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Original Article

Il4 and Il6 Gene Expression among Egyptians with Type 2 Diabetes and Chronic Kidney Disease

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ABSTRACT

Background: The gene expression of several cytokines can affect the clinical outcome of type 2 diabetes mellitus (T2DM) with chronic kidney disease (CKD). This study aim was to assess the effect of IL-4 and IL-6 gene expression in T2DM cases with CKD.

methods: This case control study was performed on 78 subjects; they were allocated into three groups; Group (1) normal individuals, Group (2) T2DM cases without CKD, and Group (3) T2DM cases with CKD having 26 subjects each. IL6 and IL-4 gene expression of all cases were assessed by real-time PCR.

Results: A notable positive association was detected between IL-6 and body mass index, duration of diabetes, blood pressure, blood glucose levels, HbA1c, kidney function measures like albumin/creatinine ratio, creatinine, urea, and estimated GFR, lipid levels like total cholesterol, LDL-C and triglycerides ($p<0.001$) while there was a strong negative relationship between IL-6 and serum albumin and IL-4 levels ($p<0.001$). However, there was a strong negative correlation between interleukin-4 (IL-4) levels and body mass index, duration of diabetes, blood pressure, glucose levels, HbA1c, kidney dysfunction markers, total cholesterol, LDL-C, triglycerides ($p<0.001$). On the other hand, a positive correlation between interleukin-4 (IL-4) levels and serum albumin, estimated GFR, and HDL-C was found ($p<0.001$).

Conclusions: IL6 and IL4 expression could contribute to CKD progression in cases with T2DM. Screening genes that influence the risk of CKD and T2DM could eventually allow the identification of individuals at risk, further multicenter studies are needed to confirm these findings.

Keywords: IL4; IL6; gene expression; type 2 diabetes; chronic kidney disease

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder causing atypical protein, lipid, and hyperglycemia, ranking as the seventh-leading cause of death globally [1]. It is also a growing public health concern in Egypt, with an estimated 9.8 million in 2019, expected to reach 16.9 million by 2045 [2].

Chronic kidney disease (CKD) is a permanent kidney dysfunction, diagnosed utilizing estimated glomerular filtration rate (eGFR) from serum creatinine with two or more readings below 60 mL/min [3]. Between 2009 and 2019, the burden of CKD in Egypt increased by 36%, ranking fifth among leading causes of death. [4]. Diabetic kidney disease (DKD) is a common DM microvascular consequence. It is distinguished by reduced eGFR and raised urinary albumin excretion due to hypertrophy, elevated basement membrane thickness, and protein accumulation in the extracellular matrix [5,6]. Kitada and Koya [7] classified "CKD with diabetes" as "DKD", "non-DKD (NDKD) with diabetes", or "combined disease of DKD and NDKD". They recommend DKD be classified as "CKD with diabetes" for clinical treatment, considering the hazy distinction between the two. Numerous studies indicate that DKD is genetically influenced, with familial aggregation indicating a significant role for genetic factors in its etiology [8]. In addition, chronic inflammation is a

common cause of DKD, which in turn leads to ESRD [9].

Cytokines, including anti-inflammatory and pro-inflammatory, have been identified as crucial in various physiological processes in DKD [10]. IL-6, initially a pro-inflammatory cytokine, now functions as a hormone-like cytokine, promoting B cell development, activating T cells, and controlling the acute-phase response [11]. IL6 is generated by numerous cell types such as lymphocytes, macrophages, fibroblasts, monocytes, and endothelial cells [12]. Various studies proved that diabetic kidney disease (DKD) patients have high serum IL-6 levels affecting the glomerular basement membrane breadth leading to diabetic glomerulopathy [13]. They also have IL-6 mRNA expression on glomerular and interstitial cells, potentially causing renal damage and mesangial proliferation [14].

