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Original article

Evaluation of miR-181a in Diabetes Mellitus Type-2 and its relation to Diabetic Nephropathy

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Abstract

A chronic consequence of diabetes mellitus (DM), diabetic nephropathy (DN) typically advances to end-stage renal disease (ESRD). However, the pathophysiology of DN is not fully understood. It has been revealed that several miRNAs have been considered as risk or protective factors in DN. Inflammation is a key determining factor in the development and progression of diabetes. The aim of the current study was to investigate the association of serum miR-181a, serum, and urine IL-6, and IL- 18 with DN. miR-181a was estimated by RT-qRT-PCR, IL-6, and IL- 18 were estimated by enzyme-linked immunosorbent assay (ELISA). 120 patients were divided into three groups; Group 1: 40 DM type 2 without DN, group 2: 40 DM type 2 with diabetic DN, and group 3: 40 control patients. Group 2 was further subdivided into 20 macroalbuminuria and 20 microalbuminuria patients. When DN patients were compared to other groups, their levels of miR-181a, IL-6, and IL-18 increased significantly. Higher levels of miR-181a in patients with macroalbuminuria than patients with

microalbuminuria in DN group. Serum miR-181a significantly positively correlated with fasting insulin, fasting glucose, HOMA IR, ACR, serum IL-6 and BMI in DN group. The ROC curve analysis revealed high significant specificity and sensitivity of serum miR-181a, serum, and urine IL-6, and IL-18 suggesting their possible utility for the primary diagnosis of DN in DM. It is concluded that miR-181a may prove to be a valuable biomarker for DN prognostic and diagnostic applications.

1. Introduction:

Type 2 Diabetes Mellitus (T2DM) is a prevalent health problem that is most likely linked to obesity. People afflicted with T2DM are subject to complications involving micro and macro vasculature due to elevated glycemic level and insulin resistance [2]. Diabetic Nephropathy (DN) occurs as a side effect to long standing hyperglycemia in T2DM individuals. DN occurs as a macrovascular complication due to macrovascular damage caused by glycation affecting renal vasculature. DN can eventually lead to end-stage renal disease due to poor glycemic control [1]. Multiple factors contribute to the incidence of T2DM and its complications. Among these factors is insulin resistance which involves the inability of insulin to exert its actions throughout the body. Micro RNA (miRNA) is a type of RNA that post-transcriptionally regulates gene expression. Dysregulated miRNA has been suggested to contribute to conditions such as obesity and insulin resistance [3]. miRNA

181a is one such miRNA that has been suggested to be linked to DM and more specifically to the incidence of DN in type 2 diabetic patients. miRNAs have the potential to become a new axis for understanding the pathogenesis and risk factors of diseases as well as contribute to new methods of management of such diseases [3].

Anti-inflammatory therapies have a great role nowadays in treatment of such chronic diseases, so studies to assess the levels of pro-inflammatory cytokines as interleukin IL-6 & interleukin IL-18 are essential [14].

IL-6 and IL-18 are associated with an increased inflammatory infiltrate with subsequent more severe kidney lesions [15].

Levels of IL-6 and IL-18 are strong predictors of diabetic nephropathy and their genetically down-regulated expression is associated with a favorable cardiometabolic profile [16].

Among epigenetic modifications, microRNAs (miRNAs) are important mediators of posttranscriptional feedback control mechanisms that are involved in modulating metabolism, as well as inflammation, which provides unique molecular and cellular insights into the pathophysiology of DN [31]. The aim of the current study was to investigate the association of serum miR-181a, serum, and urine IL-6, and IL- 18 with DN

2. Patients and Methods:

This was a randomized study performed in in Beni-Suef University Hospital within six months from February to August 2022 involving 120 patient verbal consents were obtained. The Study was approved by FM-BSU REC, approval number FMBSUREC/01022022/Hussain

2.1 Inclusion criteria:

Inclusion criteria were diabetes type 2 patients without diabetic nephropathy, diabetes type 2 patients with diabetic nephropathy who had persistent albuminuria (>300 mg/d or >200 μg/min) that is confirmed on at least 2 occasions 3-6 months apart, a relentless decline in glomerular filtration rate, and absence of signs or symptoms of other primary causes of kidney damage.

2.2. Exclusion criteria:

The study excluded all patients with serum creatinine above 2.5 mg/dl, liver disorders, chronic inflammatory disease., infectious disease, patients with malignancy, type 1 diabetes, and other renal diseases.

