

# Comparative Study of Novel Biomarkers of Kidney Injury; Kidney Injury Molecule and Liver Type Fatty Acid Binding Protein in Experimentally Induced Diabetic Nephropathy in Rats, and their Correlation with Renal Histopathological Changes

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## Abstract

**Background:** Diabetic nephropathy is a serious microvascular complication of type 2 diabetes mellitus that can progress slowly to end-stage renal disease that can be fatal. It is characterized by highly specific, slowly progressing histopathological changes. As the standard biomarkers lack the adequate sensitivity, novel biomarkers are needed for the early detection and risk stratification of diabetic nephropathy.

**Aim of Study:** To investigate the histopathological changes occurring in diabetic nephropathy, to study the associated changes of two novel biomarkers; Liver Fatty Acid Binding Protein (L-FABP) and Kidney Injury Molecule (KIM), to correlate their gene expression with the degree of histopathological changes and to compare their sensitivity and degree of correlation against the standard biomarkers, notably blood urea and serum creatinine.

**Material and Methods:** 24 experimental male rats were divided into two groups, a control group, and a diabetic nephropathy group. Type 2 diabetes mellitus was induced by High Fat Diet (HFD) followed by Streptozotocin (STZ) intraperitoneal injection. The presence of diabetes was confirmed by fasting plasma glucose & insulin levels, and the induction of diabetic nephropathy was confirmed by the presence of albuminuria.

Histological examination was performed for all experimental rats using hematoxylin and eosin stain (H & E) staining as well as Periodic Acid Schiff (PAS) stain. These results were expressed as the mean optical density of the PAS-positive reaction.

The gene expression for L-FABP and KIM were measured by Polymerase Chain Reaction (PCR) reverse transcriptase method and the levels of blood urea and serum creatinine, as standard biomarkers were measured. Statistical correlation analysis was performed by Pearson's correlation method to investigate the correlation of their gene expression and the histopathological changes.

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**Results:** Significant histopathological changes occurred in diabetic nephropathy manifested by several changes in the H & E staining as well as PAS-positive reactions. All biomarkers are elevated in the diabetic nephropathy group compared to the control group. However, no statistically significant correlation was found between the standard renal biomarkers (blood urea and serum creatinine) and gene expression of KIM. Only statistically significant correlation was found between the gene expression of L-FABP and the degree of histopathological changes.

**Conclusion:** The standard biomarkers (blood urea & serum creatinine) are lacking the adequate sensitivity for correlation with the histopathological changes in diabetic nephropathy. L-FABP is a promising future biomarker that can potentially be used for early detection as well as the follow-up of cases of diabetic nephropathy.

## Abbreviations:

AKI	: Acute kidney injury.
cDNA	: Complementary deoxyribonucleic acid.
CKD	: Chronic kidney diseases.
DN	: Diabetic nephropathy.
FABPs	: Fatty acid-binding proteins.
FFA	: Free fatty acids.
H&E stain	: Hematoxylin and eosin stain.
HFD	: High fat diet.
HOMA-IR	: Homeostatic model assessment of insulin resistance.
kDa	: KiloDalton.
KIM	: Kidney injury molecule.
LCFAs	: Long chain fatty acids.
L-FABP	: Liver fatty acid binding protein.
PAS stain	: Periodic Acid Schiff stain.
PCR	: Polymerase chain reaction.
PPAR	: Peroxisome proliferator-activated receptors.
RNA	: Ribonucleic acid.
SD	: Standard deviation.
STD	: Standard dietary chow.
STZ	: Streptozotocin.
TZDs	: Thiazolidinediones.

**Key Words:** *Diabetic nephropathy – Liver type fatty acid binding protein – Kidney injury molecule.*

### Introduction

**DESPITE** the marked advances in the therapeutic maneuvers for chronic kidney diseases such as medications, dialysis and renal replacement therapy. The incidence and prevalence of Chronic Kidney Disease (CKD) are progressively increasing and reaching epidemic proportions. With a high risk of acute exacerbations and fatal complications leading to high morbidity and mortality rates especially in critically ill patients, early intervention can significantly delay the pathogenesis and markedly improve the prognosis [1].

Such a progressive increase in the morbidity and mortality rates from CKD may be caused by the lack of early, accurate, predictive and non invasive biomarkers for the early detection and accurate diagnosis of CKD. Serum creatinine is widely used as a biomarker for renal injury and the prediction of end-stage renal disease. However, the question of whether serum creatinine is a sensitive and valid tool for such function is currently under debate [2]. This poor sensitivity may be related to the changes in creatinine production or excretion resulting from variations in skeletal muscle mass or activities, or other factors directly linked to the creatinine metabolism. However, it is currently believed that these variations are multifactorial in etiology, questioning the use of serum creatinine as a reliable, valid screening test for the detection or follow-up of CKD. The net results after the meta-analysis of recent researches reported that the serum creatinine generally has a low sensitivity as a marker of renal failure. This effect is more marked in the elderly population, or those with abnormal skeletal muscle metabolism [2].

Diabetic Nephropathy (DN) is one of the most serious microvascular complications of type 2 diabetes mellitus, and it is one of the leading causes of end-stage renal failure and death in diabetic patients. It is one of the most frequent causes of CKD worldwide, this is due to the fact that once proteinuria occurs; the rate of decline of renal function in diabetic nephropathy is considerably higher than the rate of decline in other renal diseases leading to accelerated renal damage associated with histological and structural changes as well as a rapid effect in the renal functional parameters. Given this fact, together with the poor sensitivity of the standard renal functional biomarkers, notably serum urea and creatinine. So, novel markers are needed for the early diagnosis and risk stratification of diabetic nephropathy [3].

Some recent studies found that renal impairment in diabetic nephropathy is associated with changes in the gene expression of several renal proteins that can be used as accurate functional biomarkers for diabetic nephropathy. Therefore, it will be valuable in future studies to validate the specificity and sensitivity newly discovered renal biomarkers in clinical samples from large cohorts and multiple experimental as well as clinical situations. Such studies should be facilitated by the anticipated widespread availability of well-standardized innovative tools in the near future [6].