On the other hand, IL-4, a member of the immunological recognition-induced lymphokines family, has a crucial effect in the immune system by promoting Th2 and Th1 cell development, enhancing proliferation, and controlling B cell differentiation. It also affects other cell types, including lymphoid cells, and can induce differentiation and apoptosis [15–17].

IL-4 and its signaling pathways have been connected to the progression of autoimmune and allergic disorders, with autoimmune disorders relying on Th1 cells and monocyte-generated cytokines, and allergy diseases relying on Th2 cells and associated cytokines.IL-4 has been suggested to have an anti-inflammatory function in autoimmune disorders, as shown in rheumatoid arthritis and DM mouse models [18–20].

Arababadi et al. [21] and Neelofar [22] found significant differences in IL4 -590C/T SNP genotype and allele frequencies between T2DM cases with DKD and normal controls. Heterozygous genotypes were risk factors, while Cilenšek et al. found no link between it and proliferative Diabetic retinopathy in Caucasians [23].

The current study aims to evaluate the connections between IL-6, IL4 gene expression, and renal impairment in cases with T2DM with CKD.

METHODS

This study was a case-control study performed at Internal medicine department and Medical Biochemistry and Molecular Biology Departments, Faculty of Medicine, Zagazig University, Egypt. Written informed consent was obtained from all participants, the study was approved by the research ethical committee of Faculty of Medicine (IRB#10923/5-7-2023), Zagazig University. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Inclusion Criteria: Seventy-eight participants were enrolled, and they were allocated into three groups; Group (1) (n=26) healthy individuals, Group (2) (n=26) T2DM cases without CKD, and Group (3) (n=26) T2DM cases with CKD. They were 32 males and 46 females, age (25-75 years). T2DM cases with or without CKD were chosen using the characteristics developed by the American Diabetes Association [ADA, 2023] [24]: Fasting blood glucose values ≥ 126 mg/dL, 2-hour postprandial blood glucose values >200 mg/dL, HbA1c > 6.5% in individuals with characteristic symptoms of hyperglycemia, and atypical oral glucose tolerance tests. CKD cases are characterized according to the consensus obtained by ADA [24]: GFR < 60 mL/min, or albumin creatinine ratio (ACR) > 30 mg/g for more than 3 months. GFR was calculated with measured serum creatinine value by the following formula: GFR= (140 – Age) x Mass (kg) x [0.85 if female] / 72 x [Serum Creatinine (mg/dL)].

Exclusion Criteria: We excluded all cases with the following conditions; Patients aged below 25 or above 75 years, patients with T1DM and other metabolic disorders except T2DM, patients with major diabetes complications including leg amputation, and uremia under dialysis, history of hospitalization in the previous year as the result of cerebrovascular accident (e.g. stroke), myocardial infarction, and heart failure, pregnant women, and patients who refused to give consent and lack of cooperation.

All participants underwent a complete history, clinical assessment, and laboratory examinations, which included measurements of serum total cholesterol (TC), triglycerides (TG), LDL-c, HDL-c, creatinine, serum urea, glycated hemoglobin (HbA1c), fasting and postprandial blood glucose, ACR, and expression of the IL6, IL4 genes evaluated via real-time PCR.

After 6-8 hours after the last meal, 8 ml of blood was obtained and split as follows. 1 ml was transferred to an EDTA tube for analysis of glycated he HbA1c. 1 ml was put in sodium fluoride tube for measurement of blood glucose. 2 ml was transferred to a plain tube, allowed to clot at room temperature, and then centrifuged for 10 min. at 4000 rpm. The

clear supernatant preserved at -80 °C for colorimetric analysis of serum urea, creatinine, TC, TG, and HDL-c. LDL-c was evaluated from TC, HDL-c, and TG using the Friedewald algorithm.

The rest of the 2 ml of blood were put to an EDTA tube and employed for gene expression analysis. Trizol was used to extract IL-6 and IL-4 mRNA, which was then kept at -20°C for gene expression. The first-morning urine samples were taken in a completely sterile environment. The pH was adjusted to 7.0.