2.3. Study procedure

2.3.1. At the clinic visit;

All patients were subjected to history taking. Anthropometric measurements including waist and hip circumference in cm, weight in kg and height in cm were performed. The body mass index (BMI) has been calculated [34].

2.3.2. At the laboratory;

Estimation of estimate biochemical variables (fasting and post prandial glucose, HbA1c, insulin, c- peptide, total cholesterol, triglycerides, LDL-c, HDL-c, urea, creatinine) and 24 h urinary protein after an overnight fasting of at least 10 hours. For diagnosis of DM and diabetic nephropathy. Calculation of HOMA-IR on basis of fasting value of plasma glucose and insulin according HOMA model formula [35].

2.4.Real-Time Quantitative Reverse Transcription PCR

2.4.1. MiRNA Isolation

The miRNeasy Mini Kit (cat. no. 217004, QIAGEN, Germany) was used to purify extracted total RNA, including miRNA, from the serum samples in accordance with the manufacturer's instructions. 260 nm was the measurement point for the extracted RNA using spectrophotometry (JENWAY, USA).

2.4.2. Reverse transcription of isolated mRNA

The isolated mRNA was reverse transcribed into cDNA using the TOPscriptTM RT DryMIX (dT18/dN6 plus) kit (Cat. No.: RT220, economics, Life Technologies, India) in accordance with the manufacturer's instructions. 100 ng of the isolated mRNA was added to the dissolved pellet of the strips, and the mixture was then incubated at 42 °C for five minutes.

2.4.3. Reverse transcription of isolated total RNA, including miRNA

Reverse transcription into cDNA was performed using the miScript II RT Kit (cat. no. 218161, Qiagen, Valencia, CA) on the extracted total RNA, including miRNA. One microgram of total RNA was added, and then 20 µl RT reactions were made. The manufacturer's instructions were for an initial 60 minutes of incubation at 37°C and a final five minutes at 95°C.

2.4.4. Real-time quantitative PCR using SYBR Green

Real-time PCR study used cDNA that was made from miRNA in a reverse transcription process with miScript HiFlex Buffer as the template. then combined with the RNU6, which served as the reference miRNA, in the miScript SYBR Green PCR Kit (Cat. No. 218073, Qiagen). The primer sequence was created using GenBank RNA sequences with Tm: 60–65 °C, and they were supplied by Qiagen, Germany. Prior to test preparation, cDNA was diluted 1:5 in a net volume of 25 µl using nuclease-free H_2O. The Applied Biosystems StepOneTM Real-Time PCR System (software V.2.0.1) was used to analyze the samples under ideal conditions. This included an initial phase of 15 minutes at 95°C, followed by three phases of 40 cycles lasting 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 70°C.

2.4.5. Calculation of Relative Quantification (RQ) (relative expression)

Using the comparative cycle threshold (Ct) approach, the relative expression levels of mRNA and miRNA were determined in relation to the expressions of GAPDH and RNU6 snRNA. Using the 2-ΔΔCT method [36], the fold changes in mRNA and miRNA expression were determined.

2.5. Measurements of both serum, and urine IL-6, IL- 18

Serum and urine samples were centrifuged for 20 min at 1000 rpm to measure levels of IL-6, IL- 18 by enzyme-linked immunosorbent assay (ELISA).

2.6. Statistical methodology

Analysis of data was done by IBM computer using SPSS (statistical program for social science) as follows; Description of quantitative variables as mean, SD and range, Description of qualitative variables as number and percentage, Unpaired t-test was used to compare quantitative variables, in parametric data (SD < 50 % mean), p value>0.05

insignificant, p<0.05 significant, p<0.01 highly significant [20].

3.Results:

3.1. demographic and clinical data

Table 1 showed highest levels of BMI, and disease duration in DN than other groups.

3.2. Levels of miR-181a in studied groups

When DN patients were compared to other groups, their levels of miR-181a, IL-6, and IL-18 increased significantly (Figure 1). Higher levels of miR-181a in DN patients with macroalbuminuria than DN patients with microalbuminuria (Figure 2).

3.3. Estimation of serum and urine IL-6, IL- 18 levels in studied groups

Figure 3 showed that patients with DN showed significantly higher serum and urinary IL-6, IL-18 levels versus other groups.