FABPs are a group of small cytosolic proteins which are members of the superfamily of the intracellular lipid-binding proteins. They are primarily involved in the binding of Long Chain Fatty Acids (LCFAs), the regulation of intracellular lipid trafficking and the regulation of gene expression of different signaling molecules involved in the lipid metabolism [5]. All isoforms of the FABPs share some common structural and biochemical characteristic features as; all of them are composed of peptide structure of 120 to 130 amino acid residues in length with a molecular weight ranging from 14-15kDa and the primary structural element is a beta chain structure linked to the ligand-binding domain which is a water-filled cavity which can accommodate one or two hydrophobic ligands such as fatty acids [6].

FABP1, also known as liver FABP is a member of the intracellular FABP family. It is highly expressed in the liver, kidney, lung, and intestine. It binds free fatty acids, and their coenzyme A derivatives, and may be involved in intracellular lipid transport. Its high expression in the renal tissue makes it a candidate for future use as a novel biomarker in Acute Kidney Injury (AKI) [7] as well as chronic renal conditions [8].

Kidney Injury Molecule (KIM) is another candidate molecule for such purpose which was recently discovered to be a potential biomarker for kidney injuries, as its gene expression and plasma level increases early in renal injuries, even before the appearance of symptoms or deterioration of renal functions. Kidney injury molecules are a family of transmembrane proteins, the most crucial member of this family is the Kidney Injury Molecule type-1 (KIM-1), which is a membrane protein. It was first described by Ichimura et al., in 1998 [10], It is expressed in various tissues of the body, notably the kidney and liver. In terms of the molecular structure, it is a 104kDa peptide, comprising of a 14kDa trans-membrane bound fragment and another 90kDa soluble portion. The extracellular

fragment constitutes a six-cysteine immunoglobulin-like domain bound to a Thr/Ser-Pro-rich domain, which is a typical structure of a superfamily of proteins known as mucin-like O-glycosylated proteins [11]. While the cytoplasmic domain is shorter than the transmembrane domain, with two different splice sites variants, named KIM-1a and KIM-1b respectively, the KIM-1b molecule is the variant expressed mainly in the renal tissues; it contains two tyrosine residues and a tyrosine kinase phosphorylation site. It is cleaved by the metalloproteinases family of enzymes and its constitutive shedding is mediated by extracellular signal-regulated kinase activation and accelerated by p38 mitogen-activated protein kinase activation [12]. The KIM-1a variant is mainly expressed in the liver and it is slightly different in structure from the KIM-1b molecule as it lacks the tyrosine kinase phosphorylation site. It is involved in some pathological conditions such as infections with the hepatitis virus, which is associated with a marked increase in its gene expression. The KIM-1 genes have high homology the hepatitis A virus cell receptor 1 (HAVcr-1), which is expressed by hepatocytes and could promote cellular entry of the virus in certain conditions [13].

KIM-1 is also expressed in some populations of T lymphocytes, and because of its low expression by some subpopulations of T-cells. TIM-1 molecule can function as a co-stimulatory molecule of T-cells; so, it can enhance T-cell proliferation and cytokine production in certain infective and immune conditions [14]. Also, the role of gene expression of KIM as a reliable accurate biomarker in acute renal conditions is almost established; however, such a role in chronic conditions still needs further investigations [15].

#### *Aim of the study:*

The early detection of the occurrence of the renal histopathological changes in DN may become possible due to some of promising early markers which may assist in early detection, differentiation and prognosis of DN. In this study, we have investigated the role of Kidney Injury Molecule (KIM) and Liver-type Fatty Acid-Binding Protein (L-FABP) in predicting the occurrence and the severity of DN in type 2 DM and to correlate between the changes in their gene expression and the histopathological changes occurring in diabetic nephropathy.

### **Material and Methods**

#### *Experimental animals and study material:*

24 adult male albino rats, weighing 180-200 grams were purchased from the Animal House Unit

of Kasr Al-Ainy Faculty of Medicine, Cairo University. All animals were housed in wire mesh cages and were acclimatized to the experimental conditions for one week before the first day of the experiment in a conditioned atmosphere at 25°C, humidity 55%-56%, in a regular 12 hours light-dark cycle with food and water ad libitum.

All experimental procedures were approved by the Ethical Committee of Kasr Al-Ainy Faculty of Medicine, Cairo University.

This study was conducted in the laboratories of the Physiology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University. It lasted 3 months from May 2018 till August 2018. All chemical agents and medications were purchased from the Egyptian international pharmaceuticals and chemicals company, 10<sup>th</sup> of Ramadan City, industrial zone B (E.I.P.I.C.O).

#### *Study design:*

After one week of acclimatization, rats were randomly divided into two groups; twelve rats each, which were assigned as follow:

- *Group 1:* Control group.
- *Group 2:* Streptozocin (STZ) induced DM (Diabetic Nephropathy Group).

In the Control Group (1), all rats were fed a standard dietary chow (STD: 6.5% kcal fat), and on the 14<sup>th</sup> day, 0.5ml of citrate buffer was injected intraperitoneally, followed by another similar dose after 24 hours, then they received only STD for eight weeks.

In the Diabetic Nephropathy Group (2), beginning on day 0, animals were fed a High-Fat Diet (HFD: 60% kcal fat) for two weeks. On the 14<sup>th</sup> day, rats were injected intraperitoneally with the first low dose of STZ (40mg/kg in 0.01M citrate buffer, pH 4.3), then followed by a similar second dose after 24 hours to induce type 2 DM [16]. Then, rats of group 2 were left diabetic, receiving only STD just as the control group for eight weeks, this duration was supposed to be enough to develop DN.

#### *Confirmation of type 2 DM and diabetic nephropathy in Group 2:*

After one month, type 2 DM was confirmed by measuring fasting plasma glucose and insulin levels, blood samples from the retro-orbital plexus under diethyl ether anesthesia were used to measure plasma glucose and insulin concentrations. Rats that had high blood glucose level were considered as diabetic and were used for further study.

The onset of DN was confirmed by the presence of microalbuminuria [17].

#### Parameters and study procedures:

All rats were subjected to overnight fasting at the end of the duration of the study and blood samples were collected from the retro-orbital plexus as well as urine samples for the Biochemical assessment:

Serum urea was measured using Quanti-Chrom™ Urea Assay kit (DIUR-500) [18].