Assay of IL-6, IL-4 gene expression by RT-PCR

Complementary DNA (cDNA) was formed from mRNA employing the reverse transcription PCR (rt-PCR) system from HiSenScript™ RH (-) cDNA synthesis kit (iNtRON Biotechnology, Korea). The rt-PCR was carried out at 45 °C for 60 min and 85 °C for 10 min. The second step of RT-PCR was done on Mx3005P Real-Time PCR System (Agilent Stratagene, USA) using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Cat. # P725 or P750) (Enzynomics, Korea) following the manufacturer's instructions. The PCR cycling conditions included an initial denaturation at 95°C for 12 min followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Relative expression levels of cytokine mRNAs were normalized by GAPDH as a housekeeping gene and calculated by the 2ΔΔCt method.

Using Primers sequences for IL4 mRNA:

Forward: CTCCAAGAACACAACTGAGAA, Reverse: ACTCTGGTTGGCTTCCTTCA,

Primers sequences for IL6 mRNA: Forward: CCACCGGGAACGAAAGAGAA, Reverse: TCCTGGGGGTATTGTGGAGA, Primers of GADPH (endogenous control) Forward: GACAGTCAGCCGCATCTTCT, Reverse: GCGCCCAATACGACCAAATC.

STATISTICAL ANALYSIS

We used SPSS version 24 (Spss Inc, Chicago, ILL Company) to tabulate and evaluate the data that we collected. There was a presentation of percentages and numbers for the categorical data. Categorical variables were analyzed using a chi-square test (X2). The median, range, and mean ± standard deviation where the ways quantitative data was presented. The two groups' normally distributed variables were analyzed using the student "t" test. Nonparametric variables were evaluated for correlation using Spearman's correlation coefficient (rho). To examine the potential for association, regression analysis was employed. This work's stated level of significance was 0.05, with P <0.05 considered significant.

RESULTS

There was no statistically significant difference between Cases and Controls regarding age (years) and Gender, there were statistically significant increase in BMI among cases than controls (31.95 versus 26.95 respectively, p. value <0.001). Mean value of DBP, FBS, 2 HR PPBG, HbA1c, Alb/ Creat ratio, were significantly higher among cases than controls (p<0.001). Mean value of Estimated GFR was significantly lower among cases than controls (p<0.001) (Table 1).

In the comparison between Cases and Controls regarding IL-6 and IL-4, the relative mRNA expression of IL6 was statistically significantly higher in patients with diabetes (10.09± 2.65) than normal individuals (1.01± .115) (Table 2) and (Figure1). The relative mRNA expression of IL4 was statistically significantly lower in patients with diabetes (.365± .158) than normal individuals (1.01± .111) (Table 2) and (Figure 2).

The highly significant expression of IL-6 gene was found among patients with diabetes with CKD (12.10 ± 2.02) than diabetes without CKD (8.08 ± 1.38) than normal individuals (1.006 ± 0.115) (Table 3) and (Figure 1).

The Correlation between IL6 and other variables showed that there are significant positive correlations between IL-6 and body mass index, duration of diabetes, blood pressure, blood glucose levels, HbA1c, kidney function measures like albumin/creatinine ratio, creatinine, urea, and estimated GFR, lipid levels like total cholesterol, LDL-C and triglycerides (p<0.001). Strong negative correlations between IL-6 and serum albumin as well as IL-4 levels (p<0.001). (Table 4)

However, there was a strong negative correlation between interleukin-4 (IL-4) levels and body mass index, duration of diabetes, blood pressure, glucose levels, HbA1c, kidney dysfunction markers, total cholesterol, LDL-C, triglycerides (p<0.001). Positive correlations between interleukin-4 (IL-4) levels and serum albumin, estimated GFR, and HDL-C (p<0.001). (Table 5).

Table (1): Comparison between Cases and Controls regarding baseline data.