3.4. The levels of metabolic, and renal Parameters in the subjects

Table 1 showed that there were significant (p<0.001) increases in the mean levels of serum triglycerides, cholesterol, HBA1c, fasting glucose, fasting insulin, HOMA-IR, in diabetics with nephropathy and diabetics without nephropathy than those in the control group with the highest serum levels of triglycerides, cholesterol, HBA1c, fasting glucose, fasting insulin, HOMA-IR, levels in DN. It also showed that there were significant increases in the mean levels of Creatinine, urea, ACR in diabetics with nephropathy than those in other groups.

3.4.1. Correlations of miRNA 181a

In Table 2, miRNA 181a levels showed significant(P<0.001) positive correlations with each of ACR, age, BMI, fasting insulin, fasting glucose, HOMA-IR, and significant negative correlations with serum IL-6 levels, and eGFR in the DN group but in the DM without DN patients the significant positive correlations were with fasting insulin, and HOMA-IR only.

3.6. ROC Curve analysis for miRNA 181a, IL-6, and IL-18 in the early diagnosis of diabetic nephropathy in diabetic patients

Table 3, and Figure 4 showed that miRNA181a, serum and urinary markers IL-6 and IL-18 had significant sensitivity and specificity in the early detection of diabetic nephropathy in diabetic patients.

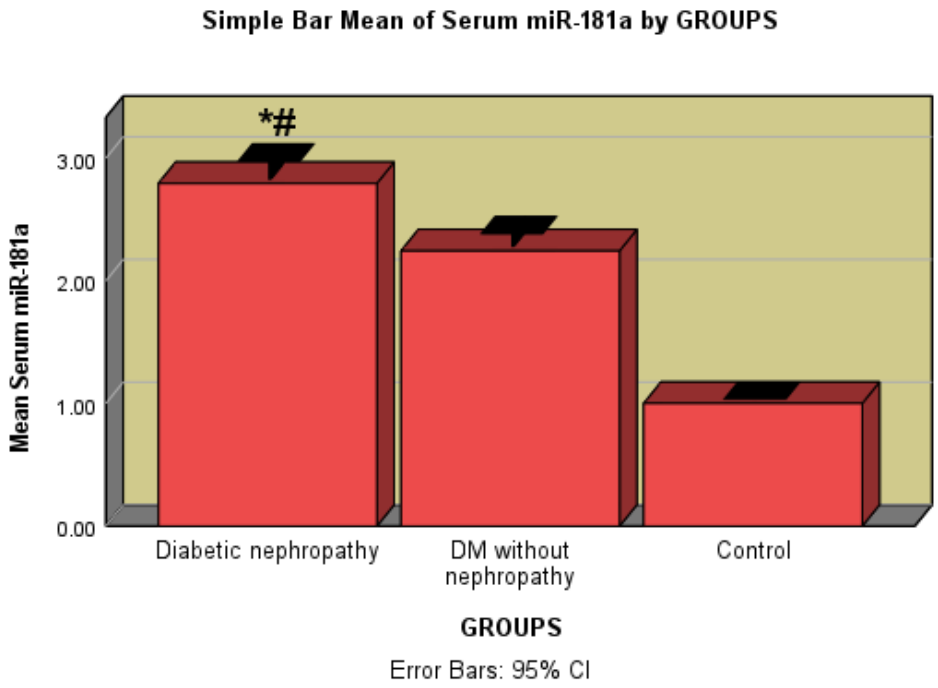

Fig 1. miRNA 181a levels in the studied groups.

Significant (p-value <0.001) increases in miRNA 181a levels in patients with DN, and diabetic patients without nephropathy compared to control group, whereas they were higher in patients with DN compared to diabetic patients without nephropathy.

