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RESEARCH ARTICLE

Association of serum and urinary tumor necrosis factor-alpha (TNF-α) with albuminuria in diabetic nephropathy.

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Abstract

Background:
Diabetic nephropathy is the leading cause of end stage renal disease in diabetes and albuminuria is the most common indicator of progressive glomerular damage. Several markers of diabetic nephropathy were discovered but not sensitive for its early detection. **Aim of the study**: was to evaluate the levels of serum and urinary TNF-α in patients with diabetic nephropathy and to clarify its possible relationship as well as other parameters to different stages of albuminuria in diabetic patients.
Methods: This cross-sectional study was conducted on 100 patients with diabetes and diabetic nephropathy and seventy healthy control subjects. The enrolled patients were divided into three groups according to the level of albuminuria (49 normoalbuminuric patients, 30 microalbuminuric patients, and 21 macroalbuminuric patients).Tumor necrosis factor-alpha was measured in all subjects using immunoenzymatic ELISA kits.
RESULTS: Type 2 diabetic patients (T2DM) with diabetic nephropathy had significantly higher values of clinical and biochemical parameters of metabolic syndrome. Serum and urinary TNF-α levels were significantly higher in patients with type 2 diabetes compared to control group. Moreover when we stratified diabetic nephropathy patients according to albumin excretion ratio, we found that patients with macroalbuminuria had higher levels of serum and urinary TNF-α than normoalbuminuric and microalbuminuric patients and for further evaluation, serum and urinary TNF-α levels in type 2 diabetic patients were positively correlated to systolic and diastolic blood pressure, total cholesterol, LDL.c, WBC's count, fasting blood glucose, HbA1c, uric acid and albumin excretion ratio. **Conclusion:** Serum and urinary TNF-α level were elevated in Type 2 diabetes mellitus patients with diabetic nephropathy with positive correlation between serum and urinary levels. Their levels were significantly positively correlated to albuminuria and may be used as a strong predictive factor for progression of glomerular disease in type 2 diabetes.

Introduction:-

Increase in prevalence of diabetes has been associated with high numbers of patients with diabetic nephropathy (DN) which affects 30–40 % of the patients with type 1 and type 2 diabetes mellitus. Diabetic nephropathy (DN) is the major microvascular complication of diabetes mellitus (DM), the most common cause of end stage renal disease and associated with increased morbidity and mortality in diabetic patients [1]. The pathogenesis of DN is multifactorial includes; metabolic, genetic, environmental and inflammatory factors which are contributing to the progression of diabetic nephropathy [2].

415 million people have diabetes in the world and more than 35.4 million people in the MENA Region (the Middle East and North Africa region) and by 2040 this will rise to 72.1 million. There were over 7.8 million cases of diabetes in Egypt in 2015 **(International diabetes federation IDF, 2015).** In our Egyptian population, diabetic nephropathy is one of the most common causes of morbidity and mortality.

The management of DN has a significant economic burden on health care budgets [3]. Subjects with type 2 diabetes, particularly those with high urinary albumin concentrations are at high risk of renal disease [4].

TNF-α is a pleotropic cytokine that is produced by many cell types including leukocytes, adipocytes and endothelial cells [5]. ADAM-17 release TNF-α into circulation as a functional 17kDa soluble form. TNF-α acts through its receptors; TNF-α receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2). In plasma, TNF-α may be either free or bound to circulating TNF-R1 and TNF-R2 [5,6,7].

In response to stimulating factors such as inflammation, TNF-α and its receptors are expressed and stimulate the production of other cytokines as (IL-8), acute phase proteins, growth factors and chemokines [monocyte chemo attractant protein (MCP)-1, and macrophage-colony stimulating factor (M-CSF)] by adjacent cells [8]. Moreover, TNF-α up regulates the expression of adhesion molecules on leukocytes and endothelial cells that mediate adhesion of monocytes, lymphocytes and granulocytes to activated endothelium and their subsequent migration [8,9]. TNF-α appears to have a direct apoptotic and cytotoxic effect on glomerular cells [8,10].

It is important to screen patients at risk for diabetic nephropathy for early detection of progressive renal disease as early intervention can slow loss of kidney function and improve patient outcomes. In clinical practice, the most commonly used markers of renal disease and its progression are serum creatinine, estimated glomerular filtration rate and proteinuria or albuminuria. Unfortunately, they are all insensitive. To the best of our knowledge, only few researches studied the association between albuminuria and TNF-α in diabetic patients and results were controversial.