Serum creatinine was estimated by Quanti-Chrom™ creatinine Assay Kit [19].

#### Measurement of albumin in blood and urine:

AMS albumin kit was used, it utilizes the colorimetric method for the quantitative determination of albumin in serum and urine using AMS SAT 450 Chemistry System.

#### Fasting serum glucose:

The serum glucose was assessed by Trinder [20] method.

#### Fasting serum insulin:

Insulin concentrations were measured in the serum samples by Enzyme-Linked Immunosorbent Assay (ELISA) using the rat insulin ELISA kits (Linco research).

*HOMA-IR index was calculated as follow to assess the peripheral resistance to insulin:*

$$\text{HOMA-IR} = [\text{Glucose (mmol/dL)} \times \text{insulin (gIU/mL)}] / 22.5.$$

HOMA-IR number >2.0 was taken as an evidence of insulin resistance [21].

#### Histological examination:

After sacrifice, specimens from kidney were immediately dissected out, fixed in 10% buffered formalin, then embedded in paraffin. The procedures were performed in the Histology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University. Sections of 5  $\mu\text{m}$  thickness were subjected to the following:

- 1- Hematoxylin & eosin (H & E) stain [22].
- 2- Periodic Acid-Schiff (PAS) reaction to demonstrate basement membrane and brush border as a measure of glomerular and tubular injuries [22].

#### Morphometric study:

Using Leica Qwin 500 LTD computer-assisted image analyzer, the area percent represented the

areas of the positive reaction or staining, which were masked by a binary color to the area enclosed within a standard measuring frame (it was 7286.783  $\mu\text{m}^2$ ) on using magnification X400. The optical density of PAS reaction was measured [23].

#### Determination of KIM-1, L-FABP by reverse transcriptase-PCR in kidney tissue:

The levels of mRNA were analyzed in renal tissue using reverse transcription (RT)-PCR method. Briefly, a complementary DNA (cDNA) was synthesized from a single stranded RNA template in a reaction catalyzed by reversetranscriptase enzyme. Amplification of b-actin was used as a control for sample loading and integrity.

The primer sequences for KIM-1, L-FABP and b-actin were selected. PCR products were identified by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The size of amplicons was confirmed using a 100-bp ladder as a standard size marker. The amplicons were visualized, and images were captured using a gel documentation system (Alpha Innotech Inc., San Leandro, CA). Gene expression then was measured by generating densitometry data for band intensities in different sets of experiments, by analyzing the gel images on the Image J program semi-quantitatively [23]. The resulting band intensities were compared with constitutively expressed b-actin. The intensity of mRNAs was standardized against that of the b-actin mRNA obtained from each sample, and the results were expressed as the PCR-product/b-actin mRNA ratio.

The primer sequences for KIM-1, L-FABP and b-actin, were as follows:

Gene symbol	Primers sequence (5'-3')	Gene Bank accession number
KIM-1	<ul style="list-style-type: none"> <li>• Forward primer 5'-ACTCCTGCAGACTGGAATG G-3'</li> <li>• Reverse primer 5'-CAAAGCTCAGAGAGCCCAT C-3'</li> </ul>	NM-012556
L-FABP	<ul style="list-style-type: none"> <li>• Forward primer 5'-CTGAGGACCTCATCCAGAA-3'</li> <li>• Reverse primer 5'-CACCTCCATCTTAACCAC-3'</li> </ul>	NM-173149
B-actin	<ul style="list-style-type: none"> <li>• Forward primer 5'-AGGCATCCTGACCCTGAAG TAC-3'</li> <li>• Reverse primer 5'-TCTTCATGAGGTAGTCTGTC AG-3'</li> </ul>	NM-007393

**Statistical analysis:**

Data were statistically described and compared regarding mean  $\pm$  standard deviation (mean  $\pm$  SD) using the two-sided Student *t*-test for independent samples. All statistical calculations and analysis were done using the computer program SPSS release 22 of Microsoft Windows.

The *p*-value or calculated probability was determined; *p*-values  $<0.05$  were considered statistically significant, while *p*-values  $<0.01$  were considered highly significant and *p*-values  $>0.05$  were considered insignificant.

**Results**

Table (1): Measurement of the plasma glucose, plasma insulin and HOMA-IR index in the studied groups.

	Group				<i>p</i> -value
	Control Group		DM Group		
	Mean	Standard Deviation	Mean	Standard Deviation	
• Plasma glucose (mmol/L)	5.45	0.53	16.18	1.69	$<0.001^*$
• Plasma insulin ( $\mu$ U/L)	10.29	1.10	5.40	0.96	$<0.001^*$
• HOMA-IR index	1.87	0.21	4.30	0.90	$<0.001^*$

\*: Statistically significant. Values are represented as mean + SD. *p*-value  $<0.05$  compared to the control group is considered statistically significant.

Table (2): Measurement of the blood urea, serum creatinine, plasma albumin/creatinine ratio, urinary albumin excretion in the studied groups.

	Group				<i>p</i> -value
	Control Group		DM Group		
	Mean	Standard Deviation	Mean	Standard Deviation	
• Blood urea (mg/dl)	47.00	6.70	96.27	12.57	$<0.001^*$
• Serum creatinine mg/dl	0.31	0.23	1.54	0.38	$<0.001^*$
• Plasma Albumin/creatinine ratio	140.55	1.69	40.57	2.09	$<0.001^*$
• Urinary Albumin excretion (gm/dl)	0.03	0.01	0.53	0.06	$<0.001^*$

\*: Statistically significant. Values are represented as mean + SD. *p*-value  $<0.05$  compared to the control group is considered statistically significant.

Table (3): Measurement of the mean optical density of PAS +ve reaction, PCR for KIM, PCR for L-FABP in the studied groups.

	Group				<i>p</i> -value
	Control Group		DM Group		
	Mean	Standard Deviation	Mean	Standard Deviation	
• Mean optical density of PAS +ve reaction	0.38	0.04	0.73	0.07	$<0.001^*$
• PCR for KIM	1.00	0.01	6.11	0.54	$<0.001^*$
• PCR for L-FABP	1.01	0.02	6.94	1.45	$<0.001^*$

\*: Statistically significant. Values are represented as mean + SD. *p*-value  $<0.05$  compared to the control group is considered statistically significant.

