Study of Serum Tumor Necrosis Factor Alpha and Interleukin 6 in Type 2 Diabetic Patients with Albuminuria

Ahmed Zahran[1], Enas S. Essa[2] Waleed F. Abd Elazeem[2]

[1]Internal Medicine Department, Nephrology Unite and [2]Clinical Pathology Department Faculty of Medicine, Menoufia University, Egypt
ahmed173@hotmail.com

Abstract:Background: Chronic kidney disease (CKD) is one of the major complications of type 2 diabetes and is the leading cause of end stage renal disease (ESRD). There are Growing evidences indicating that chronic low-grade inflammatory response is a recognized factor in the pathogenesis of development and progression of diabetic renal injury. The aim of this study was to analyze the relationship between inflammatory markers tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) with urinary albumin excretion (UAE) as a marker of renal injury. **Methods:** A total of 73 type 2 diabetic patients were divided into three groups according to urinary albumin excretion, normoalbuminuria, microalbuminuria and macroalbuminuria In addition, 10 apparently healthy subjects were included as control group. TNF-α, IL-6, Glycated hemoglobin (HbA1c) and creatinine were measured in all population. Urinary albumin excretion was measured by morning spot sample albumin / creatinine ratio (ACR). **Results:** Levels of TNF-α and IL-6 were found to differ significantly among studied groups. Both markers showed significant positive correlation with duration of diabetes, Albumin creatinine ratio and glycated hemoglobin and showed significant negative correlation with estimated glomerular filtration rate (eGFR), however TNF-α showed a better correlation with these variables when compared with IL-6. Stepwise regression analysis demonstrated that TNF-α is the independent predictors for ACR in total population with adjusted R^2 0.512 and P value less than 0.01. **Conclusion:** TNF-α and IL-6 are higher in type 2 diabetic patients with albuminuria and correlate well with the severity of albuminuria, however TNF-α was found to be a predictor of ACR suggesting the possible role of TNF-α in pathogenesis and progression of renal affection in type 2 diabetic patients.

[Ahmed Zahran, Enas S. Essaand Waleed F. Abd Elazeem**Study of Serum Tumor Necrosis Factor Alpha and Interleukin 6 in Type 2 Diabetic Patients with Albuminuria.** Life Science Journal 2012; 9(1):877-882]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 128

Keywords: Diabetic nephropathy; Albuminuria; tumor necrosis factor alpha and interleukin 6.

1. Introduction

The global diabetes burden is predicted to rise to 366 million by 2030 and would present itself as a major health challenge (1). Chronic kidney disease (CKD) is one of the major complications of type 2 diabetes and is the leading cause of end stage renal disease (ESRD) (2).The literatures are replete with studies about the involvement of innate immune system and low grade inflammation in pathogenesis of DM. and there is now clear evidence that inflammatory markers, acute-phase reactants and proinflammatory cytokines are strongly associated with the risk of developing type 2 diabetes (3-10). Based on this perspective several studies were conducted to address the role of this inflammatory cytokines in the development of diabetic complications. The exact mechanisms leading to the development and progression of renal damage in diabetes are not yet completely known. Growing evidence indicates that activation of innate immunity with the development of a chronic low-grade inflammatory response is a recognized factor in the pathogenesis of this disease (11). Cytokines are a group of pharmacologically active, low molecular weight polypeptides that possess autocrine, paracrine, and juxtacrine effects. These molecules cluster into several classes (i.e. interleukins, tumor necrosis factors, interferons, colony-stimulating factors, transforming growth factors and chemokines) (12). The cytokine TNF-α is a well-known member of the TNF superfamily consisting of 157 amino-acid peptide produced mainly by monocytes, macrophages, B and T lymphocytes. TNF signals through two distinct receptors TNFR1 and TNFR2 thereby controlling expression of cytokines, immune receptors, proteases, growth factors and cell cycle genes which in turn regulate inflammation, survival, apoptosis, cell migration, proliferation and differentiation (13). Human IL-6 composed of 184 amino acid and produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangium cells, and several tumor cells (14). Different inflammatory molecules, including pro-inflammatory cytokines such as TNF-α and IL-6 play a critical role in the development of micro vascular diabetic complications, including nephropathy (15-16). The objective of the present study is to examine the relation between inflammatory cytokines TNF-α and IL-6 with urinary albumin excretion (UAE) in type 2 diabetic patients in early stages of diabetic kidney disease.