Aim of the study:-

To evaluate serum and urinary tumor necrosis factor-alpha levels in T2DM and whether their levels are associated with severity of albuminuria in diabetic nephropathy patients or not.

Subjects and methods:-

A total of 170 unrelated individuals were included in this study; 100 T2DM patients and 70 healthy volunteers. Age, sex and ethnic origin of control subjects were matched with the patients. Control subjects had normal fasting blood glucose and had no evidence of microalbuminuria or hypertension. All cases were recruited from cases admitted and followed in diabetes, endocrinology and renal outpatient clinics of Internal Medicine department, Faculty of Medicine, Zagazig University hospitals, in the period from Jan 2015 to the end of the same year.

T2DM patients were diagnosed according to their fasting blood glucose based on the American Diabetes Association criteria reported in 2015.The T2DM patients were then stratified into three subgroups according to their urinary albumin excretion ratio (AER) in 24 h urine collections. Normal AER was defined as an AER persistently <20 μg /min or <30 mg/24 h, microalbuminuria as an AER between 20 and 200 μg /min or 30 and 300 mg/24 h and macroalbuminuria as an AER>200 μg /min or >300 mg/24 h in at least two out of three urine collections. All positive cases reexamined after 3 months to confirm diagnosis and for follow up. Patients with normal AER were required to have neither anti-hypertensive medication nor signs of cardiovascular disease.

All patients who had liver, thyroid, any active inflammatory diseases, received insulin therapy for at least 2 years or on hemodialysis were excluded as well as patients with history of drug intake including antibiotics, non-steroidal anti-inflammatory drugs, corticosteroids or cytotoxic medications at the time of the study.

All patients were subjected to thorough medical history taking and full physical examination including blood pressure and anthropometric variables including body mass index (BMI) and waist/hip ratio.

The estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) equation: eGFR (mL/min per 1.73m2)=$186 * SCr^{-1.154} * Age^{-0.203} * 0.742$ (if female) [11].

Blood samples and Biochemical measurements:-
Blood samples were withdrawn from all subjects after an overnight fast and divided into 3 portions: 1 ml of whole blood was collected into tubes containing EDTA for HbA1c; 1 ml of whole blood was collected into tubes containing fluoride for fasting blood glucose and serum from remaining part of the sample was separated immediately and stored at -20^0C until analysis for other biochemical measurement.

Fasting blood glucose concentration was measured using the glucose oxidase method (Spinreact, Girona, Spain).Total cholesterol and triglycerides were measured by routine enzymatic methods (Spinreact, Girona, Spain). HDL cholesterol was determined after precipitation of the apoB-containing lipoproteins. LDL cholesterol was calculated.

Urine samples:-
Urine aliquots were stored at −80°C prior to being used for measurements of urinary markers. Twenty-four hour urine samples were collected from each participant in sterilized urine containers and used to determine albumin in 24 h urine specimen. The urine levels of the biomarkers were normalized to the urinary creatinine concentration to control for variations in hydration status.

Serum and urinary TNF-*α:* serum levels were measured by an enzyme-linked immune absorbent assay (ELISA) using commercially available standard kits (Quantikine high-sensitivity human TNF-α Research & Diagnostic Systems, Europe Ltd, Abington, UK). The urinary concentration of TNF-α was determined using an enzyme-linked immunosorbent assay (ELISA) with a Human TNF-α Quantikine ELISA kit (DTA00C; R&D systems, Minneapolis, MN, USA).

The study protocol was approved by the Ethical Committee of Faculty of Medicine, Zagazig University and informed written consent was obtained from each individual.

Statistical analyses
Were performed using the Statistical Package for the Social Sciences for Windows (version 22.0; SPSS Inc., Chicago, IL, USA). Data were expressed using descriptive statistic (mean ± standard deviation) and were analyzed. One-way analysis of variance (ANOVA) test was done to compare different parameters between more than two groups. Pearson correlation coefficient was used to assess the association between serum and urinary TNF-α levels, clinical, biochemical tests and other studied metabolic parameters in patients with diabetic nephropathy. P-values were considered significant if <0.05.