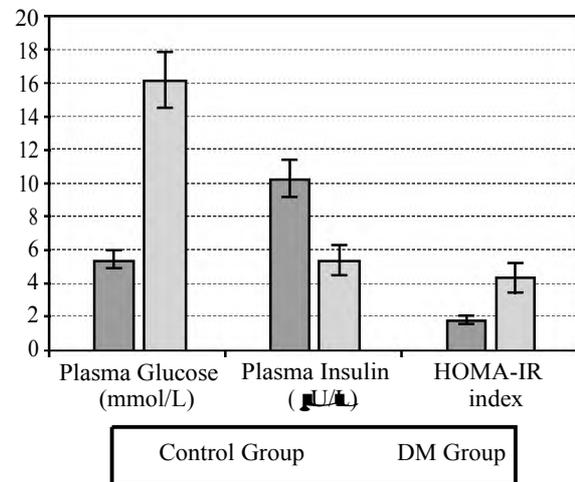


Fig. (1): Comparison between the plasma glucose, plasma insulin and HOMA-IR index among the studied groups.

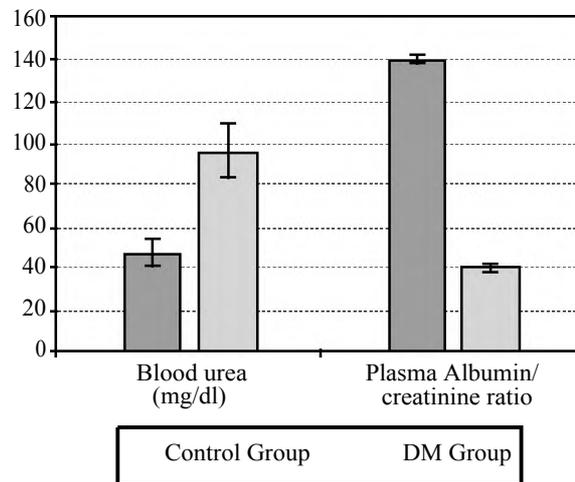


Fig. (2): Comparison between the blood urea, plasma Albumin/creatinine ratio among the studied groups.

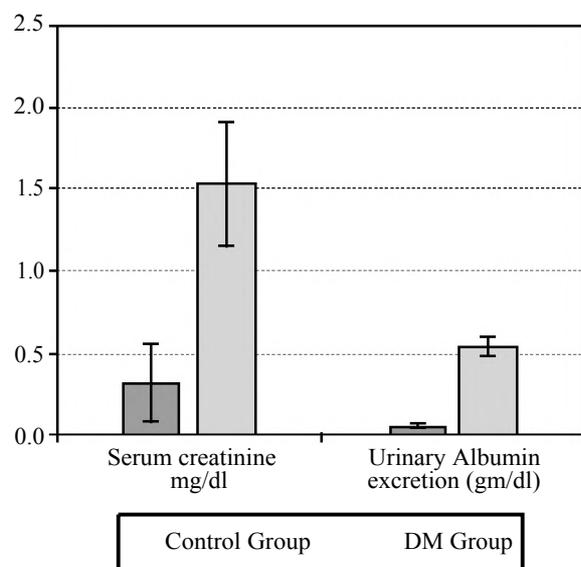


Fig. (3): Comparison between the serum creatinine, Urinary Albumin excretion among the studied groups.

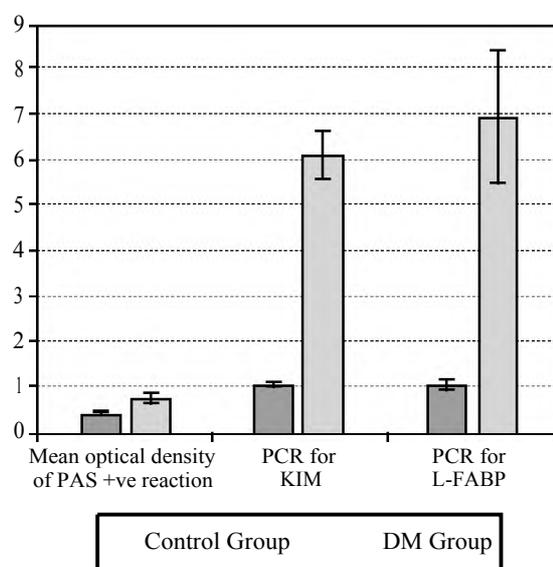


Fig. (4): Comparison between the mean optical density of PAS +ve reaction, PCR for KIM, PCR for L-FABP among the studied groups.

**Correlation analysis:**

Correlation analysis is the statistical method to evaluate the strength of a relationship between two numerically measured continuous variables. It is particularly useful when possible connections between different study variables are needed to be established. It is represented as the *r*-value (the correlation coefficient), where *r* > zero indicates positive correlation and *r* < zero indicates a negative correlation [24].

Correlation analyses of the histopathological changes (mean optical density of PAS +ve reaction) with blood glucose level, blood insulin level, HOMA-IR index, blood urea, serum creatinine,

albumin excretion, albumin creatinine ratio, L-FABP and KIM mRNA expressions.

Correlation coefficient by linear regression analyses was carried out between the histopathological changes (mean optical density of PAS +ve reaction) and the various parameters. A direct statistically significant positive correlation was observed only between the histopathological changes and the expression of L-FABP (*r*=0.809, *p*<.05), all other correlations were statistically insignificant (Table 4), Fig. (5).

Thus, as a conclusion, all renal markers tested (apart from the L-FABP) failed to show significant degree of correlation.

Table (4): Correlation between the histopathological changes (mean optical density of PAS +ve reaction) and the different parameters in the studied groups.

	Blood urea (mg/dl)	Serum creatinine (mg/dl)	Plasma Albumin/creatinine ratio	Urinary Albumin excretion (gm/dl)	Plasma Glucose (mmol/L)	Plasma Insulin (µU/L)	HOMA-IR index	PCR for KIM	PCR for L-FABP
<i>r</i>	-0.592	-0.600	0.556	0.210	-0.410	-0.254	-0.286	0.140	0.809
<i>p</i> -value	0.071	0.067	0.095	0.560	0.240	0.479	0.423	0.700	0.005*
N	10	10	10	10	10	10	10	10	10

\*= Statistically significant.  
*p*-value <0.05 is considered statistically significant.

