely, IL-41 demonstrated notably higher specificity, making it more suitable for confirming disease presence when a positive result was obtained. This diagnostic pattern aligned with the findings of **Donate *et al*., (2021)**, who emphasized the clinical value of inflammatory cytokines as complementary tools in the diagnosis and management of DKD.

The overall diagnostic accuracy and predictive values of both biomarkers remained modest. IL-40 exhibited slightly better accuracy and a higher positive predictive value, whereas IL-41 achieved superior specificity and a comparable negative predictive value. These differences might have been influenced by the prevalence of DKD in the study cohort and other demographic variables, underscoring the need for contextual interpretation of performance indicators.

The results were consistent with previous literature emphasizing the role of inflammation and immune dysregulation in the pathogenesis of DKD. Studies by (**Pichler *et al*., 2017 ;Campion, Sanchez-Ferras, and Batchu, 2017)** described how pro-inflammatory cytokines such as IL-6, IL-1, IL-18, and TNF-α contributed to both glomerular and tubular injury in diabetic patients. **Donate *et al*., (2021)** reported increased urinary levels of IL-6, IL-8, and IL-18 in DKD patients, which were associated with early renal function decline. **Khanijou *et al*., (2022)** also highlighted the importance of early detection of inflammatory and renal biomarkers for timely diagnosis and therapeutic intervention.

In contrast to many pro-inflammatory cytokines, both IL-40 and IL-41 were found to be decreased in DKD patients compared to those with diabetes without kidney disease. This decline might have reflected chronic immune exhaustion or excessive local consumption of these cytokines in renal tissues. The reduced levels of IL-41 despite its high specificity suggested that its suppression could serve as an inverse marker of disease progression.

From a clinical perspective, IL-41 appeared more appropriate as a predictive diagnostic tool, while IL-40 might have served a useful role in preliminary screening due to its greater sensitivity.

our conclusion, although IL-40 and IL-41 did not demonstrate strong standalone predictive power in differentiating DKD from DM, the findings suggested potential utility when these biomarkers were used in combination. Their integration with conventional renal markers and other inflammatory mediators might improve early detection, risk stratification, and clinical outcomes in DKD.

Conclusions and Recommendations

Conclusions

1. The present study has demonstrated a significant reduction in serum levels of Interleukin-40 and Interleukin-41 in patients with diabetic kidney disease (DKD) and diabetes mellitus (DM) compared to the control group. This suggested a potential dysregulation in immune regulation associated with diabetes and its renal complications, offering insights into the underlying immunopathological mechanisms of DKD.

2. Results revealed that Interleukin-41 possesses excellent predictive capability for distinguishing between DKD patients and healthy individuals (area under the curve = 98.4%), outperforming Interleukin-40 (area under the curve = 89.6%). Similarly, Interleukin-41 demonstrated exceptional predictive performance in differentiating between DM patients and healthy individuals (area under the curve = 99.6%). These findings confirmed the promising potential of these immunological biomarkers in predictive diabetes and its renal complications.

3.A statistically significant positive correlation was found between Interleukin-40 and Interleukin-41 levels in DKD patients ($r = 0.335$, $p = 0.030$), suggesting potential shared inflammatory pathways or co-regulation in the pathological context of DKD. This finding represents a specific pathway warranting further investigation.

4.No statistically significant correlations were observed between Interleukin-40 or Interleukin-41 levels and other clinical parameters, including glycated hemoglobin (HbA1c), serum creatinine, blood urea, and serum ferritin. These findings imply that IL-40 and IL-41 may be involved in alternative immunological pathways not directly correlated with conventional metabolic or renal function markers.

5.DKD patients with a positive family history of diabetes exhibited significantly higher levels of Interleukin-41 compared to those without family history, suggesting potential genetic factors influencing this cytokines levels and associated inflammation in DKD.

6.Interleukin-40 and Interleukin-41 levels were not significantly affected by treatment modality (insulin, oral therapy, or mixed therapy) or by the number of weekly dialysis sessions, indicating that these cytokines may serve as state-dependent markers rather than being modified by therapeutic interventions.

7.The findings of this study confirmed the potential role of Interleukin-40 and Interleukin-41 as promising predictive biomarkers in DKD and provide novel insights into the immunological mechanisms involved in the development of this disease.

Recommendations.