			Cases	Controls	t. test	P. value
Age (years)	Mean ± SD		53.75± 9.52	51.62± 8.69	.960	.340
Gender	Female	No.	30	16	X^2 .106	.745
		%	57.7%	61.5%		
	Male	No.	22	10		
		%	42.3%	38.5%		
BMI	Mean ± SD		31.95± 4.88	26.95± 1.90	5.018	<0.001
Duration of DM (yrs)	Mean ± SD		10.16± 4.04	-	12.767	<0.001
SBP (mmHg)	Mean ± SD		122.48± 4.38	119.88± 3.39	2.647	.010
DBP (mmHg)	Mean ± SD		80.17± 4.45	74.19± 3.69	5.896	<0.001
FBS(mg/dl)	Mean ± SD		181.65± 42.06	102.08± 5.38	9.575	<0.001
2 HR PPBG(mg/dl)	Mean ± SD		284.73± 41.52	122.96± 7.36	19.649	<0.001
HbA1c(%)	Mean ± SD		8.16± 1.08	4.91± .319	14.901	<0.001
Alb/ Creat ratio (mg/g)	Mean ± SD		256.05± 313.22	7.82± 4.37	4.027	<0.001
Estimated GFR(ml/mnt/1.73m^2)	Mean ± SD		53.60± 40.07	103.15± 6.72	-6.242	<0.001

BMI: Body mass index, DM: Diabetes mellitus, SBP: Systolic blood pressure, FBS: Fasting blood sugar, 2HR PPBG: 2 hours post prandial blood glucose, HbA1c: Hemoglobin A 1c, A:b/ Create: Albumin/ Creatinine ratio, GFR: glomerular filtration rate

Table 2. Comparison between cases and controls regarding IL-6 and IL-4

		Cases (n = 52)	Controls (n = 26)	t. test	P. value
IL-6 gene expression	Mean ± SD	10.09± 2.65	1.01± .115	17.366	<0.001
IL-4 gene expression	Mean ± SD	.365± .158	1.01± .111	-18.594-	<0.001

Table 3. Comparison between type 2 diabetic patients with CKD, type 2 diabetic patients without CKD and controls regarding IL-6, IL-4

		Type 2 diabetic patients with CKD	Type 2 diabetic patients without CKD	Controls	t. test	P. value	LSD
IL-6 gene expression	Mean ± SD	12.10± 2.02	8.08± 1.38	1.006± .115	406.443	<0.001	P1<0.001 P2<0.001 P3<0.001
IL-4 gene expression	Mean ± SD	.216± 057	.514± .038	1.01± .111	724.827	<0.001	P1<0.001 P2<0.001 P3<0.001

Table 4 : Correlation between IL-6 and other variables.

Correlation	Pearson's correlation	
	R	P
Age(yrs) * IL-6	.176	.123
BMI(kg/m^2) * IL-6	.533	<0.001
SBP (mmHg) * IL-6	.387	<0.001
DBP (mmHg) * IL-6	.630	<0.001
FBS(mg/dl) * IL-6	.719	<0.001
2 HR PPBG(mg/dl) * IL-6	.841	<0.001
HbA1c(%) * IL-6	.741	<0.001
alb/ creat ratio (mg/g) * IL-6	.747	<0.001
s. albumin(g/dl) * IL-6	-.667-	<0.001
S. creat(mg/dl) * IL-6	.709	<0.001
bl urea(mg/dl) * IL-6	.717	<0.001
esti GFR(ml/mnt/1.73m^2) * IL-6	-.782-	<0.001
T.cholesterol(mg/dl) * IL-6	.620	<0.001
HDL-C (mg/dl) * IL-6	-.521-	<0.001
LDL-C (mg/dl) * IL-6	.602	<0.001
TG (mg/dl) * IL-6	.807	<0.001
IL-4 * IL-6	-.942-	<0.001