2. Patients and Methods

The protocol for this study followed the ethical standards and approved by the ethical committee of our institution and all subjects gave informed consent to participate in this study. This study was conducted on 73 type 2 diabetic patients, 29 males and 44 females. In addition, 10 apparently healthy, age and gender matched, subjects were involved in this study as a control group (5 males, 5 females).

Patients with the following criteria were excluded: age over 70 years, body mass index (BMI) above 30, current acute infection or any acute illness, systemic hypertension defined as blood pressure more than 140/90, history of ischemic heart disease, cerebrovascular stroke, malignancy, dyslipidemia, hepatic disorders, smokers, hematological diseases and patients with serum creatinine more than 1.5 mg/dl.

Patients were divided according to UAE which was measured by early morning spot urine sample for albumin creatinine ratio (ACR) into 3 groups, diabetic without microalbuminuria (ACR less than 30 mg/gm), diabetic with microalbuminuria (ACR between 30 – 300 mg/gm) and diabetic with macroalbuminuria (ACR more than 300 mg/gm).

All subjects underwent full history taking and clinical examination including measuring blood pressure weight and height. Mean arterial pressure (MAP) was calculated as {(2 x diastolic blood pressure (mmHg) + systolic blood pressure (mmHg)}/3. BMI Was calculated as weight (Kg)/ {Height (m)} ^{2} GFR was estimated using Modification of Diet in Renal Disease Abbreviated Equation (MDRD):

[GFR = 186 × (serum Cr)$^{-1.154}$× (age)$^{-.203}$× (0.742 if female) × (1.210 if African American)] (17).

Laboratory assessment

Blood samples were collected by sterile venipuncture and divided into 2 parts; the first part was collected on dipotassiumethelenediamine tetra-acetic acid (EDTA) tube for glycated hemoglobin (HbA1c). The second part was transferred into another plain vacutainer tube, left to clot, then centrifuged for 10 minutes at 4000 r.p.m. and the serum obtained for determination of TNF α and IL-6 was kept frozen at -20 ºC till analysis. Early morning 10- 20 ml of midstream urine was collected for measurement of urinary albumin and creatinine for measurement of albumin creatinine ration (ACR). ACR was calculated using the following equation: ACR = Albumin mg/ d l ÷ Creatinine g/ dl

HbA1c was measured using quantitative colorimetric measurement of glycohemoglobin as percent of total hemoglobin using kits supplied by STANBIO LABORATORY, USA.

Albumin in urine was estimated by Beckman's microalbumin test kit on Synchron CX9 autoanalyser.Beckman.Urine creatinine is measured by a modified rate Jaffe method.

Serum TNF-α was determined by enzyme linked immunosorbent assay method, using IDELISA^{TM} Human TNF-α ELISA kit. It utilizes a monoclonal antibody (capture antibody) specific for human TNF α coated on a 96-well plate. Standards and samples are added to the wells, and any human TNF α present binds to the immobilized antibody. The wells are washed and biotinylated polyclonal anti-human TNF-α antibody (detection antibody) is added. After a second wash, avidin-horseradish peroxidase (avidin-HRP) is added, producing an antibody-antigen-antibody sandwich. The wells are again washed and a substrate solution is added, which produces a blue color in direct proportion to the amount of human TNF-α present in the initial sample. The stop buffer is then added to terminate the reaction. This results in a color change from blue to yellow. The wells are then read at 450 nm

Serum IL-6 was determined by enzyme linked immunosorbent assay method using AviBion Human IL-6 ELISA kit, Ani Biotech Oy, Orgenium Laboratories Business Unit, FINLAND. The assay employs an antibody specific for human IL6 coated on a 96-well plate. Standards, samples and biotinylated anti-human IL6 are pipetted into the wells and the IL6 present in a sample is captured by the antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed. Following this second wash step, TMB substrate solution is added to the wells resulting in color development proportional to the amount of IL6 bound. The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm.