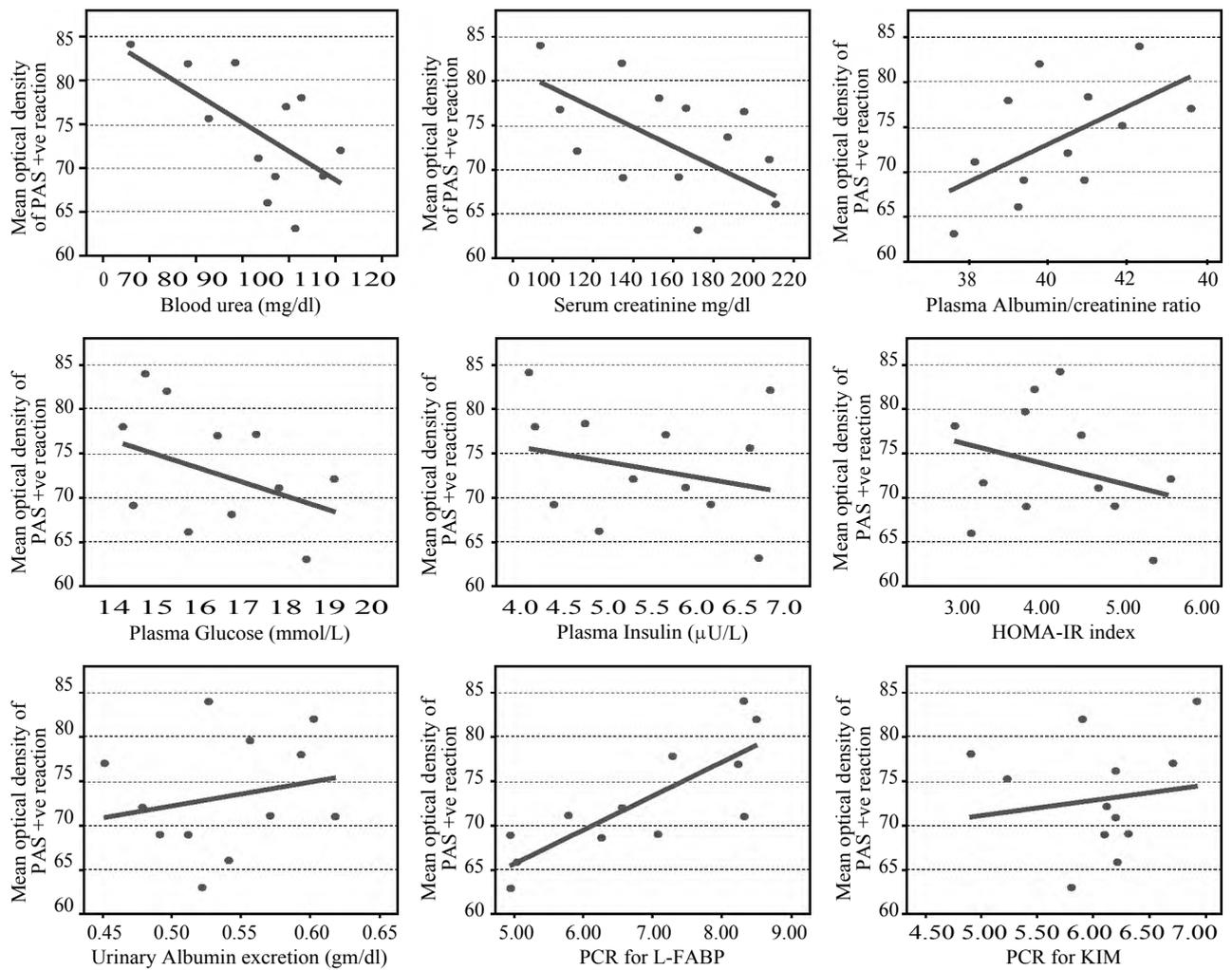


Fig. (5): Correlation analysis curves.

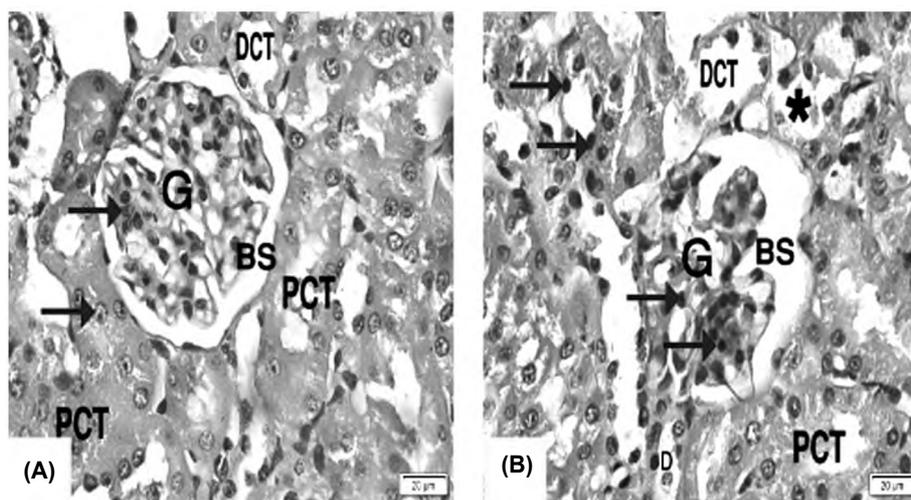


Fig. (6): Photomicrograph of a section in the renal cortex from: (A) A control rat shows strong Malpighian renal corpuscle containing glo-meruli (G) with many capillaries surrounded with Bowman's Space (BS), Proximal Convoluted Tubules (PCT) with apical brush border and Distal Convoluted Tubules (DCT). Pale nuclei with prominent nuclei are seen (arrows); (B) A diabetic rat shows segmented glomerulus (G) with markedly dilated Bowman's Space (BS). Many tubular and glo-merular cells have small darkly stained condensed nuclei (arrows). Most of tubules lost their brush border (PCT) & (DCT). Some renal tubules show casts within the lumen (asterisks). Other tubules show desquamation of cells (D). (H & E X400).