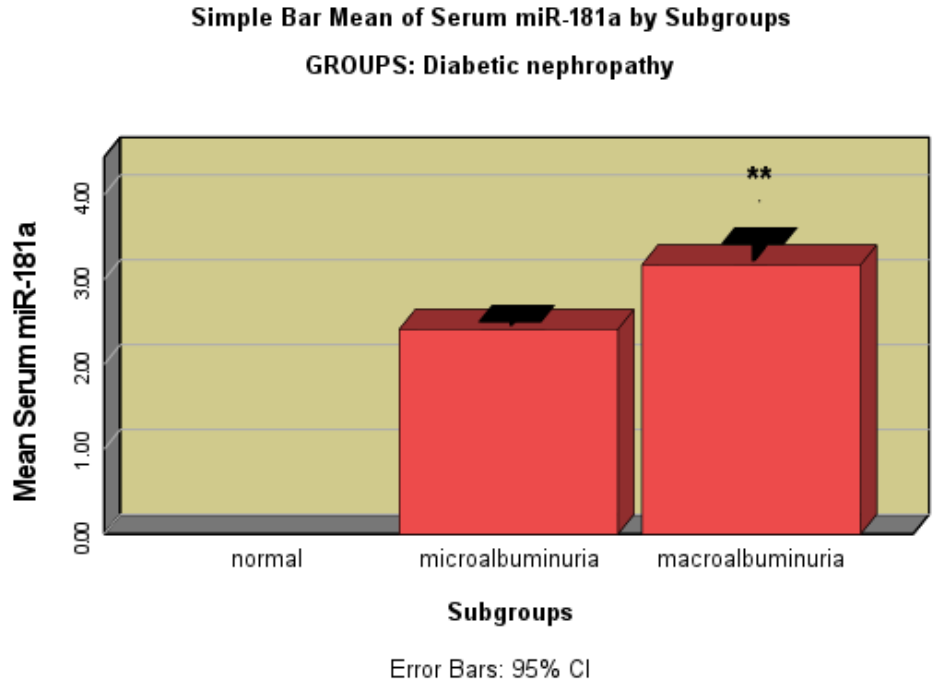

Fig 2. comparison between miRNA 181a levels between DN group's subgroups.

Significant increases in miRNA 181a levels in patients with macro-albuminuria than those in patients with microalbuminuria within the patients with DN.

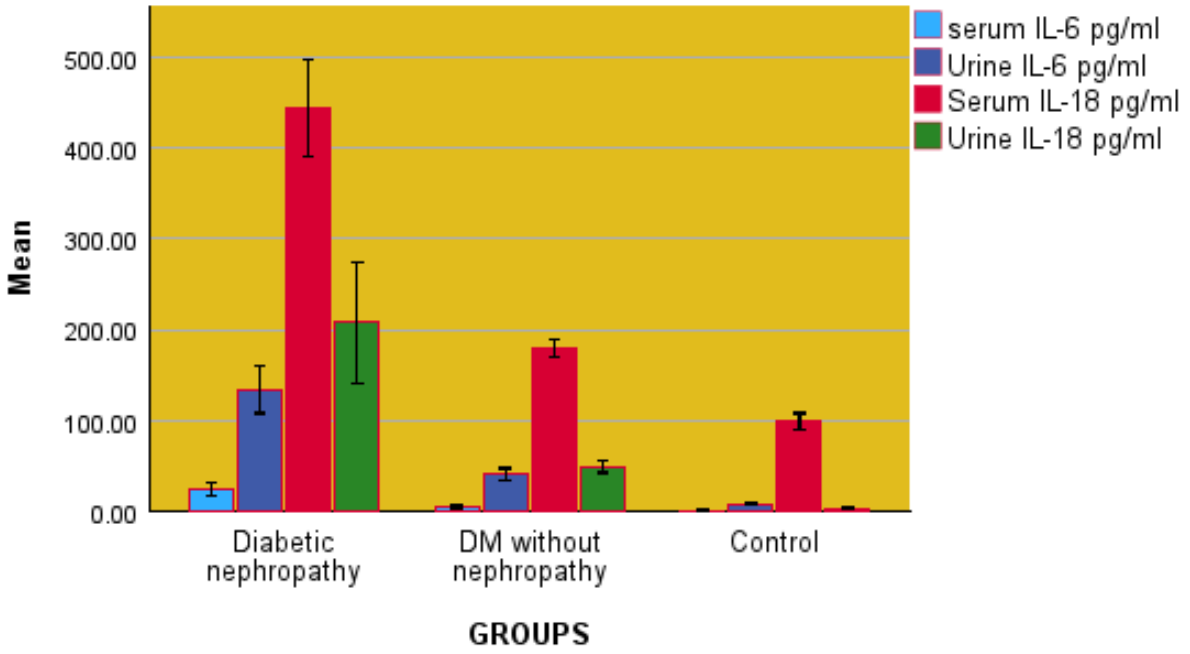

Fig 3. Levels of serum, and urine IL-6, and IL-18 in the studied groups.