Based on the findings of this study, several recommendations can be proposed to guide future research and improve clinical practice:

1. Longitudinal Studies: Monitor the levels of interleukin-40 and interleukin-41 in diabetic patients over an extended period of time, paying particular attention to changes in these levels at various phases of the progression of DKD. Whether these cytokines can function as predictive markers for the onset and severity of disease may be ascertained with the aid of such investigations.
2. Animal Models and Molecular Mechanisms: Investigate the molecular mechanisms controlling the production of interleukin-40 and interleukin-41 in relation to diabetes and its renal complications using animal models. Understanding the underlying mechanisms and locating possible therapeutic targets may be made easier with the help of these studies.
3. Gene Function Studies: Examine the effects of genetic and environmental factors on the levels of these cytokines in patients with DKD in order to determine the regulatory factors affecting the expression of interleukin-40 and interleukin-41.

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Appendices

Appendices 1.

Role of immunological biomarkers Interleukin-40 and Interleukin-41 in serum of Diabetic Kidney Disease			
Sample No.: Case – Control	Type of study:	Gender:	Age:
Disease Diagnosis:			
Inclusion Criteria: Diabetes mellitus with 5 stage of nephropathy with out of Current Inflammation & infection			
Family history to disease	Yes		
	No		
Type of Rx: Oral…… insulin……. Mixed…… **Kidney disease stage:** **stage5…….**			

Biomarkers		
Biomarker	**Levels**	**Unit**
- Interleukin 40		
- Interleukin 41		
- HbA1c		
- R. B. S.		
Kidney Function Test		
- B.Urea		
- S.Creatinine		
-S.protein		
- S.Albumin		
Liver Function Test		
-AST		

-ALT		
-ALP		
-T.S.B		
Electrolytes profile		
-Na^{+2}		
-Ca^{+2}		
-Po4		
Other test		
-UIBC		
-S.FERRITIN		
-IRON		
CBC		
-HB		

Appendices 2.