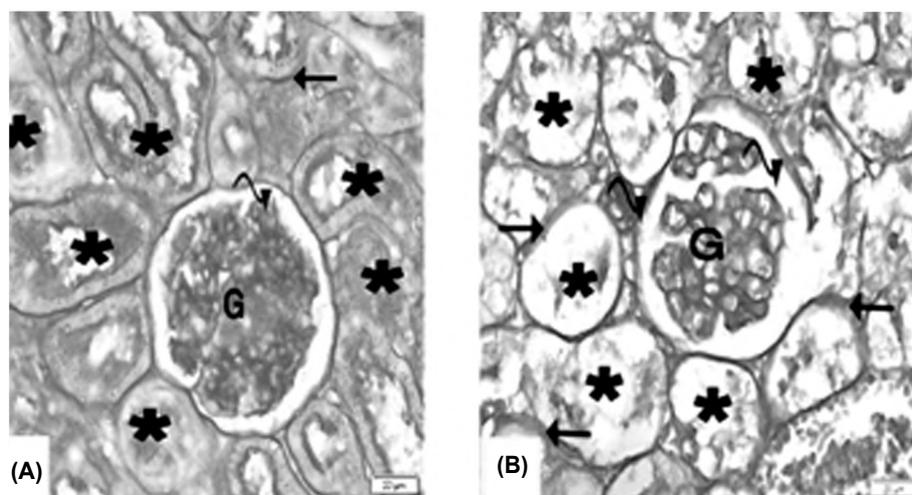


Fig. (7): Photomicrograph of a section in the renal cortex from: (A) A control rat shows normal staining of mesangial matrix (G), capsular basement membrane (curved arrow), tubular basement membrane (arrow) and brush border (asterisks). (B) A diabetic rat shows dense staining of mesangial matrix (G), thickened capsular basement membrane (curved arrows) and tubular basement membrane (arrows). Most of the tubules are seen with areas of lost brush border (asterisks). (PAS X400).

### Discussion

Our results showed a significant deterioration of renal functional parameters in Group 2 which is evidenced by a marked increase in blood urea and serum creatinine, together with albuminuria, resulting in a net decrease in the albumin-creatinine ratio. In addition, Group 2 showed an evidence of DN, manifested by increased blood glucose associated with insulin resistance (Tables 1,2) & Figs. (1,3).

Diabetic Nephropathy (DN) is one of the most serious microvascular complications of type 2 diabetes mellitus, as well as it is one of the leading causes of chronic renal impairment slowly progressing to end-stage renal failure and death [25]. Recent evidences suggest that more than 30% of all diabetic patients develop DN within 10-20 years after the onset of diabetes [26]. In these patients, the incidence of development of microalbuminuria is 20-40% if diabetic nephropathy remains untreated. Moreover, within 20-25 years almost 20% of these patients will develop end-stage renal failure, which further requires chronic hemodialysis and even possibly, renal transplantation [27].

So, given that, and given the fact that current biomarkers that are used for screening and follow-up of chronic renal diseases in general are lacking the adequate sensitivity, therefore, novel biomarkers are needed for the early detection and risk stratification of diabetic nephropathy [28].

The mechanism of renal impairment in diabetes is multifactorial and involves multiple pathological pathways; chronic hyperglycemia causes overproduction of hydrogen peroxide which in turn increases lipid peroxidation together with the glycosylation of proteins in blood and tissues, leading to the overproduction and accumulation of Advanced Glycosylated End products (AGEs) [29]. In addition to the AGEs, chronic hyperglycemia causes elevated levels of Transforming Growth Factor Beta-1 (TGF- $\beta$  1) together with the increased production of reactive oxygen species and renal polyol formation which contributes to increased renal albumin permeability and Extracellular Matrix (ECM) accumulation leading to elevated proteinuria, disruption of the basement membrane, and tubulointerstitial fibrosis [30].

The histological changes occurring in diabetic nephropathy are the hallmark of the pathophysiological alterations including; both glomerular and tubular structural and functional disruption, mesangial cell activation, structural disruption of the glomerular basement membrane, podocyte injury and tubular abnormalities [31].

The Streptozotocin (STZ)-induced diabetes is a widely used, well established and highly reproducible animal model to experimentally induce and study diabetic complications including retinopathy, neuropathy, and nephropathy. A high-fat diet followed by intraperitoneal administration of STZ leads to the development of diabetic nephropathy

athy. This model mimics almost all pathophysiological features of clinical diabetic nephropathy [32].

Knowing the exact mechanisms for the development of diabetic nephropathy, including the alteration in the signaling molecules, and their correlation with the histopathological changes occurring in diabetic nephropathy is of utmost importance for the development of new pharmacological therapies that can reverse such alterations, potentially leading to the attenuation of the disease progression and better clinical outcome.

Our results showed that renal tissue from normal rats stained with the H & E stain showed the typical well-organized architecture of the renal histology. It showed the normal glomerular structure surrounded by the Bowman's capsule, proximal, and distal convoluted tubules without any evidence of fibrosis, basement membrane changes, inflammatory infiltration, or necrosis. Whereas, different grades of various pathological changes were observed in the renal tissue of diabetic rats. It showed distorted glomerular symmetry, thickening of the basement membrane, darkly stained nuclei, distorted tubular structure in the form of loss of the brush border together with luminal cast infiltration Fig. (6).

Periodic acid-Schiff staining is one of the most commonly used histological methods for detection of the histopathological changes occurring in diabetic lesions. It is highly sensitive for the detection of carbohydrate deposition in the tissues. Periodic acid oxidizes the diol groups in different sugars leading to aldehyde formation by breaking up the bonds between the adjacent carbon atoms in the monosaccharide subunits, the product then reacts with the Schiff reagent to form the purple-magenta color characteristic of the PAS staining [33].

Diabetic nephropathy is associated with an expansion in the mesangial matrix with the development of mesangial sclerosis, associated with eosinophilic PAS positive nodules called nodular glomerulosclerosis, leading to prominent vascular changes which are seen with these cases and represents the hallmark of the pathological process [34].

