Study of Toll-like Receptors 4 in Type 2 Diabetic Patients with and without nephropathy

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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. ***Objective:*** The aim of this work is to study the relation of Toll-like receptor 4 (TLR4) in diabetic nephropathy in patients with type 2 diabetes (T2DM). ***Methods:*** A total of 50 type 2 diabetic (T2DM) patients were divided into three groups according to urinary albumin/creatinine ratio (UACR) normoalbuminuria, microalbuminuria and macroalbuminuria. In addition, 10 apparently healthy subjects were included as a control group. Fasting blood glucose (FBG), Glycated hemoglobin (HbA1c), blood urea and serum creatinine were measured in all population. Urinary albumin excretion was measured by morning spot sample and urinary albumin/creatinine ratio (UACR) was calculated. Quantification of CD14 and TLR4 expression on monocytes subsets was done by flowcytometry. ***Results:*** Levels of CD14 were found to be significantly increased in patients with macroalbuminurea while TLR4 levels were increased in T2DM patients with further elevation in patients with macroalbuminurea. Both markers showed significant positive correlations with the duration of diabetes, HbA1c, serum creatinine and UACR and significant negative correlations with estimated glomerular filtration rate (eGFR). Multivariate regression analysis demonstrated that CD14 and TLR4 are independent predictors for the occurrence microalbuminurea in T2DM patients. ***Conclusion:*** TLR4 levels were higher in T2DM patients compared to normal subjects. These observations significantly add to the emerging role of TLRs in T2DM development. TLR4 were also found to correlate well with the severity of albuminuria in T2DM and to be good predictors of microalbuminurea suggesting their possible role in pathogenesis and progression of DN.
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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 mellitus (T2DM) and is the leading cause of end stage renal disease (ESRD). [2] Diabetic kidney disease is associated with enhanced morbidity and mortality, particularly with accelerated cardiovascular disease. [3] The earliest clinical evidence of nephropathy is elevated urine albumin level > 30 mg/24hours (i.e. microalbumiurea).[4] Microalbuminuria is generally considered as the earliest non-invasive marker for the development of diabetic nephropathy (DN). [5]

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. [6] Activation of the innate immune system via toll-like receptors (TLRs) is implicated in the pathogenesis of insulin resistance, diabetes, and atherosclerosis. [7-9] Complimentary genetic studies link TLR4 polymorphisms to T2DM, suggesting a casual relationship between TLR function and diabetes and its complications. [10]

TLRs are evolutionarily preserved pattern-recognition receptors, [11] expressed on several cell types including monocytes, predominant cells of the innate immune system that are pivotal in diabetes and atherogenesis. [12] TLRs play an important role in the activation and regulation of the innate immune system and inflammation. [11] Toll-like receptors in these cells efficiently transduce the inflammatory signals. [13] Each TLR family member recognizes a specific pathogen component, upon activation, triggers a signaling cascade leading to cytokine production and adaptive immune response. [11] Among the TLRs, TLR4 plays a critical role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis in both clinical and experimental conditions. [7-9,14] Ligands for TLR4 include high-mobility group B1 protein (HMGB1), heat shock protein (HSP) 60, HSP70, endotoxin, hyaluronan,

advanced glycation end products, and extracellular matrix components. [15] However, TLR4 does not interact directly with the most potent inflammatory signals. Upstream to TLR4 is the multifunctional lipopolysaccharide (LPS) receptor CD14. CD14 is a 55-kDa protein that is expressed in two forms: glycosylphosphatidylinositol-anchored membrane protein (mCDI4) and a soluble serum protein (sCD14) lacking the glycosylphosphatidylinositol anchor. [16-18] Both circulating sCD14 and cellular CD14 receptor interact with the inflammatory signals; LPS is one of the most potent stimuli known. An excess of circulating sCD14 is known to buffer these signals, avoiding their exposure with cell (macrophage)-anchored CD14. [19] CD14 is in close interaction with TLR4, and LPS induces physical proximity between TLR4 and CD14 before nuclear translocation of nuclear factor-kB and triggering of the inflammatory cascade. [18]