Table 5: Correlation between IL-4 and other variables

Correlation	Pearson's correlation	
	R	p
Age(yrs) * IL-4	-.166-	.147
BMI(kg/m^2) * IL-4	-.507-	<0.001
SBP (mmHg) * IL-4	-.400-	<0.001
DBP (mmHg) * IL-4	-.657-	<0.001
FBS(mg/dl) * IL-4	-.701-	<0.001
HbA1c(%) * IL-4	-.724-	<0.001
s. albumin(g/dl) * IL-4	.702	<0.001
S. creat(mg/dl) * IL-4	-.750-	<0.001
bl urea(mg/dl) * IL-4	-.752-	<0.001
esti GFR(ml/mnt/1.73m^2) * IL-4	.809	<0.001
HDL-C (mg/dl) * IL-4	.484	<0.001
LDL-C (mg/dl) * IL-4	-.592-	<0.001
TG (mg/dl) * IL-4	-.807-	<0.001
PLT (x103/uL) * IL-4	-.078-	.495
MPV (fL) * IL-4	-.745-	<0.001
PDW (fL) * IL-4	-.625-	<0.001
PCT(%) * IL-4	-.346-	.002

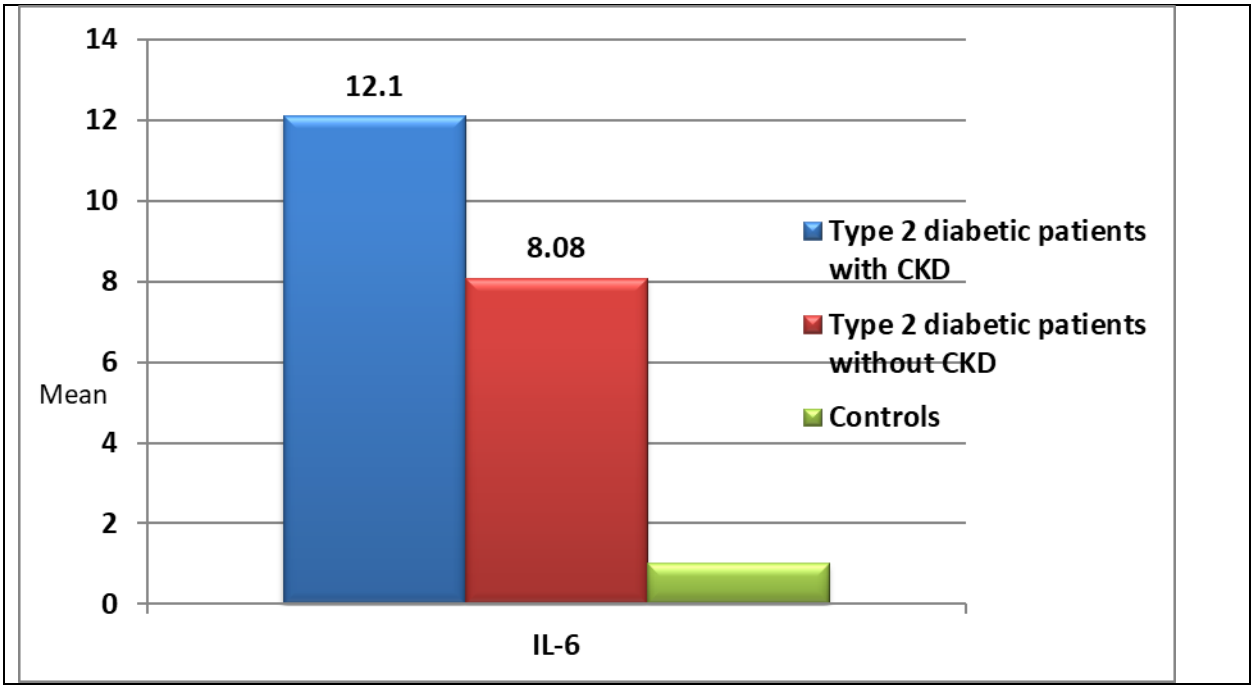

Figure. 1: Comparison between Type 2 diabetic patients with CKD, Type 2 diabetic patients without CKD and Controls regarding IL-6.