Statistical Evaluation

We used the statistical package of social signs (SPSS, version 16) to perform the analysis. Correlation between inflammatory markers (TNF-α and IL-6) with duration of diabetes, HbA1c, albuminuria and eGFR was performed by pearson correlation, one way ANOVA test or Kruskalwalis test was used as appropriate for comparison of quantitative variables among more than two independent groups. Multiple stepwise regression analysis was performed to determine the possible predictor for ACR among potential risk factors including inflammatory markers. P value ≤ 0.05 was considered significant.

3. Results

The cohort was divided according toACR into 3 groups. Group I; diabetics with normoalbuminuria, group II; diabetics with microalbuminuria and group III; diabetics with macroalbuminuria in addition to group IV; control group. Baseline characteristics and comparison of the studied groups are shown in table 1.

The groups are matched regarding age, sex, BMI and mean arterial blood pressure. Serum TNF-α and IL-6 differed significantly among the studied groups (Figure 1). Pearson's correlation coefficients (r) between serum TNF-α and Duration of diabetes mellitus, HbA1c & ACR showed a significant positive correlations with *P* value less than 0.001, while it showed a significant negative correlation with eGFR with *P* value less than 0.001.(Figure 2). IL-6 also showed significant correlations with the same parameters but with less r value (*P*< 0.01) (Figure 3). Interestingly both TNF-α and IL-6 correlated significantly positive with r value 0.521 and P value less than 0.001. Stepwise regression analysis demonstrated that TNF-α is an independent predictor for ACR in total population with adjusted R^2 0.512 and *P* value less than 0.01.

Table 1: Baseline characteristics and comparison of the studied groups.

	(Group I) Diabetics with normoalbuminuria ACR (mg/gm) 17.74 + 9.32 (n= 23)	(Group II) Diabetics with microalbuminuria ACR (mg/gm) 77.74 + 65.02 (n= 34)	(Group III) Diabetics with macroalbuminuria ACR (mg/gm) 468.69 + 57.40 (n=16)	(Group IV) Control group ACR (mg/gm) 4.40 + 4.01 (n= 10) Mean ± SD	*P* value
*Age (years)	55.96 ± 6.20	57.12 ± 7.65	61.31 ± 6.42	55.10 ± 6.72	> 0.05
**Sex (M/F)	7/16 (30.4/69.6%)	16/18 (47.1/52.9%)	6/10 (37.5/62.5%)	5/5 (50/50%)	> 0.05
*Duration of D M (years)	7.17 ± 2.95	14.85 ± 5.59	21.25 ± 6.01	-	< 0.001
*MAP (mmHg)	94.78 ± 4.09	94.36 ± 4.14	93.33 ± 3.16	91.50 ± 5.58	> 0.05
*BMI	27.35 ±1.76	28.29 ± 1.50	28.42 ± 1.36	27.63 ± 1.40	> 0.05
*HbA1c	7.33 ± 1.56	9.25 ± 1.52	11.80 ± 1.55	5.57 ± 0.42	< 0.001
*eGFR (ml/min/1.73m^2)	81.96 ± 26.41	68.32 ± 18.15	56.14 ± 12.92	83.56 ± 25.40	< 0.01
*TNF-α (pg/ml)	65.56 ± 69.39	173.24 ± 84.95	312.69 ± 75.56	13.50 ± 3.31	< 0.001
*IL-6 (pg/ml)	96.52 ± 151.29	214.91 ± 165.40	346.75 ± 191.70	5.20 ± 3.36	< 0.001

*: Mean ± Standard Deviation, **: Number and percentage, M/F: Male/Female
D M: Diabetes Mellitus, MAP: Mean arterial pressure, BMI: Body mass index, HbA1C: glycated hemoglobin, ACR: Albumin creatinine ratio, eGFR: Estimated glomerular filtration rate, TNF-α: Serum tumor necrosis factor alpha, IL-6: Interleukin 6.