2. Subjects and Methods

The protocol for this study followed the ethical standards and was approved by the ethical committee of our institution and all subjects gave informed consent to participate in this study. This study was carried out on a number of 50 T2DM patients (26 males and 24 females). In addition, 10 healthy subjects (5males and 5 females) with matched age and gender were involved as a control group. Patients were divided according to urinary albumin/creatinine ratio (UACR) which was measured by early morning spot urine sample into 3 groups, diabetic without microalbuminuria (UACR <30 mg/gm) (n=10), diabetic with microalbuminuria (UACR between 30–299 mg/gm) (n=14) and diabetic with macroalbuminurea (UACR ≥300 mg/gm) (n=26). 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 = 175 × (serum Cr)$^{-1.154}$× (age)$^{-203}$× (0.742 if female) × (1.212 if African American)]. [20]

Laboratory assessment

Blood samples were collected by sterile venipuncture and divided into 2 parts; the first part was collected on EDTA tube for glycated hemoglobin (HbA1c) and for flow cytometry analysis. The second part was delivered into a plain tube in which serum was separated by centrifugation on 3000 rpm for 10 minutes and used for assessment of serum urea and serum creatinine and fasting blood glucose. Early morning 10- 20 ml of midstream urine was collected for measurement of albumin creatinine ration (ACR). ACR was determined by Beckman microalbumin test kit on Synchron CX9 autoanalyzer (Beckman Coulter Inc., CA, USA). HbA1c was measured using quantitative colorimetric measurement of glycohemoglobin as percent of total hemoglobin using kits supplied by STANBIO LABORATORY (San Antonio, Texas, USA).

Laboratory quantification of TLR4 expression on monocytes subsets

Principle of the test:

Flow cytometry measures cells bearing TLR4 within a population of monocyte cells

Procedures:

Isolation of peripheral blood mononuclear cells (PBMC), blood was collected in sterile collection tubes containing EDTA. Two ml of Ficoll were placed in a centrifuge tube and layered by 1 ml of blood sample on top placed very carefully ensuring that the blood and Ficoll do not mix, centrifugation at 1800 rpm for 20 min. the WBCs were isolated by Ficoll gradient. The cell suspension was washed three times in PBS with Centrifuge for 5 minutes at 3200 RPM, then the samples were adjusted to a final concentration of 10^6/ml by PBS. To block Fc receptors, 34 ul of a 3 mg/ml solution of normal mouse IgG (Caltag /Burlingame, Ca) was added to different tubes and incubated on ice for 10 minutes. Samples were then liquated into different tubes of 100 ul each and stained with both monoclonal antibody antiTLR4 (FITC) *(mouse monoclonal anti-human antibodies against TLR4 Clone 76B357.1, mouse anti-human IgG2b, Abcam, Cambridge, MA, USA)* and antiCD14 (PE) *(Clone 61D3, mouse anti-human immunoglobulin G, Abcam, Cambridge, MA, USA)* (10 ul of each monoclonal antibodies). After gentle mixing, cells were incubated for 15 minutes on ice. Then the cells were washing with PBS twice. After discarding the supernatant and re-suspending the cells in residual buffer the cells were fixed in 200 ml of 2% ultra-pure formaldehyde

- Data were acquired on a FACS caliber flowcytometer (BD immune cytometry systems, San Jose, CA). - The instrument set up was checked weekly using QC windows beads (Flowcytometry standard, San Juan, PR).
- Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers and flowcytometric two parameters dot plots and quadrant statistics were generated by cell quest software (Becton Dickinson immune-cytometry systems).
- Analysis was performed after manual gating around a monocytes population on a forward scatter versus side scatter dot-plot. The monocytes were

further evaluated as CD14 positive cells. In the gated population, the percentages of positive cells for TLR-4 were made by dual platform technique. Results were expressed as percentages of monocytes positive for TLR-4 marker. [22, 23] Figure [1].