Results:-

Clinical, anthropometric and biochemical characteristics of the studied groups:-
Among cases, 47% were males and 53% were females and in control individuals 51% were males and 49% were females. The mean age of case group was 40.3±9.88 years and in controls 40.9± 9.8years. The case and control individuals were thus matched in terms of age and sex.

There were significant higher values in case group compared to control group as regards systolic blood pressure, diastolic blood pressure, BMI, Waist-hip ratio, total cholesterol,TG, LDL.c, HDL.c, fasting blood glucose, HbA1c,WBC's count, serum creatinine, uric acid and albumin excretion ratio (AER) ($p < 0.05$). On the other hand diabetic patients and patients with diabetic nephropathy had significantly lower values of HDL.c and estimated glomerular filtration rate (eGFR) compared to healthy control group ***(table 1).***

Clinical, anthropometric and biochemical characteristics of type 2 diabetic patients stratified according to urinary albumin excretion ratio (AER):-
Macroalbuminuric patients had significantly higher levels of WBC's count, fasting blood glucose, HbA1c, uric acid and albumin excretion ratio (AER) compared to normoalbuminuric and microalbuminuric patients ($p < 0.05$).On the other hand there were no significant differences between studied subjects as regards other clinical and laboratory parameters ***(table 2)***.

Table 1: Clinical ,anthropometric and biochemical characteristics of the studied groups

Characteristics	Control(n=70)	Type2 diabetic patients(n=100)
Age (years)	40.9± 9.8	40.3±9.88
BMI(kg/m^2)	22.27±1.32	31.8±5.09*
Waist/hip ratio	0.79±0.04	1.13±0.18*
SBP (mmHg)	117.7±3.84	130±11.86*
DBP (mmHg)	75.9±4.10	83.74±10.70*
T-Cho (mg/dl)	183.3±18.25	211.04±31.34*
TG (mg/dl)	176±13.68	228.69±37.44*
LDL.c (mg/dl)	100.4±21.48	128.48±30.64*
HDL.c (mg/dl)	47.7±5.99	36.82±5.09*
FBG (mg/dl)	87.85±3.69	130.66±57.72*
HbA1c (%)	5.69±0.55	8 ±3.27*
WBC's	4.57±0.55	7.77±2.24*
Serum creatinine (mg/dl)	0.916±.047	2.11±0.18*
eGFR (mL/min)	89.19±11.2	68.66±9.52*
Serum TNF-α (pg/ml)	1.75±0.18	6.42±1.93*
Urinary TNF-α (pg/mg)	1.41±0.18	3.64±1.09*
AER (µg/mg)	13.74±0.71	174.97±153.98*
Uric acid (mg/dl)	2.92±0.55	8.41±0.93*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; Total-C, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; eGFR, Estimated glomerular filtration rate; AER, Albumin/excretion ratio.

Table 2: Clinical, anthropometric and biochemical characteristics of type 2 diabetic patients stratified according to urinary albumin excretion ratio (AER)

Characteristics	Patients with normoalbuminuria (n=49)	Patients with Microalbuminuria (n=30)	Patients with Macroalbuminuria (n=21)
Age (years)	37.65±8.51	45.50±11.20	39.09±8.28
BMI(kg/m^2)	31.93±5.27	31.0±5.23	32.71±4.48
Waist/hip ratio	1.14 ±0.18	1.10±.186	1.168±0.16
SBP (mmHg)	129.30±11.09	133.03±13.26	127.3±11.09
DBP (mmHg)	83.63±11.10	85.80±9.89	81.04±10.75
T-Cho (mg/dl)	214.89±28.93	208.30±35.73	205.95±30.4
TG (mg/dl)	231.32±33.92	229.13±43.36	221.90±37.2
LDL.c (mg/dl)	131.69±27.44	126.47±37.05	123.8±28.23
HDL.c (mg/dl)	36.93±5.27	36.0±5.23	37.7±4.48
FBG (mg/dl)	102.7±13.59	167.9±52.05*	189.4±28.02*
HbA1c (%)	6.92±1.87	10.08±3.00*	11.29±1.67*
WBC's	6.22±0.98	8.9±2.35*	9.71±1.63*
S. creatinine (mg/dl)	2.02±0.18	2.093±0.18	2.137±0.16
eGFR	67.32±9.78	69.07±7.86	69.63±8.48
AER (µg/mg)	28.49±2.53	256.9±23.08*	399.61±30.5*
Uric acid (mg/dl)	7.08±2.6	9.42 ±2.03*	10.08±1.40*

Levels of serum and urinary TNF-α in diabetic patients and patients with diabetic nephropathy stratified according to AER.