Significant (p-value <0.001) increases in serum, and urine IL-6, and IL-18 in patients with DN, and diabetic patients without nephropathy compared to control group, whereas they were higher in patients with DN compared to diabetic patients without nephropathy.

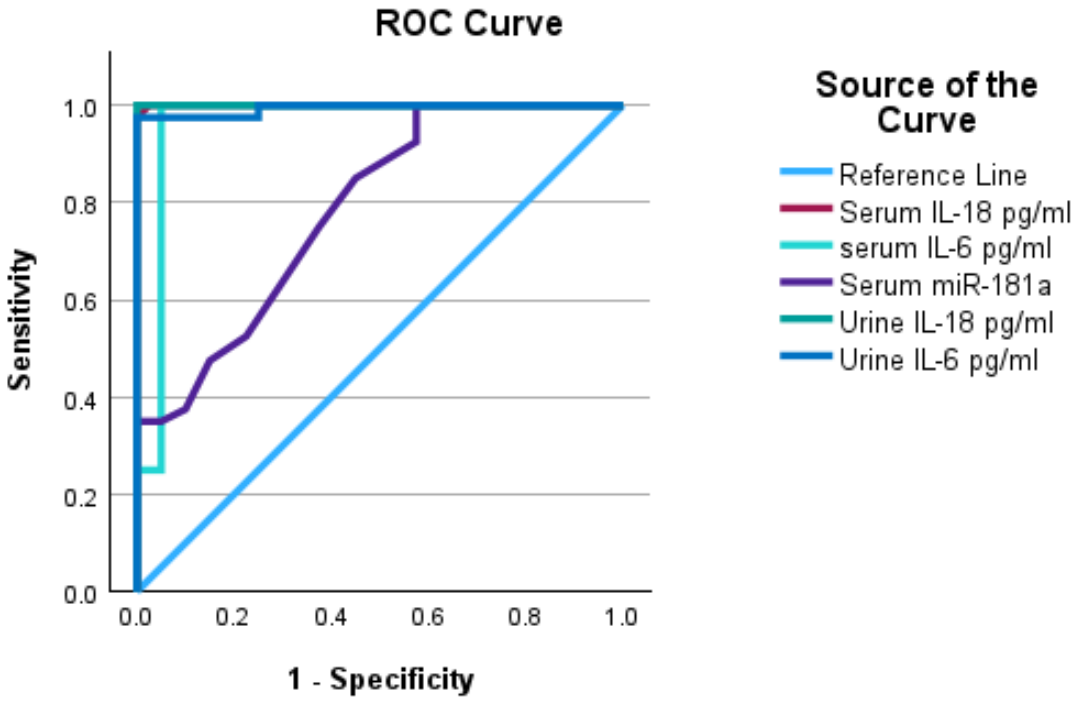

Fig 4. ROC Curve analysis for miRNA 181a, IL-6, and IL-18 in the early diagnosis of diabetic nephropathy in diabetic patients.

Table 1. Comparison between the studied groups regarding demographic, and clinical data

	DN N=40	DM without nephropathy N=40	Control N=40
Age (Years) (M ± SD)	48.1 ± 5.51	49.2 ± 5.45	48.55 ± 6.36
Gender N/%	Male: 22/55 Female: 18/45	Male: 24/60 Female: 16/40	Male: 22/55 Female: 18/45
BMI (M ± SD)	26.3 ± 1.64 b** c**	24.68 ± 1.99 a** c**	22.38 ± 1.53 a** b**
Duration of DM (M ± SD)	15.38 ± 1.60 b** c**	7.88 ± 1.71 a** c**	_
Serum TG(mg/dL) (M ± SD)	220.38 ± 18.61 b** c**	203.58 ± 23.23 a** c**	113.33 ± 12.38 a** b**
Serum HDL-C (mg/dL) (M ± SD)	43.45 ± 2.43 b* c**	45.28 ± 3.34 a* c**	63.62 ±1.92 a** b**
Serum cholesterol (mg/dL) (M ± SD)	176.21 ± 6.58 c**	174.15 ± 10.07 c**	164.63 ± 12.69 a** b**
HBA1c(%) (M ± SD)	8.42 ± 0.65 b** c**	7.45 ± 0.56 a** c**	4.71 ± 0.48 a** b**
Fasting glucose (mg/dL) M ± SD	174.73 ± 12.55 b** c**	205.38 ± 24.35 a** c**	79.45 ± 6.19 a** b**
Fasting Insulin(µ U/L) (M ± SD)	22.03 ± 2.91 b** c**	13.53 ± 2.75 a** c**	5.10 ± 1.24 a** b**
HOMA-IR (M ± SD)	9.48 ± 1.48 b** c**	6.85 ± 1.54 a** c**	1.00 ± 0.25 a** b**
Urea (mg/dL) (M ± SD)	55.2 ± 7.42 b** c**	20.38 ± 3.04 a**	18.45 ± 3.08 a**
Creatinine(mg/dL) (M ± SD)	2.11 ± 0.27 b** c**	0.82 ± 0.32 a**c*	0.72 ± 0.02 a**b*
GFR(ml/min/1.73m2) (M ± SD)	38.20 ± 10.70 b** c**	76.85 ± 3.42 a** c**	96.63 ± 1.81 a** b**
ACR (mg/g) (M ± SD)	469.08 ± 272.33 b** c**	24.40 ± 2.99 a**	15.88 ± 2.63 a**