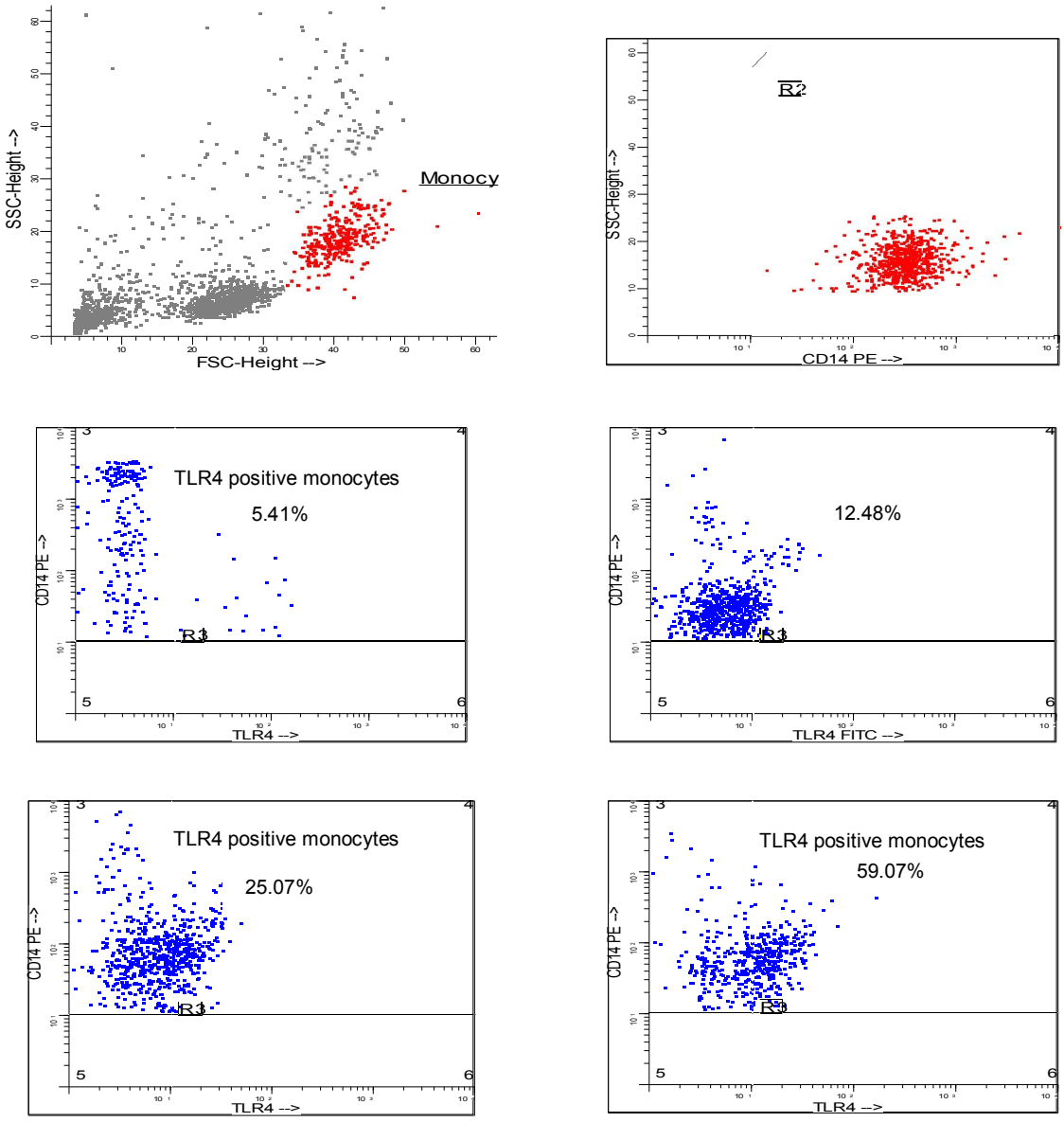


Figure (1): Flow cytometry Gating Strategy for analyze the TLR-4 expression on monocytes in different groups

Statistical analysis

We used the statistical package of social signs (SPSS, version 16) to perform the analysis. Categorical data were presented as number and percentages and continuous variables as means ± standard deviation (SD). One way ANOVA test or Kruskal-Wallis test was used as appropriate for comparison of quantitative variables more than two independent groups. Intergroup comparisons were performed using the chi-square test, t-test, and Mann–Whitney U test as appropriate. Pearson's correlation coefficient, r, was used to assess the relationship between CD14 and TLR4 and other variables in the three patient groups (i.e. 1, 2 and 3). Multivariate regression analysis was performed to identify variables that were independently associated with microalbuminurea. The values of Odds ratio (OR) and 95% confidence interval (CI) are summarized. P value <0.05 was considered significant.