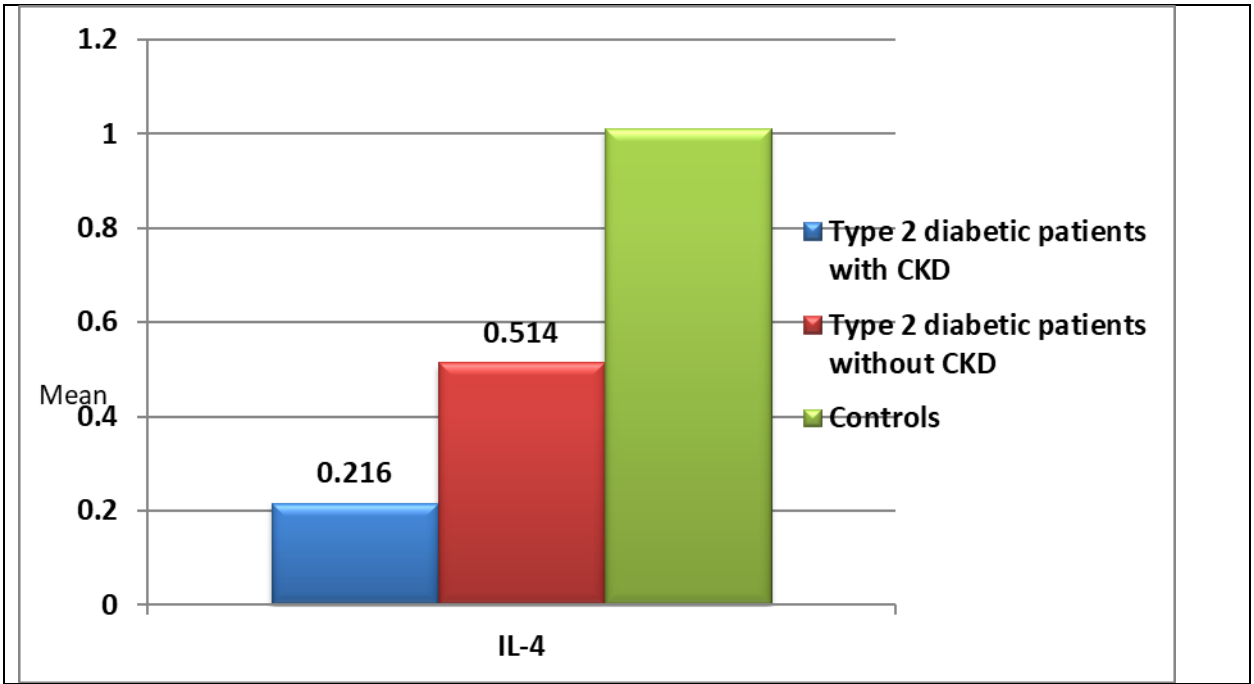

Figure. 2: Comparison between Type 2 diabetic patients with CKD, Type 2 diabetic patients without CKD and Controls regarding IL-4.

DISCUSSION

Diabetes mellitus, a metabolic disease-causing hyperglycemia due to insulin defects, is predicted to affect 366 million people globally by 2030, affecting nearly 5% of the global population [25].

CKD is a common DM microvascular consequence that could lead to dialysis need. Not all DM develop kidney disease, however, around one-third of DM cases eventually acquire this condition [26].

Research indicates that inflammation in T2DM may contribute to the progress and advancement of

DKD, as elevated levels of inflammatory cytokines are found in cases with both conditions [27]. This raises questions about the extent of inflammatory mechanisms contributing to renal injury secondary to diabetes, with higher cytokine expression linked to severity [28].

Interleukin 6, a pro-inflammatory cytokine, controls the adhesion of cells and chemotactic molecule synthesis, producing other cytokines that enhance the response of inflammation [29,30]. Moreover, It promotes mesangial cell growth and matrix expansion, and its expression is favorably connected to the pathogenic score and prognosis of DKD [31].