DN: diabetes with nephropathy, TG: triglycerides, LDL-c: low density lipoprotein – cholesterol, HDL-c: high density lipoprotein – cholesterol, HBA1c: glycated hemoglobin, HOMA-IR: Homeostatic model assessment for insulin resistance, GFR: glomerular filtration rate, ACR: albumin – creatinine ratio, a: statistically significant with DN group, b: statistically significant with DM group, c: statistically significant with control group, *: $p<0.05$, **: $p<0.001$

Table 2. Significant Correlation between miRNA 181a and clinical & laboratory parameters in the diabetic patients.

Parameter		Group 1 (n =40) DN group	Group 2 (n=40) DM without nephropathy
BMI (kg/m2)	**r** **P**	0.535** <0.001	0.063 0.701
Serum IL-6 (pg/ml)	**r** **p**	- 0.372 0.018	-0.284 0.05
ACR (mg/g)	**r** **p**	0.836** <0.001	0.128 0.432
Fasting Insulin (µ U/L)	**r** **p**	0.511** <0.001	0.671** <0.001
Fasting Glucose (mg/dl)	**r** **p**	0.445* 0.004	0.135 0.406
HOMA-IR	**r** **p**	0.651** <0.001	0.651** <0.001

DN: diabetes with nephropathy, r: Pearson Correlation, p: Significance, ACR: albumin – creatinine ratio, BMI: body-mass index, HOMA-IR: Homeostatic model assessment for insulin resistance *: p<0.05, **: p<0.001

Table 3. ROC Curve analysis for miRNA 181a, IL-6, and IL-18 in the early diagnosis of diabetic nephropathy in diabetic patients.

Variable(s)	AUC	Cut off value	Sensitivity	Specificity
serum IL-6 pg/ml	.963	≥ 4.8	97.5 %	95%
Urine IL-6 pg/ml	.994	≥78.5	97 %	95 %
Serum IL-8 pg/ml	1.000	≥205	97 %	100 %
Urine IL-8 pg/ml	1.000	≥ 88	100 %	100%
Serum miR-181a	.786	≥ 2.45	75 %	62.5 %

4. Discussion:

Diabetic nephropathy, a complication of T2DM, is a major cause of end-stage renal disease eventually requiring dialysis or even renal transplantation thus requiring urgent diagnosis and management [4].

This study sought to update knowledge on the role of miRNAs, more specifically miRNA 181a, in the pathogenesis of DN and to propose new diagnostic, prognostic, or therapeutic approaches for the management of DN that would use or target miRNA 181a. miRNA 181a was selected as it hasn't been, to the best of our knowledge, thoroughly researched into its applicability to the management of DN.

In the current research, miRNA 181a mean levels exhibited a significant increase in diabetic patients with DN and those without DN compared to those in control patients. The highest levels were observed in diabetic patients with DN. These results indicated a substantial relationship between miRNA 181a and the development and pathophysiology of diabetic nephropathy.

This association was suggested by **Zhang, Wu et al. (2018)** shown to play an antifibrotic role through induction of fibroblast apoptosis and reduction of collagen type 1 expression. Further analysis revealed that miRNA 181-a targets the 3' untranslated region of fibroblast growth factor-1 in DN. **Zhang, Wu et al. (2018)** determined that overexpression of miRNA 181a could reduce synthesis of fibroblast growth factor 1 and its associated proteins thus contributing to renal fibrosis in DN.