3. Results

This study included 50 T2DM patients and 10 healthy persons as a control group. The diabetic patients were divided into 3 groups according (UACR). So the whole cohort was divided into 4 groups. *Group 1*: Control group consists of 10 healthy persons, 5 (50%) males and 5 (50%) females; *Group 2*: Diabetic patients with normoalbuminuria (UACR < 30 mg/gm) consists of 10 patients, 5 (50%) males and 5 (50%) females; *Group 3*: Diabetic patients with microalbuminuria (UACR between 30 - 299 mg/gm) consists of 14 patients, 6 (42.9%) males and 8 (57.1%) females; *Group 4*: Diabetic patients with macroalbuminuria (UACR ≥ 300 mg/gm) consists of 25 patients, 14 (53.8%) males and 12 (46.2%) females.

Table 1: Demographic and laboratory findings of the studied groups

Variable	Group 1 Control (n=10)	Group 2 Diabetes with normoalbuminurea (n=10)	Group 3 Diabetes with microalbuminurea (n=14)	Group 4 Diabetes with macroalbuminurea (n=26)	*P* value
Age (years)	62.1±7.26	61.5±5.44	63.86±7.08	66.62±8.03	>0.05
Gender (M/F)(No/%)	5/5 (50/50%)	6/4 (60/40%)	6/8 (42.9/57.1%)	14/12 (53.8/46.2%)	>0.05
BMI (kg/m)	25±1.62	26.3±2.5	25.8±2	26.4±2	>0.05
MAP (mmHg)	112.3±7.13	115.33±9.05	144.52±18.22	155.0±13.8	**<0.05**$^{**,***,\#,\#\#,\$}$
Duration of DM (months)		49.2±14.37	67.71±19.18	79.92±17.36	**<0.05**$^{\#,\#\#,\$}$
FBG (mg/dl)	83.6±7.75	142.0±37.06	205.71±55.15	239.46±50.02	**<0.05**$^{*,**,***,\#,\#\#,\$}$
Hb A1c%	4.67±0.51	8.3±0.95	8.93±1.05	9.63±1.20	**<0.05**$^{*,**,***,\#,\#\#,\$}$
Urea (mg/dl)	24.4±5.76	28.3±10.22	59.71±13.04	73.73±33.51	**<0.05**$^{**,***,\#,\#\#,\$}$
Creatinine (mg/dl)	1.01±0.12	1.05±0.14	1.17±0.29	1.68±0.79	**<0.05**$^{**,***,\#,\#\#,\$}$
eGFR (ml/min/1.73m^2)	108.6±11.60	102.0±10.33	81.57±7.1	51.46±12.17	**<0.05**$^{**,***,\#,\#\#,\$}$
UACR(mg/mg creatinine)	15.9±4.25	11.1 ±2.64	85.43±29.45	428.85±67.37	**<0.05**$^{**,***,\#,\#\#,\$}$
CD14	6.90±2.23	8.75±3.56	9.44±4.09	14.77±5.83	**<0.05**$^{***,\#\#,\$}$
TLR4	4.77±2.27	21.0±6.30	20.5±5.41	65.25±15.83	**<0.05**$^{*,**,***,\#\#,\$}$

*: Control VS Normoalbuminuria, **: Control VS Microalbuminuria, ***: Control VS Macroalbuminuria, #: Normoalbuminuria VS Microalbuminuria, ##: Normoalbuminuria VS Macroalbuminuria, $: Microalbuminuria VS Macroalbuminuria., M/F: Male/Female, **BMI:** Body mass index, **MAP:** Mean arterial pressure, **HbA1c:** Glycated hemoglobin, **eGFR:** estimated glomerular filtration rate, **UACR:** Urinary albumin creatinine ratio

Table 2: Pearson correlation between serum CD14 and other variables in T2DM patients (groups 2, 3, and 4)

	CD 14	
	Correlation coefficient (r)	**P value**
Age	+ 0.11	>0.05
BMI	+ 0.10	>0.05
MAP	+ 0.48	**<0.001**
Duration of DM	+ 0.34	**<0.05**
Fasting blood sugar	+ 0.43	**<0.001**
Hb A1c	+ 0.46	**<0.001**
Urea	+ 0.45	**<0.001**
Creatinine	+ 0.33	**<0.05**
eGFR	- 0.50	**<0.001**
UACR	+ 0.54	**<0.001**
TLR 4	+ 0.49	**<0.001**