Serum and urinary TNF-α were significantly higher in cases compared to healthy controls (p <0.001) ***(table1)***. Also there were higher significant values of serum TNF-α in macroalbuminuria (8.09±1.40) and microalbuminuria (7.43±2.03) compared to patients with normoalbuminuria (5.9±1.34), (p < 0.001) ***(Fig.1)*** and higher significant

values of urinary TNF-α in patients with macroalbuminuria (4.82±0.79) and microalbuminuria (4.21±1.1) compared to patients with normoalbuminuria (2.88±1.85), ($p < 0.001$) ***(Fig.2).***

Correlation between serum and urinary TNF-α and clinical, anthropometric and biochemical characteristics in diabetic nephropathy patients:-

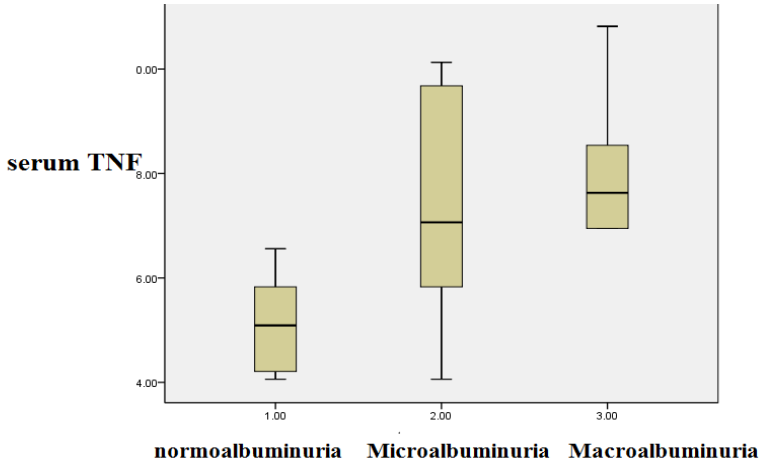
In patients with diabetic nephropathy, serum and urinary TNF-α were positively correlated to systolic and diastolic blood pressure, total cholesterol, LDL .c, WBC's count, fasting blood glucose, HbA1c, uric acid and albumin excretion ratio. On the hand there were no significant correlation between serum & urinary TNF-α to other clinical and biochemical characters ($p > 0.05$) ***(table 3).***

Also positive linear correlation was found between serum and urinary TNF-α in diabetic nephropathy patients was detected (***Fig.3).***

Table 3: Pearson's correlation between serum & urinary TNF-α(pg/ml) and clinical, anthropometric and biochemical characteristics in diabetic nephropathy patients (n = 100)

Characteristics	S.TNF-α		U.TNF-α	
	r	p	r	p
BMI(kg/m^2)	0.107	NS	0.103	NS
Waist/hip ratio	0.112	NS	0.172	NS
SBP (mmHg)	0.507	<0.001	0.617	<0.001
DBP (mmHg)	0.328	<0.001	0.378	<0.001
T-Cho (mg/dl)	0.494	<0.001	0.591	<0.001
TG (mg/dl)	0.173	NS	0.012	NS
LDL.c (mg/dl)	0.480	<0.001	0.476	<0.001
HDL.c (mg/dl)	-0.107	NS	-0.134	NS
FBG (mg/dl)	0.960	<0.001	0.756	<0.001
Uric acid (mg/dl)	0.945	<0.001	0.874	<0.001
HbA1c (%)	0.947	<0.001	0.893	<0.001
WBC's	0.786	<0.001	0.819	<0.001
Serum creatinine (mg/dl)	0.112	NS	0.103	NS
eGFR	0.113	NS	0.121	NS
AER (µg/mg)	0.666	<0.001	0.673	<0.001