Furthermore, **Liang and Xu (2020)** determined that elevated miRNA 181a as a result of hyperglycemia is associated with the induction of cellular apoptosis. As a result, a new theory for DN in T2DM patients hinted that miRNA 181a might be implicated in Renal pathological changes.

In addition, **Liu, Chen et al. (2022)** deduced from their research that, in the context of renal inflammation in DN, miRNA 181a demonstrates anti-inflammatory effects by targeting TNF-α.

On the contrary, **Zha, Qu et al. (2019)** found that the influence of maternally expressed gene (MEG3) RNA through acting as a sponge for miRNA 181a led to downstream effects that promoted fibrosis and inflammatory response in DN.

The current investigation also determined a strong positive correlation between miRNA 181a levels and fasting insulin, fasting glucose, & HOMA-IR levels in diabetics patients. The current outcome is further supported by **Williams and Mitchell (2012)** who found that elevated glycemic levels where associated with increased expression of miRNA 181a. **Mononen, Lyytikäinen et al. (2019)** as well determined that elevated miRNA 181a expression appeared to activate a

pathway leading to insulin resistance in T2DM through negatively correlating with genes directing the transfer of GLUT4 to the plasma membrane in the insulin signaling pathway which support the correlation reached in this research between elevated miRNA 181a levels and insulin laboratory results.

Also, according to **Zhou, Li et al. (2012)** miR-181a could improve hepatic insulin sensitivity and glucose homeostasis, thereby offering a new therapeutic approach for treating insulin resistance and type 2 diabetes which further supports the theory that miRNA 181a plays a role in the pathogenesis of DM and its complications, most importantly DN.

In the current investigation, it was discovered that patients with macroalbuminuria had mean levels of miRNA 181a that were higher than those with microalbuminuria in diabetic patients with nephropathy. Additionally, in diabetic patients with nephropathy, there was a strong positive correlation between miRNA 181a and urine ACR.

According to **Gross, de Azevedo et al. (2005)** who found that staging of diabetic nephropathy was dependent on the categorization of the degree of albuminuria exhibited by diabetic patients thus establishing a clear positive correlation in the present work between miRNA 181a levels and the degree of albuminuria in diabetic nephropathy revealed the association of miRNA 181a with the subsequent DN staging, and severity. Not only were miRNA 181a levels starkly distinguishable among diabetic patients with DN and DM patients without nephropathy but also distinguishable among macro and micro albuminuria patients with DN.

The current study observed that the serum miRNA 181a showed high considerable sensitivity and specificity with extremely accurate diagnosis of diabetic nephropathy by using the ROC curve analysis for the early detection of diabetic nephropathy in diabetes mellitus. These findings which suggested utilization of serum miRNA 181a in the early diagnosis and progression monitoring of diabetic nephropathy in diabetes mellitus have not been previously studied.

In the current study it was determined that diabetic patients generally exhibited higher BMIs than control patients. Furthermore, diabetic patients with diabetic nephropathy exhibited the highest BMIs which suggests that obesity can be associated with DN. This outcome was further supported by **Tziomalos and Athyros (2015)** being among the risk factors contributing to the incidence of DN.

Kidneys affection in patients with diabetes mellitus is due to not only hyperglycemia, advanced glycosylation products but also the effect of proinflammatory cytokines. Both IL-6 and IL-18 are involved in multiple renal pathology e.g IgA nephropathy, lupus nephritis and diabetic nephropathy [17].

Diabetic nephropathy assessment is mandatory to give an effective reference for clinical immunotherapy researchers, who work to diagnose and improve the renal function in these chronic diseases [18].

Assessment of the serum and urinary levels of IL-6, IL-18 in our study were done for the control group to know the normal values and for all diabetic subjects with and without nephropathy. Despite urine ACR measurement is the classical predictor for DN, pointing to the level of renal impairment and glomerular filtration membrane affection, urine protein can be affected by dietary habits and metabolic consumption of these patients. Therefore, urine ACR measurement is considered as a deficient tool for early recognition and prediction of DN. In contrast to IL-6 and IL-18 are significantly altered in the early stages of any kidney injury [19].