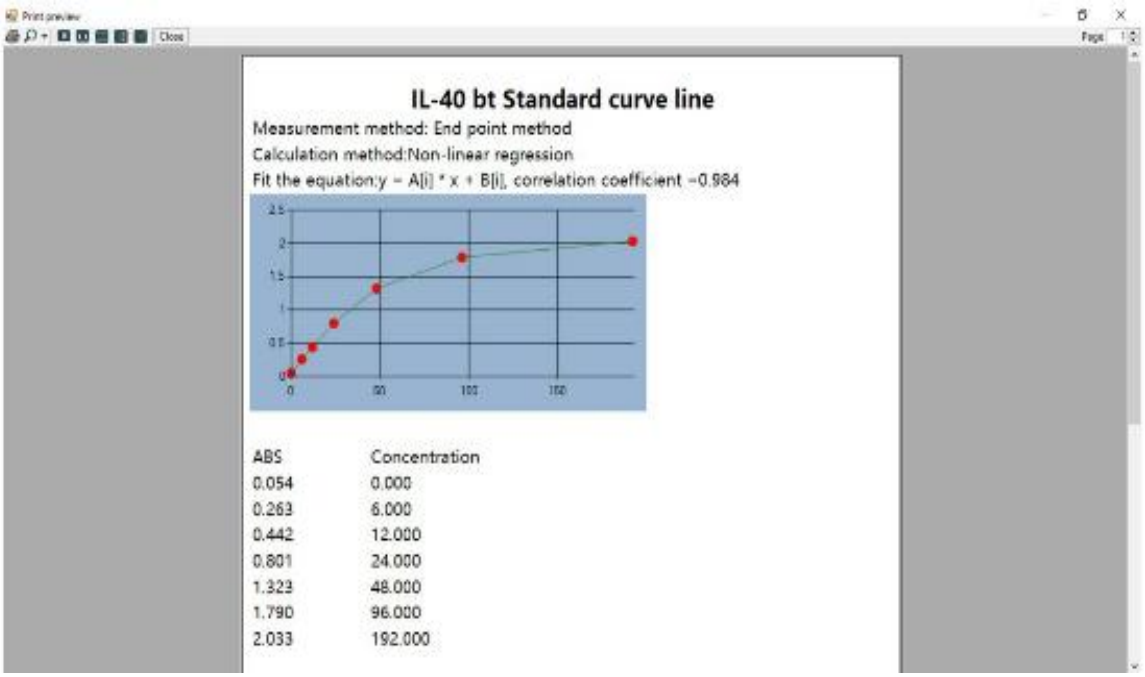

Figure 1: The standard curve of IL40

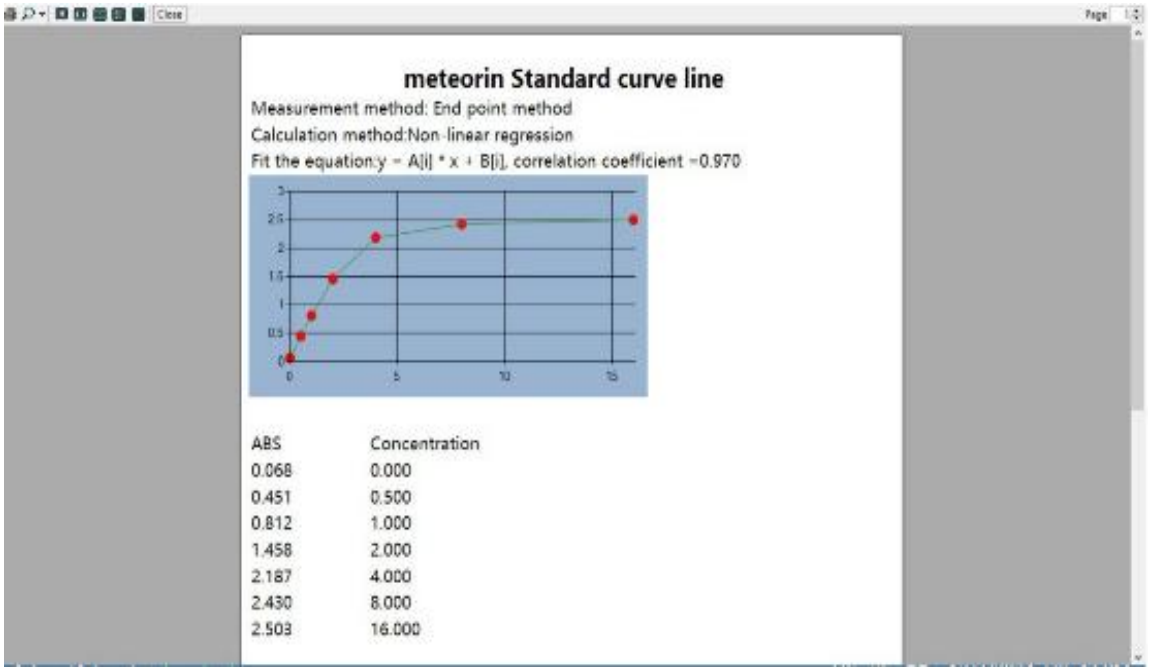

***Figure 2: The standard curve of* IL41**

Appendices.3

ELISA system with plate, kits and Laboratory Equipment

Appendices.4

ELISA system reader/washer

Appendices.5

Mindray BS-230 system

Appendices.6

Complete blood count system

الخلاصة.

يمثل مرض الكلى السكري (DKD) إحدى المضاعفات المزمنة والخطيرة لداء السكري، ويُعد أحد الأسباب الرئيسية للفشل الكلوي في مراحله النهائية على مستوى العالم. ، يؤدي مرض الكلى السكري كذالك إلى تغييرات في الديناميكا المتجانسة لمكونات الخلايا المناعية والالتهاب الفيزيولوجي المرضي، والتي تساهم في إنتاج أو تثبيط أو تقليل تنظيم الوسطاء (mediators)، مع اهتمام خاص بالوسطاء المكتشفين حديثًا مثل الإنترلوكين-40 والإنترلوكين-41، واستكشاف إمكانيتهم في الاستخدام كمؤشرات حيوية جديدة في التنبؤ ومراقبة تطور مرض الكلى السكري. يُعتبر كل من IL-40 وIL-41 مهمين لأنه تم تحديدهما مؤخرًا كالسايتوكينات قد تلعب دورًا في الحالات المناعية الذاتية والالتهابية، وتُعد هذه أول دراسة لتقييم دور IL-40 وIL-41 في مرض الكلى السكري في منطقة الشرق الأوسط.

هدفت الدراسة إلى تقييم مستويات IL-40 وIL-41 لدى مرضى السكري بشكل عام، ولدى المصابين بمرض الكلى السكري بشكل خاص، مقارنةً بالأشخاص الأصحاء، وتحديد القيمة التنبؤية لهذه المؤشرات الحيوية، كما يهدف إلى دراسة العلاقات بينها وبين المؤشرات المختبرية التقليدية (HbA1c، الكرياتينين، اليوريا، الفيريتين).