Fig.1: Serum TNF-α in diabetic nephropathy patients stratified according to AER



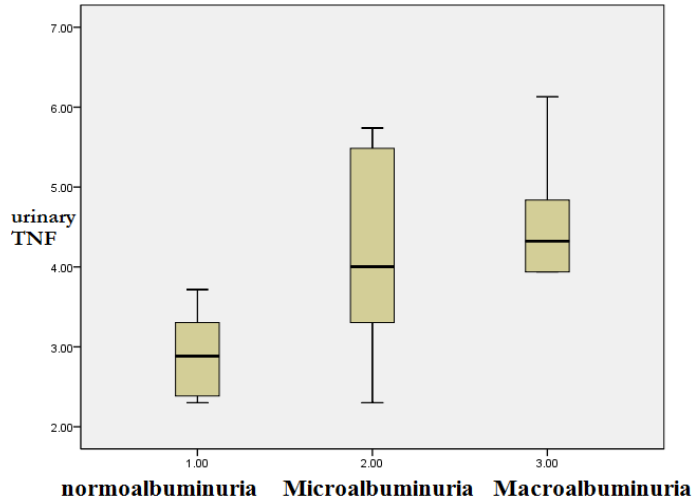


Fig.2: Urinary TNF-α in diabetic nephropathy patients stratified according to AER

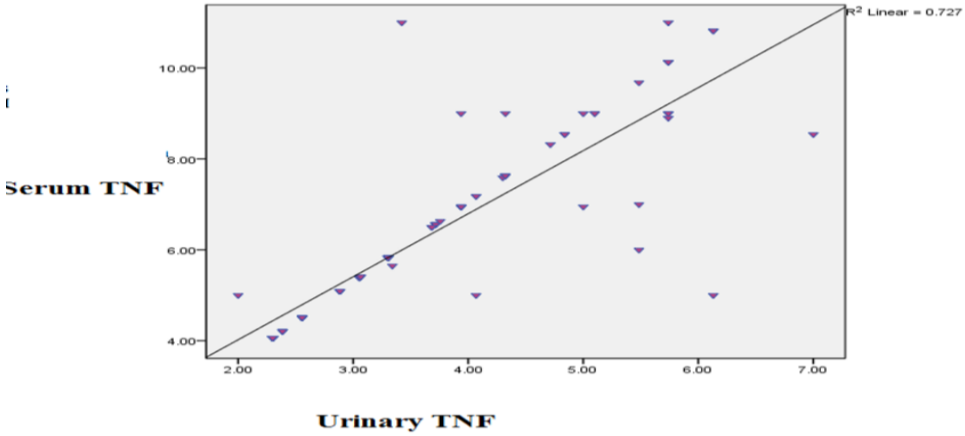


Fig.3: Correlation between serum and urinary TNF-α in diabetic nephropathy patients

Discussion:-

Diabetic nephropathy (DN) is a common microvascular complication of diabetes mellitus as well as the leading cause of end stage renal disease worldwide [12,13]. Prevalence about 30-40% in type 2 diabetic patients [12,14].

The prognosis of DN is poor due to late diagnosis. Many researches have been made to develop novel diagnostic and therapeutic approaches, but DN is still one of the most common causes of morbidity and mortality in type 2 diabetes [12,14] so, the early diagnosis of DN is critical to prevent progressive renal damage in these patients [15,16].

Glomerulopathy is considered to be an early sign of DN and microalbuminuria is a strong predictor of DN progression [17,18]. Different mechanisms may contribute to the pathogenesis of the disease. Accordingly, biomarkers with a high specificity to different abnormalities are required to predict the onset and progression of DN in type 2 diabetes [19]. Albuminuria is not mandatory to develop DN as many diabetic patients with normoalbuminuria may develop progressive and end stage renal damage [20].

TNF-*α* may promote renal damage in diabetic nephropathy through several mechanisms; production of endothelin-1, cytotoxic effect on glomerular cells, stimulation of apoptosis, disruption of the intracellular junctions of the glomerular filtration barrier and increases its permeability resulting in the development of albuminuria [21]. Urinary TNF-*α* serves as a biomarker of renal inflammation and a predictor of ESRD in diabetic patients [22].

In this study, we investigated the correlation of the serum and urinary inflammatory marker TNF-α with albuminuria as well as other clinical and laboratory parameters of nephropathy, moreover to assess the efficacy of these biomarkers in predicting a decline in kidney function.