Our results revealed a highly significant different in serum and urinary levels of IL-18, IL-6 comparative between both patients and control subjects. Meanwhile, serum and urinary IL-6 and IL-18 levels were higher in diabetic patients with nephropathy as compared with those without nephropathy. **Wu R et al., 2018** research is in accordance with our results suggesting the involvement of IL-6 and IL-18 in the progression of diabetic complications.

Also other researchers; **Cui J et al., 2020, Ishikado et al., 2020 and Zhang et al., 2021** agreed with our results reporting that both IL-6 and IL-18 in serum and urine are associated with microalbuminuria and clinical albuminuria in diabetic patients. These researchers also stated that their levels are increased compared with diabetic patients who are without albuminuria. Therefore, serum and urinary levels of IL-18, and IL-6 may have great pathogenic roles in diabetic nephropathy and are good predictors for urinary albumin excretion in diabetes mellitus. **Hadeel et al., 2021** stated that, besides the metabolic and hemodynamic factors, it is important to consider the great participation role of inflammatory cytokines on both pathogenesis and development of diabetic nephropathy.

Hong., 2022 reported that there are major changes in the cellular proinflammatory cytokines e.g IL-18, and IL-6 in patients with diabetes as they play a significant critical role in the pathogenesis of diabetic complications through multiple biochemical and hemodynamic pathways.

Emanuela et al., 2017 proved that the expression of IL-6 and IL-18 were increased more in patients with diabetic nephropathy and with overall disease exacerbation. This research explained that by the ability of both IL-6 and IL-18 to activate of numerous inflammatory signaling pathways; promoting the disruption of both the charge barrier and physiological barrier of the renal filtration

membrane allowing the leakage of urinary protein.

Serum and urinary markers of IL-6 and IL-18 had a higher sensitivity and specificity compared to miRNA181a.

LaPierre et al., 2017 stated AUC cutoff values of both miR-181a, miR-144 expression levels had a considerable specificity and sensitivity, as these diagnostic biomarkers for diabetes are varying according the glycemic control status of the patients.

Meanwhile, a study done by **Anker et al., 2012** concluded that there are a great anti-inflammatory actions of miR-181a by targeting and blocking the TNF-α in the context of renal inflammation in DN.

This were illustrated by **Tang et al., 2020** who stated that the increased serum levels of IL-18 and IL-6 in diabetes mellitus didn't reflect only the chronic inflammation but it consider apart from atopic and autoimmune conditions.

This was in concordance with the study of **Tuttle et al., 2022** who stated that IL-6 and IL-18 are the most abundant cytokines associated with metabolic disorders. It causes major toxic effects on beta cells by receptor-mediated signaling inducing cell apoptosis.

Regarding the Pearson Correlation, our results showed there is a important significant negative correlation between miRNA 181a and serum IL-6. This explained by **Sheinerman et al., 2018** who stated that increased levels of miR-181a could be seen in both inflammatory conditions, beside the miR-181a has a role as an anti-inflammatory miRNA, likely depending on the cellular and physiological context of the patients.

Moreover, **Karlsson et al., 2020** reported that miR-181a play an important significant role in the aging process; identifying it as multimorbidity-associated miRNA. Therefore, it can be used as a biomarker of health status because it has different effects at different ages.

5. Conclusion and Recommendations :

Diabetic nephropathy patients had higher serum levels of miRNA 181a. As opposed to non-diabetics, all diabetic patients had higher levels. Furthermore, patients with diabetic nephropathy who had macroalbuminuria had higher levels of miRNA 181a than those who had microalbuminuria. The levels of miRNA 181a in the diabetic nephropathy group showed a positive correlation with BMI, serum fasting insulin, serum fasting glucose, HOMA IR, and urine ACR. Serum miRNA 181a demonstrated a significant sensitivity and specificity with an incredibly accurate diagnostic of diabetic nephropathy for the early detection of diabetic nephropathy in diabetes mellitus. According to all current research, miRNA 181a may serve as a biomarker for diabetic nephropathy diagnosis and prognosis. The study recommends that the same study be conducted upon a larger number of patients of different ages and genders,

evaluating other types of miRNAs that might be involved in Diabetic nephropathy. Serum and urinary IL-6, IL-18 levels are closely matched to renal injury. Their elevated levels reveal poor prognosis in patients with diabetes mellitus. Combined assay is more valuable for assessing patients' condition and prognosis. In addition, serum and urinary IL18 and IL-6 levels can be used as markers for diagnosing and follow up the progression of DM.

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