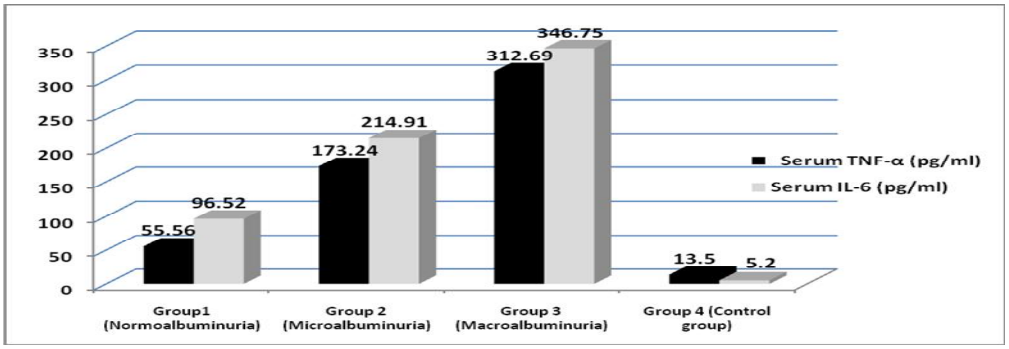
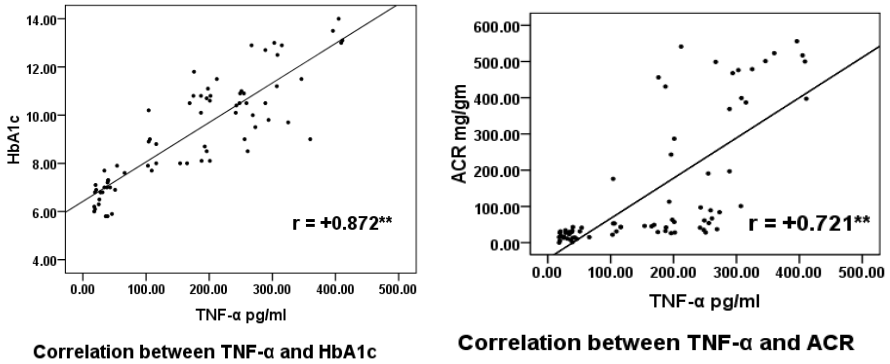
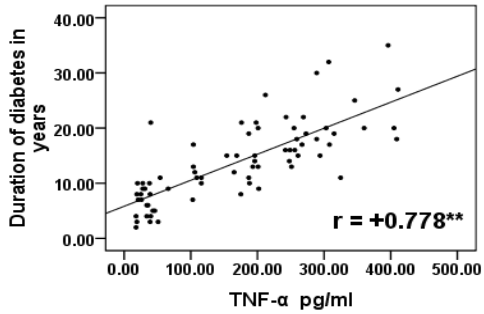
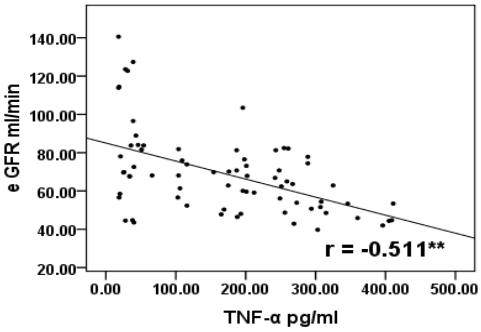


Figure 1: Comparison of TNF-α and IL-6 among the studied groups





correlation between TNF-α and duration of diabetes in years

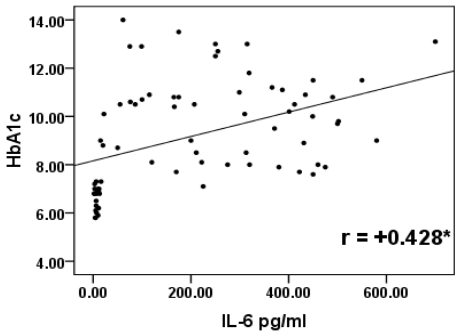


Correlation between TNF-α and e GFR

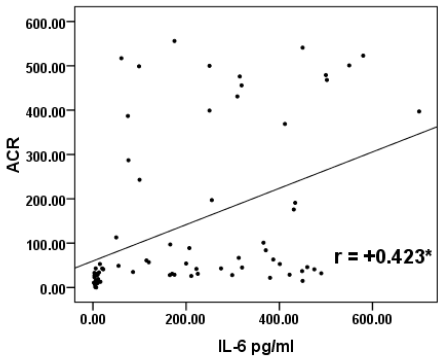
**: P value < 0.001

TNF-α: Serum tumor necrosis factor alpha,HbA1C: glycated hemoglobin, ACR: Albumin creatinine ratio e GFR: Estimated glomerular filtration rate