In our study we found that, type 2 diabetic patients with diabetic nephropathy had significantly higher values of systolic, diastolic blood pressure, BMI, waist-hip ratio, total cholesterol ,TG ,LDL.c ,HDL.c ,fasting blood glucose, HbA1c ,WBC's count, serum creatinine, uric acid and albumin excretion ratio (AER) than control group ($p < 0.05$). On the other hand diabetic patient with diabetic nephropathy had significantly lower values of HDL.c and estimated glomerular filtration rate (eGFR) compared to healthy control group.

According to our results, diabetic nephropathy patient when stratified according to albumin excretion ratio there were significantly higher levels in macroalbuminuric compared to normoalbuminuric and microalbuminuric as regards WBC's count, fasting blood glucose, HbA1c, uric acid and albumin excretion ratio, on contrary there were no significant differences as regard other clinical and laboratory parameters between studied groups ($p > 0.05$).

Similar to our results **Molitch et al., (2004)** found that glycosylated hemoglobin levels were higher in microalbuminuric compared to normoalbuminuric patients. The above findings reflect the association between poor glycemic control and the development of diabetic nephropathy [23]. Our findings agree with the conclusion of the UKPDS study (United Kingdom Prospective Study), in which a reduction of glycosylated hemoglobin levels by 0.9% was correlated with a reduction in the relative risk of albuminuria by 30% [24,25].

In agreement to our results, **Cooper et al., (2001)** observed that, hypertension is more in patients with diabetic nephropathy. It is well known that hypertension and diabetes coexist in the majority of patients and that systolic blood pressure is a major hemodynamic mechanism of proteinuria in diabetic nephropathy [26].

We found that serum and urinary TNF-α levels were significant higher in diabetic patients compared to controls and this was similar to results obtained by **Moriwaki et al., (2003)** who observed that there were significant differences in serum levels of TNF-α between the type 2 diabetic patients and control subjects [21].

We also found that serum and urinary TNF-α levels were significantly higher in macroalbuminuric and microalbuminuric group compared to normoalbuminuric patients, similar results detected by **Kalantarinia et al., (2003)** they showed higher serum TNF-α levels both in macroalbuminuric and in microalbuminuric compared with normoalbuminuric patients [27]. In agreement with our results also, **Wong et al., (2007)** reported that the serum and urinary concentrations of TNF-α were elevated in DN as compared with non-diabetic individuals or with diabetic subjects without kidney disease and that these concentrations increase concomitantly with the progression of DN. These results indicate the role of elevated levels of this inflammatory cytokine with the development and progression of renal injury in DM [28]. Also **Navarro et al., (2003)** found that by applying Multivariate analysis there were a significant and independent relationship between urinary TNF-α and urinary albumin excretion [29].

Interestingly, **Lampropoulou et al., (2014)** found that, compared to patients with normoalbuminuria, patients with microalbuminuria had significantly higher urinary TNF-α, but similar serum TNF-α levels [30] and showed that urinary TNF-α was the only independent predictor of the degree of microalbuminuria and no correlation was observed between serum and urinary TNF-α level in their study suggesting mainly intra renal production of this cytokine and therefore local and non systemic activation of the inflammatory response and this was not in agreement with our study, we found significant linear correlation between serum and urinary TNF-α that was in agreement with **Wong et al** study .They supported their results by evaluation of the correlation between microalbuminuria and CRP, fibrinogen and serum TNF- α levels in their results. However, it should be noted that it remains unclear whether TNF- α urinary excretion is a marker of glomerular permeability or reflects tubulointerstitial damage [30]. Also **Navarro et al., (1999)** didn't find any significant correlation between serum and urinary concentrations and explained these results by an intra renal production of TNF-α [31].

Considering the results of our study, in patients with diabetic nephropathy, serum and urinary TNF-α were positively correlated to systolic and diastolic blood pressure, total cholesterol, LDL.c, WBC's count, fasting blood glucose, HbA1c, uric acid and albumin excretion ratio. On the contrary, there were non-significant correlation between serum and urinary TNF-α and serum creatinine or eGFR; similar results confirmed by **Lampropoulou et al**, **(2014)**, they observed no correlation between eGFR and serum or urinary TNF- α [30].

Conclusion:-

Serum and urinary TNF-α level were elevated in type 2 diabetic patients with diabetic nephropathy with positive correlation between serum and urinary levels. Their levels were significantly positively correlated to albuminuria and may be used as a strong predictive factor for progression of glomerular disease in type 2 diabetes.

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