T2DM: Type 2 diabetes mellitus, **BMI:** Body mass index, **MAP:** Mean arterial pressure, **HbA1c:** Glycated hemoglobin, **eGFR:** estimated glomerular filtration rate, **UACR:** Urinary albumin creatinine ratio

Base line characteristics and comparison between studied groups are shown in (Table 1). All groups were matched regarding age, gender and BMI. There was significant differences among the studied groups regarding the CD14 and TLR4 with *P* value <0.05. Analysis between each two groups regarding the CD14 showed that there was a significant difference between group 4 (diabetes with macroalbuminurea) and each group separately while the first three groups did not show significant difference among themselves. On the other hand, analysis between each two groups regarding the TLR4 showed that there were significant differences between group 1 (control group) and each group separately while there was no significant difference between group 2 (diabetes with normoalbuminurea) and group 3 (diabetes with microalbuminurea). Finally, TLR4 levels were significantly elevated in group 4 (diabetes with macroalbuminurea) compared to group 2 (diabetes with normoalbuminurea) and group 3 (diabetes with microalbuminurea).

Pearson correlation between CD14 and the other variables showed significant positive correlations with the MAP, duration of DM, FBG, HbA1c, blood urea, serum creatinine and UACR and significant negative correlation with eGFR in the three patient groups (i.e. groups 2, 3 and 4) (Table 2 and Figure 2). Similarly, TLR4 showed significant positive correlations with the MAP, duration of DM, FBG, HbA1c, blood urea, serum creatinine and UACR and significant negative correlation with eGFR in the three patient groups (i.e. groups 2, 3 and 4) (Table 3 and Figure 3). A multivariate regression model including age, BMI, MAP, FBG, HbA1C, duration of diabetes, eGFR, CD14 and TLR4 showed that both CD14 and TLR4 were independently associated with the occurrence of microalbuminurea in T2DM patients (OR; 1.8 and CI; 0.56-7.6) and (OR;2.1 and CI; 0.88-5.8) respectively and *P* value <0.05 (Table 4).

Table 3: Pearson correlation between serum TLR4 and other variables in T2DM patients (groups 2, 3, and 4)

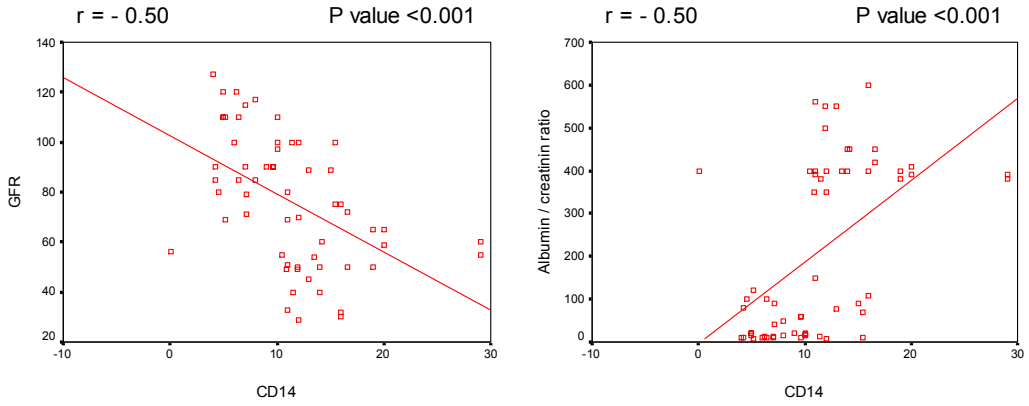
	TLR4	
	Correlation coefficient (r)	**P value**
Age	+ 0.14	>0.05
BMI	+ 0.05	>0.05
MAP	+ 0.59	**<0.001**
Duration of DM	+ 0.42	**<0.05**
Fasting blood sugar	+ 0.58	**<0.001**
Hb A1c	+ 0.58	**<0.001**
Urea	+ 0.43	**<0.05**
Creatinine	+ 0.41	**<0.05**
eGFR	- 0.77	**<0.001**
UACR	+ 0.88	**<0.001**
CD 14	+ 0.49	**<0.001**

T2DM: Type 2 diabetes mellitus, **BMI:** Body mass index, **MAP:** Mean arterial pressure, **HbA1c:** Glycated hemoglobin, **eGFR:** estimated glomerular filtration rate, **UACR:** Urinary albumin creatinine ratio