Our results showed normal mesangial matrix in the control rat, together with normal basement membrane and tubular structures with PAS staining. While in the diabetic nephropathy group, PAS-stained section showed dense staining of the mesangial matrix, as well as distorted brush bor-

ders and basement membrane structure. (Table 4), Fig. (7).

Several new biomarkers have been studied in recent researchers, but results are still inconclusive in general, however, among the most studied biomarkers, liver-type Fatty Acid Binding Protein (L-FABP) and Kidney Injury Molecule (KIM) are considered two of the most promising biomarkers for the detection and follow-up of CKD. While more studies and extensive correlation analyses are still needed for further elucidation of their accuracy. Complete data about the changes in the expression of these molecules, their blood and urinary levels consequently and their relation to the progression of nephropathy in diabetic patients are not yet fully available but will be of considerable interest.

Our results and statistical analysis showed a statistically significant positive correlation between the histopathological changes in DN Group and the gene expression of L-FABP in the renal tissues of these rats ( $r=0.809$ ,  $p<0.05$ ), while there is no statistically significant correlation between the histopathological changes and the other study variables including; KIM, blood urea and serum creatinine. (Table 4), Fig. (5).

The urinary excretion of L-FABP, which is expressed in the proximal convoluted tubules and involved in FFA metabolism, is reported by several researches to reflect the clinical progression of chronic diseases of kidney. Kamijo et al., [35] reported that urinary L-FABP shows a promising clinical biomarker to predict and monitor the progress of chronic glomerular diseases. But, a little is known about the role of L-FABP blood level or gene expression in cases of diabetic nephropathy and its correlation with glomerular as well as tubulointerstitial injuries regarding animal studies or in vivo in human diabetic nephropathy studies [35].

Our results are also consistent with Nakamura et al., [36] who studies the effect of oral hypoglycemic drugs on the urinary levels of L-FABP and found that it increases in cases of diabetic nephropathy and proved that the proper control of diabetes mellitus is effective in reducing urinary L-FABP concentrations in patients with early diabetic nephropathy compared to the uncontrolled diabetes mellitus [36].

The role of L-FABP as a signaling molecule in the renal tissues and its mechanisms of action or its protective effects in the different kinds or renal injury are not clear yet. However, it is hypothesized

that its mechanism of action most probably involves the activation of Peroxisome Proliferator-Activated Receptors (PPARs) expression and augmenting its effects. Some animal studies demonstrated that increased levels of L-FABP are associated with higher activity and increased levels of expression of PPAR- $\psi$ . However, this effect is still lacking enough evidence derived from human studies [37].

The PPAR- $\psi$  expression is predominant in the kidney, and it is specifically implicated in the tubulointerstitial response to different kinds of renal injury especially those characterized by progressive nephropathy [38]. Zafiriou et al., [39] reported that PPAR- $\psi$  agonist therapy in patients with metabolic inherent renal disease might decrease proteinuria and may delay progression of tubulointerstitial diseases by inhibiting the inflammatory and fibrous responses [39]. The proinflammatory and profibrotic effects of albumin on the proximal tubular cells are well recognized and are mediated by activation of the PPAR- $\psi$  signaling pathway [40].

PPARs are ligand-activated transcription factors of the superfamily of nuclear hormone ligand receptors. Three PPAR isoforms have been cloned and are differentially expressed in several tissues with a variable degree of expression, with a strong expression in the renal tissues. The three subtypes are PPAR- $\alpha$ , PPAR- $\psi$ , and PPAR- $\beta$  [41].

PPAR- $\alpha$  primarily modulates the inflammatory response [42], while PPAR- $\beta$  participates in embryonic development [43], placental implantation [44], and bone formation [45]. PPAR- $\psi$  activation is involved in energy homeostasis and it plays important roles in the regulation of glucose and fat metabolism, it is also an essential regulator of adipocyte differentiation and it stimulates lipids uptake and adipogenesis by fat cells [46]. PPAR- $\psi$  is the most important isoform regarding the glucose and lipid metabolism, it is also involved in other diversity of actions including; cell cycle regulation, cellular repair and differentiation, anti-inflammatory and antiatherogenic responses [47], as well as being a key transcriptional factor in adipogenesis and it plays an essential role in the regulation of insulin sensitivity [48].

In the past few years, tremendous progress has been achieved in the control of type 2 diabetes mellitus by the discovery and the frequent use of the novel classes of oral hypoglycemic drugs, including antidiabetic thiazolidinediones (TZDs), such as troglitazone and rosiglitazone, which are specific ligands of PPAR- $\psi$  signaling pathway [49], and their use resulted in a marked decrease in the

incidence and prevalence of the microvascular complications associated with type 2 diabetes mellitus including diabetic nephropathy. Also, a significant advance has been reached towards understanding the role of PPAR pathway regarding different metabolic disorders including obesity, dyslipidemia and hypertension [50].

Given the above-mentioned facts, in addition to the fact that both PPAR signaling pathway and L-FABP are actively involved in the regulation of lipid and glucose metabolism, therefore, the hypothesized linkage between them, although not fully proven yet, it is of a logical interest.

The other molecule we investigated in our study; Kidney Injury Molecule (KIM) showed no statistically significant correlation between its levels and the degree of structural and histopathological changes. Although it was considered a promising molecule as a marker for kidney injury, recent studies revealed that it is a sensitive marker in acute kidney injuries, including ischemic, septic injuries and acute renal failure [51]. However, results regarding its role as a marker for chronic kidney injuries is still inconsistent and inconclusive [52].

Many researchers have proved that the factors that initiate acute tubular epithelial cell injury result in KIM-1 protein expression. KIM-1 gene and protein products are up-regulated 3 hours after experimental renal ischemia/reperfusion injury [53], cisplatin [54], cyclosporine [55], iodinated contrast agents [56], and other nephrotoxic agents that cause acute tubular cell damage and result in KIM up-regulation.

Being expressed at very low levels in the normal physiological conditions, the exact physiological functions of KIM in healthy individuals are not very clear yet. However, it is postulated that it may have a role as a regulator of phagocytosis and apoptosis in the renal proximal tubular cells [57].