Interleukin 4 is an anti-inflammatory cytokine that inhibits cell immunity, raising concerns about its potential in the renal damage development in DM individuals, as it is crucial in inhibiting autoimmunity and inflammation [32,33].

Interestingly, this is the first study which was designed to assess IL-6 gene and IL-4 gene expression in T2DM cases with CKD. Our study showed highly significant expression of IL6 gene among patients with diabetes with CKD (12.10 ± 2.02) than diabetes without CKD (8.08 ± 1.38) than normal individuals (1.006 ± 0.115). However, insignificant expression of IL4 gene was found among patients with diabetes with CKD (0.216 ± 0.057) than diabetes without CKD (0.514 ± 0.038) than normal individuals (1.01 ± 0.111).

Our findings supported by Wu et al. [34], who demonstrated activated pro-inflammatory cytokine IL-6, but not IL-4 production in patients with DKD. Our study contradicts Neelofar et al. [22] report, which found IL-4 expression higher in CKD cases compared to normal individuals with no variance between DM cases without CKD and control cases. However, the study found a substantial variance in the expression of IL-6 gene between control group individuals and CKD cases with no remarkable variances between DM cases without CKD and normal individuals in the north Indian population.

There were various studies concerned with IL6 production in DKD, Suzuki et al. [14] assumed that IL-6 is expressed in glomerular resident cells in DKD, and its expression is increased in renal tissues. Also, Shikano et al. [35], proved that increased plasma IL-6 concentrations are correlated with atherosclerotic parameters, and diabetic kidney disease. These studies highlight the importance of understanding the association between IL-6 and complications in DM patients.

Reports revealed that T2DM cases with renal function deterioration have lower serum IL-4 values compared to the normal individuals [36]. In another study IL-4 levels are elevated in diabetic cases with DKD compared to control group [37]. In addition to the study of Arababadi et al. [21] in south-east Iranian patients which studied polymorphisms at -590 region of IL-4 in T2DM cases with CKD showed a substantial variation between normal controls and T2DM with CKD.

Mittal et al. [38] studied association of IL-2, IL-4, and IL-6 polymorphisms with the risk of ESRD compared to normal participants. IL-4 and IL-6 were shown to be strongly related with ESRD. The researchers next looked at the combined influence of IL-4 and IL-6 genotypes on the probability of ESRD. Cases with IL-4low-IL-6 elevated levels were at a greater risk.

The current study provided a possible insight about IL4 and IL6 gene expression among Egyptians at Zagazig University Hospitals with type 2 diabetes and chronic kidney disease.

Limitations:

Since this study is a small-scale, case control study involving only 72 participants, there is bound to be some selection bias in the results. Adjusting for all potential confounders, such as the unknown intervention outside of Zagazig University Hospitals, is challenging. Second, our findings only apply to the Egyptian population. Further studies are needed with a large sample size that could produce significant results. Also, additional further research, such as prospective cohort studies or randomized controlled trials, is needed to establish effect of IL-4 and IL-6 gene expression in T2DM cases with CKD.

Author contribution: All authors contributed to the study. AFA was responsible for selecting the subject, BAM, EAM was accountable for laboratory revisions and analysis, AEAS was responsible for data collection, statistical analysis, and initial writing, and MGH was responsible for collecting the data of the studied cases and all shared for the formulation of the study design, editing, revision, and preparation of the final manuscript.

CONCLUSIONS

We concluded that IL6 and IL4 expression could contribute to CKD progression in cases with T2DM. Screening genes that influence the risk of CKD and T2DM could eventually allow the identification of individuals at risk, further multicenter studies are needed to confirm these findings. Genetic investigations of CKD and T2DM will also provide additional knowledge about their root cause, characterize heterogeneity within DM case

subgroups, and give rise to new treatments and prevention strategies.

No potential conflict of interest was reported by the authors.

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