اتبعت الدراسة تصميم الحالة والشاهد (Case-Control) وتضمنت فيها 128 حالة: 42 مريضًا بمرض الكلى السكري، 43 مريضًا بالسكري دون مرض كلوي، و43 شخصًا سليمًا كضوابط. تم جمع مرضى DKD وDM من مستشفى الإمام الحسين في كربلاء، بينما تم جمع العينات من الأصحاء في مستشفى المجر الكبير في ميسان.للفتره بين تشرين الثاني 2014 لغاية كانون الثاني 2025

تم تحليل عينات الدم باستخدام تقنية ELISA(كواشف من شركة HUMAN الألمانية) لقياس مستويات IL-40 وIL-41، إلى جانب مؤشرات مختبرية أخرى. وقد تم استخدام اختبارات إحصائية مثل ANOVA، واختبار كروسكال-والِس، وتحليل الارتباط، ومنحنيات ROC.

أظهرت النتائج الرئيسية أن مستويات IL-40 انخفضت بنسبة تُقدّر بحوالي 47.2% (15.8±4.0 بيكوغرام/مل) لدى مرضى DKD، و52.8% (14.1±3.7 بيكوغرام/مل) لدى مرضى السكري (DM) مقارنةً بالأصحاء (29.9±8.7 بيكوغرام/مل)، في حين انخفضت مستويات IL-41 بنسبة 82.4% (0.53±0.18 بيكوغرام/مل) لدى مرضى الكلى السكري (DKD) و78.7% (0.64±0.11 بيكوغرام/مل) لدى مرضى السكري (DM) مقارنةً بالأصحاء (3.01±0.98 بيكوغرام/مل)، وكانت جميع الفروقات ذات

دلالة إحصائية عالية ($p<0.001$). أظهر IL-41 أداءً تنبؤي ممتازًا حيث بلغت المساحة تحت منحنى ROC نسبة 98.4%، ودقة التشخيص 95.3% في التمييز بين مرضى DKD والأصحاء، في حين أظهر IL-40 فائدة تنبؤية جيدة بمساحة تحت المنحنى بلغت 89.6% ودقة 90.6%. أما التغيرات الكيميائية الحيوية الأخرى لدى مرضى الكلى السكري (DKD) فقد شملت ارتفاع اليوريا والكرياتينين، انخفاض الهيموغلوبين، واضطراب مستويات الكالسيوم والفوسفات. لم يُلاحظ وجود ارتباطات ذات دلالة إحصائية بين مستويات IL-40 وIL-41 والمؤشرات السريرية التقليدية، باستثناء وجود ارتباط إيجابي بين IL-40 وIL-41 لدى مجموعة مرضى الكلى السكري DKD ($r=0.335$، $p=0.030$).

أكدت هذه الدراسة على وجود انخفاض ملحوظ في مستويات IL-40 و IL-41 لدى مرضى السكري ومرضى اعتلال الكلية السكري المشخصين مسبقًا مقارنة بالأشخاص الأصحاء، مما يشير إلى دور محتمل لهذه المؤشرات كعلامات بيولوجية تكميلية او تنبؤيه. ولا سيما أن IL-41 أظهر حساسية ونوعية عالية جدًا في التمييز بين مرضى اعتلال الكلية السكري المعروفين. ومع ذلك، تشمل القيود التي واجهتها الدراسة صغر حجم العينة، وعدم تقييم عوامل التهابية أخرى، وغياب المتابعة طويلة الأمد. في الختام، قدمت هذه الدراسة أدلة أولية تدعم الإمكانات التنبؤية ودور لكل من IL-40 وخاصة IL-41 في مرض اعتلال الكلية السكري لدى المرضى المشخصين مسبقًا. ونظرًا لعدم وجود ارتباط مع المؤشرات التقليدية، يُوصى بإجراء دراسات واسعة النطاق وطولية للتحقق من هذه النتائج واستكشاف قابليتها للتطبيق السريري، خاصة في المراحل المبكرة من المرض، لتأكيد قدرتها على التشخيص المبكر أو التنبؤ بالمرض.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية الطب

قسم الأحياء المجهرية الطبية

دور المؤشرات المناعية، الإنترلوكين-40 والإنترلوكين-41 في مصل مرضى السكّري المصابين بالمرحلة النهائية من مرض الكلى

رسالة

مقدمة إلى مجلس كلية الطب / جامعة كربلاء، كجزء من متطلبات نيل درجة الماجستير في الأحياء المجهرية الطبية.

إعداد

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بكالوريوس علوم حياة - كلية العلوم / جامعة ميسان (2014)

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أستاذ دكتور

سوسن محمد جبار

هـ 1446

م 2025