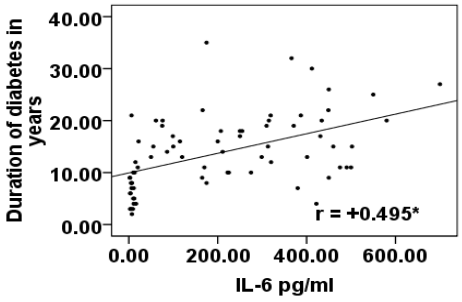
Figure 2: correlation between TNF-α with HbA1c, ACR, duration of diabetes in years and e GFR



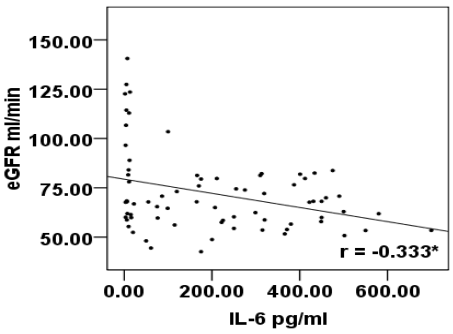
Correlation between IL-6 and HbA1c



Correlation between IL-6 and ACR



Correlation between IL-6 and duration of diabetes



Correlation between IL-6 and eGFR

*: P value < 0.01

IL-6:Serum Interleukin 6, HbA1C: glycated hemoglobin, ACR: Albumin creatinine ratio, eGFR: Estimated glomerular filtration rate,

Figure 3: correlation between IL-6 with HbA1c, ACR, duration of diabetes in years and eGFR

4. Discussion:

Diabetic nephropathy is one of the major microvascular complication of type 1 and type 2 diabetes mellitus and the leading cause of end stage renal disease. It was thought to be a result from interactions between hemodynamic and metabolic factors, however research during the past 10 years has provided insight into the etiology of diabetic nephropathy at the cellular and molecular level, and inflammation has emerged as being a key pathophysiological mechanism (18). Clinical investigations have implicated the roles of TNF-α and IL-6 in the development of diabetic nephropathy, suggesting that inhibition of those cytokines is promising remedy to ameliorate diabetic nephropathy.

In this present study we investigated the role of inflammatory cytokines TNF-α and IL-6 in relation to albuminuria in patients with type 2 diabetes. In our study we found that TNF-α and IL-6 differed significantly among studied groups. Furthermore there