Our results are consistent with Eynatten et al., [58] and Nauta et al., [59] who found that urinary KIM is not significantly associated with the pathological changes in diabetic nephropathy. However, they are inconsistent with Hosohata et al., [60] and Sabbisetti et al., [61] who found a positive correlation between the levels of urinary KIM and the progression of diabetic nephropathy in type 2 and type 1 DM, respectively.

Based on this conflict and according to our study results, it is not advisable to use the KIM as a biomarker for diabetic nephropathy. At least there

are other molecules such as L-FABP which are more promising as sensitive and specific biomarkers to be relied on for such function.

#### Conclusion:

L-FABP is the most sensitive marker with the most significant correlation with the renal histopathological changes occurring in type 2 diabetic nephropathy in rats. It is a promising as a future biomarker to be used with a good sensitivity for the early detection, risk stratification and follow-up of diabetic nephropathy cases.

Other studied parameters (blood urea, serum creatinine and KIM gene expression) failed to show statistically significant correlation with the renal histological changes.

#### Recommendations:

Future studies on human cases are recommended.

Further studies regarding the L-FABP physiological role and its signaling cascade in the physiological and in the different pathological conditions are still needed.

#### Conflict of interest:

The authors declare that there is no conflict of interest is associated with this work.

#### Funding:

This study was self-funded. All author contributed equally in the funding and no external funding resources or research grants were used in this study.

#### Acknowledgement:

I want to thank all the authors for their valuable effort in this manuscript.

Also, I would like to express my gratitude to the academic and technical staff members at the Departments of Human Physiology, Biochemistry and Histology Departments, Kasr Al-Ainy Faculty of Medicine, Cairo University for providing administrative and technical support for this work.

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## دراسة مقارنة للدلائل الجديدة لإصابة الكلى، جزء إصابة الكلى، وبروتين ربط الأحماض الدهنية من النوع الخاص بالكبد في إعتلال الكلى السكري التجريبي في الجرذان، وعلاقتهم بالتغيرات النسيجية المرضية في الكلى

إعتلال الكلى السكري هو أحد المضاعفات الخطيرة للأوعية الدموية الدقيقة من داء البول السكري من النوع الثاني والتي يمكن أن تتقدم ببطء إلى مرحلة مرضية متقدمة مؤثرة على أنسجة الكلى والتي يمكن أن تكون قاتلة. ويتميز المرض بتغيرات مرضية محددة، تتقدم ببطء في أنسجة الكلى. ونظراً لأن المؤشرات الحيوية التقليدية تقتصر إلى الحساسية الكافية، فإن هناك حاجة إلى مؤشرات حيوية جديدة للكشف المبكر وتقليل خطر إعتلال الكلى السكري.

الهدف: إستكشاف التغيرات النسيجية المرضية التي تحدث في إعتلال الكلى السكري، ودراسة التغيرات المرتبطة بأثنين من المؤشرات الحيوية الجديدة، بروتين ربط الأحماض الدهنية من النوع الخاص بالكبد (L-FABP) ومؤشر جزء إصابة الكلى (KIM) وربط درجة تعبيرهم الجيني مع درجة التغيرات الهستوباثولوجية في أنسجة الكلى، ومقارنة حساسيتها ودرجة ارتباطها بالمؤشرات الحيوية التقليدية، وخاصة اليوريا والكرياتينين في الدم.

المواد وطرق البحث: تم تقسيم أربعة وعشرون من ذكور فئران التجارب إلى مجموعتين، مجموعة ضابطة، ومجموعة إعتلال الكلى السكري. تم إحداث داء السكري من النوع الثاني بواسطة غنية بالدهون متبوعة بالحقن داخل غشاء الصفاق بعقار الستيروئيدوسين، وتم تأكيد وجود مرض البول السكري بواسطة قياس مستوى الجلوكوز والأنسولين أثناء الصيام في الدم، وتم تأكيد إعتلال الكلى السكري من خلال وجود إفراز للزلال في البول.

كما تم إجراء الفحص النسيجي لجميع الفئران التجريبية باستخدام صبغة ال (H & E) بالإضافة إلى صبغة ال (PAS) وتم التعبير عن النتائج على أنها متوسط الكثافة البصرية للتفاعل الإيجابي مع صبغة ال (PAS).

كما تم قياس التعبير الجيني لبروتين ربط الأحماض الدهنية من النوع الخاص بالكبد (L-FABP) ومؤشر جزء إصابة الكلى (KIM) بواسطة طريقة النسخ الجيني العكسي (PCR) وتم قياس مستويات اليوريا والكرياتينين في الدم كمثال تقليدي للمؤشرات الحيوية القياسية في حالات إعتلال الكلى السكري وتم قياس درجة الارتباط بواسطة طريقة بيرسون لتحليل الارتباط الإحصائي في العلاقة بين التعبير الجيني والتغيرات النسيجية المرضية.

النتائج: حدثت تغييرات نسيجية مرضية كبيرة في إعتلال الكلى السكري تتضح من العديد من التغيرات في صبغة ال (H & E) وكذلك تفاعلات صبغة ال (PAS) الإيجابية. جميع المؤشرات الحيوية كانت مرتفعة في مجموعة إعتلال الكلى السكري بالمقارنة بالمجموعة الضابطة. ومع ذلك، لم يتم العثور على علاقة ارتباط ذات دلالة إحصائية بين اليوريا أو الكرياتينين في الدم ودرجة التعبير الجيني لمؤشر جزء إصابة الكلى (KIM) تم العثور فقط على علاقة ذات دلالة إحصائية بين التعبير الجيني لبروتين ربط الأحماض الدهنية من النوع الخاص بالكبد (L-FABP) ودرجة التغيرات النسيجية المرضية في أنسجة الكلى.

الخلاصة: تقتصر المؤشرات الحيوية القياسية التقليدية (اليوريا والكرياتينين في الدم) إلى الحساسية الكافية للارتباط مع التغيرات النسيجية المرضية التي تحدث في إعتلال الكلى السكري. بروتين ربط الأحماض الدهنية من النوع الخاص بالكبد (L-FABP) هو علامة بيولوجية مستقبلية واعدة يمكن إستخدامها للكشف المبكر وكذلك متابعة حالات إعتلال الكلى السكري.