were a significant correlation between each marker and HbA1c, albuminuria, eGFR & duration of diabetes. These results are consistent with other investigators who found higher levels of inflammatory cytokines among diabetic patients with albuminuria. Moriwaki et al. reported higher levels of TNF-α and IL-6 among diabetic patients without albuminuria compared to those with albuminuria and concluded that TNF-α and IL-6 may have some etiopathogenic roles in diabetic nephropathy (19). In a study included 95 diabetic patients conducted by Refaat H. et al. they found higher levels of serum and urine TNF-α in diabetic group with proteinuria and similar to our results found a good correlation between serum TNF-α and protein / creatinine ratio, HbA1c & duration of diabetes mellitus (20). Consistent with our results Maulana Azad studied IL-6 in 60 diabetic patients divided into three groups (normo, micro and macroalbuminuria) and found that IL-6 was higher in diabetic patients with macroalbuminuria compared to patient with microalbuminuria and normoalbuminuria and they also found a good correlation between IL-6 with HbA1c and urinary albumin excretion which was similar to our results but with a better r values (21). In our study stepwise regression analysis demonstrated that TNF-α was an independent predictor of urinary albumin excretion while IL-6 was not. In accordance with us Ng Dp et al. demonstrated that TNF-α system is likely to exert independent effects on albuminuria and renal function in type 2 diabetes while C reactive protein and IL-6 did not show that (22). Serum and urine TNF-α were found to be independently and significantly associated with ACR in diabetic patients (20, 23). Many investigators demonstrated a structural damage associated with inflammatory cytokines. Dalla Versa et al studied 74 type 2 diabetic patients regarding acute phase markers of inflammation including IL-6 in relation to structural kidney damage determined by mesangial fractional volume and glomerular basement membrane (GMB) width in a kidney biopsy and they found that IL-6 is significantly higher in group with increased GBM thickness and linear regression analysis demonstrated that IL-6 is one of the predictors of GBM thickness (24). Renal IL-6 expression has been related to mesangial proliferation, tubular atrophy and the intensity of interstitial infiltrates in diverse models of renal disease, suggesting a contributing role in the progression of renal disease (25). Experimental studies have demonstrated that urinary albumin excretion significantly correlates with renal cortical mRNA levels and urinary TNF-α excretion in animal models of diabetic nephropathy (26–27). Of more interest TNF-α inhibition by Infleximab reduced urinary albumin excretion in diabetic rat (28).

Conclusion

Serum TNF-α and IL-6 are elevated in diabetic patients with albuminuria and their levels are significantly higher with increasing levels of albuminuria. Both markers correlated well with ACR however TNF-α showed a better correlation than IL-6, moreover TNF-α found to be a predictor of ACR. This can suggest a possible role of TNF-α in pathogenesis and progression of renal injury in diabetic patients. Further studies are needed to confirm our finding and to study the possible role of TNF-α inhibitor in the prevention and treatment of diabetic nephropathy.

Corresponding author
Ahmed Zahran
Nephrology Unit, Internal Medicine Department
Faculty of Medicine, Menoufia University, Egypt.
ahmed173@hotmail.com

References

1. Sarah W, Gojka R, Anders G, Richard S, and Hilary K. (2005): Global Prevalence of Diabetes. Estimates for the year 2000 and projections for 2030. Diabetes Care. 27:1047–1053.
2. Kramer H, Molitch ME. (2005): Screening for kidney disease in adults with diabetes. Diabetes Care. 28:1813-6.
3. Pickup J, Mattock M, Chusney G, Burt D. (1997): NIDDM as a disease of the innate immune system: association of acute phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia. 40: 1286–92.
4. Pickup J, Crook M. (1998): Is type II diabetes mellitus a disease of the innate immune system? Diabetologia. 41:1241–8.
5. Mu¨ller S, Martin S, Koenig W, Hanifi-Moghaddam P, Rathmann W, Haastert B, *et al.* (2002): Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-a or its receptors. Diabetologia. 45:805–12.
6. Temelkova-Kurktschiev T, Henkel E, Koelher C, Karrei K, Hanefeld M(2002): Subclinical inflammation in newly detected type II diabetes and impaired glucose tolerance. Diabetologia. 45:151.
7. Dandona P, Aljada A, Bandyopadhyay A. (2004): Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 25:4–7.
8. Alexandraki K, Piperi C, Kalofoutis C, Singh J, Alaveras A, Kalofoutis A. (2006): Inflammatory process in type 2 diabetes: the role of cytokines. Ann NY Acad Sci. 1084:89–117.
9. Dennis RJ, Maldonado D, Rojas MX, Aschner P, Rondon M, Charry L. (2010): Casas A. Inadequate glucose control in type 2 diabetes is associated with impaired lung function and systemic inflammation: a cross-sectional study. BMC Pulm Med.10:38

10. Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, Rentfro A, McCormick JB, Fisher-Hoch SP. (2012): Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels ofleptin in a population of Mexican Americans: A cross-sectional study. Cytokines. 57(1): 136-42
11. Navarro JF, Mora-Fernández C. (2011): Inflammatory Pathways.Contrib Nephrol.170:113-23.
12. Navarro JF, Mora C. (2008):The Role of Inflammatory Cytokines in Diabetic Nephropathy. J Am SocNephrol. 19(3):433-42.
13. Haider S, Knöfler M. (2009): Human tumor necrosis factor: physiological and pathological roles in placenta and endometrium. Placenta. 30(2):111-123.
14. Kishimoto T. (1989): The biology of interleukin-6.Blood. 74(1):1-10.
15. Navarro JF, Mora-Fernandez C. (2006): The role of TNF-alpha in diabetic nephropathy: Pathogenic and therapeutic implications. Cytokine Growth Factor Rev. 17: 441–450.
16. Wong CK, Ho AW, Tong PC, Yeung CY, Kong AP, Lun SW, Chan JC, Lam CW. (2007): Aberrant activation profile of cytokines and mitogen-activated protein kinases in type 2 diabetic patients with nephropathy. Clin ExpImmunol. 149(1):123-31.
17. Levey AS, Greene T, Kusek JW, Beck GJ (2000): MDRD study group.A simplified equation to predict glomerular filtration rate from serum creatinine (Abstract). J Am Soc Nephrol. 11: A0828.
18. Navarro JF, Mora C, Muros M, García J. (2011): Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy.Nat Rev Nephrol. 7(6):327-40.
19. Moriwaki Y, Yamamoto T, Shibutani Y, Aoki E, Tsutsumi Z, Takahashi S, Okamura H, Koga M, Fukuchi M, Hada T. (2003): Elevated levels of interleukin-18 and tumor necrosis factor-alpha in serum of patients with type 2 diabetes mellitus: relationship with diabetic nephropathy. Metabolism. 52(5):605-8.
20. Refaat H, Mady G, Abd El Ghany M, Abou Seif Kh, El Hadidi E, Elshahawy Y, Sany D. and Abd El Aziz H. (2010): Correlation between Tumor Necrosis Factor Alpha and Proteinuria in Type-2 Diabetic Patients. Arab Journal of Nephrology and Transplantation. 3(1):33-8.
21. Maulana Azad (2008): Interleukin-6 and C-reactive protein in pathogenesis of diabetic nephropathy: new evidence linking inflammation, glycemic control, and microalbuminuria. Iran J Kidney Dis. 2(2):72-9.
22. Ng DP, Fukushima M, Tai BC, Koh D, Leong H, Imura H, Lim XL. (2008): Reduced GFR and albuminuria in Chinese type 2 diabetes mellitus patients are both independently associated with activation of the TNF-alpha system. Diabetologia. 51(12):2318-24.
23. Navarro JF, Mora C, Muros M, García J. (2006): Urinary tumor necrosis factor-alpha excretion independently correlates with clinical markers of glomerular and tubulointerstitial injury in type 2 diabetic patients. Nephrol Dial Transplant. 21(12):3428-34.
24. Dalla Vestra M, Mussap M, Gallina P, Bruseghin M, Cernigoi AM, Saller A, Plebani M, Fioretto P. (2005):Acute-phase markers of inflammation and glomerular structure in patients with type 2 diabetes. J Am Soc Nephrol. 16 Suppl 1:S78-82.
25. Rivero A, Mora C, Muros M, García J, Herrera H, Navarro-González JF. (2009): Pathogenic perspectives for the role of inflammation in diabetic nephropathy. Clin Sci (Lond). 116(6):479-92.
26. Navarro, JF, Milena, F., Mora, C. León C, Claverie F, Flores C, García J. (2005):Tumor necrosis factor-α gene expression in diabetic nephropathy: relationship with urinary albumin excretion and effect of angiotensin-converting enzyme inhibition. Kidney Int Suppl. 68: S98–102
27. Navarro JF, Milena F.J, Mora C, Le on C. and Garc´ıa J. (2006): Renal pro-inflammatory cytokine gene expression in diabetic nephropathy: effect of angiotensinconverting enzyme inhibition and pentoxifylline administration. Am. J Nephrol. 26:562–570.
28. Moriwaki Y, Inokuchi T, Yamamoto A, Ka T, Tsutsumi Z, Takahashi S, Yamamoto T. (2007): Effect of TNF-alpha inhibition on urinary albumin excretion in experimental diabetic rats. Acta Diabetol. 44(4):215-8.

3/2/2012