Table 4: Multivariate regression analysis for independent risk factors for the occurrence of microalbuminurea in T2DM patients

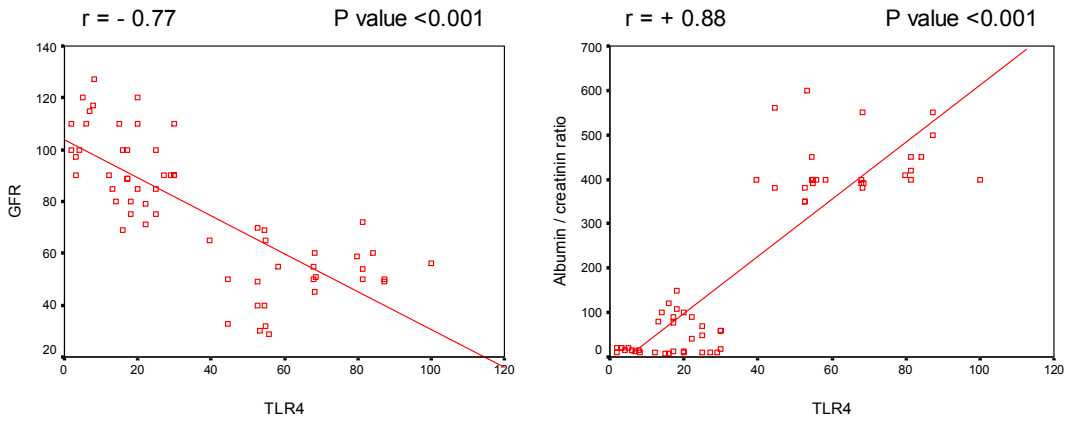
Parameters	**β**	**SE**	**Expected (β) (odds ratio)**	**P Value**	**95% CI**	
					Lower	Upper
MAP	- 0.60	49.5	0.99	0.86	0.02	5.7
Duration of DM	- 0.03	57.2	0.93	0.47	0.12	7.6
Fasting blood sugars	- 0.04	23.8	1.01	0.56	0.19	10.3
Hb A1c	1.82	79.8	0.96	0.68	0	16.9
eGFR	0.11	75.6	0.89	0.76	0.23	6.7
CD14	0.36	34.7	1.80	**<0.05**	0.56	7.6
TRL4	0.27	22.4	2.1	**<0.05**	0.88	5.8

T2DM: Type 2 diabetes mellitus, **DN:** Diabetic nephropathy, **MAP:** Mean arterial pressure, **HbA1c:** Glycated hemoglobin, **eGFR:** estimated glomerular filtration rate, **UACR:** Urinary albumin creatinine ratio



eGFR: estimated glomerular filtration rate, **UACR:** Urinary albumin creatinine ratio

Figure 2: Pearson correlation between serum CD14 and eGFR and UACR



eGFR: estimated glomerular filtration rate, **UACR:** Urinary albumin creatinine ratio

Figure 3: Pearson correlation between serum TLR4 and eGFR and UACR

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 [24]. In the present work we aimed to analyze the relationship between the inflammatory markers CD14 and TLR4 and the UACR as a marker of diabetic nephropathy in T2DM patients.

In the current study there were significant differences between the four studied groups regarding both CD14 and TLR4. When comparing each two groups together; CD14 levels were significantly elevated only in T2DM patients with macroalbuminurea. On the other hand, TLR4 levels were significantly elevated in T2DM patients compared to the control group and in T2DM patients with macroalbuminurea compared to both normoalbuminuric and microalbuminuric T2DM patients. TLRs have been considered as activators of inflammation under hyperglycemia and insulin resistance. [25-28] **Dasu and colleagues** measured the TLR mRNA, protein expression, TLR ligands, and TLR signaling in freshly isolated monocytes from 23 healthy human control subjects and 23 T2DM patients using real-time RT-PCR, Western blot, and flow cytometric assays. Consistent with our results they found that T2DM patients had significantly increased TLR2, TLR4 mRNA, and protein in monocytes compared with control subjects. They concluded that they made the novel observation that TLR2 and TLR4 expression and their ligands, signaling, and functional activation are increased in recently diagnosed T2DM and contribute to the pro-inflammatory state. [29] **Shi *et al.,*** and **Wong *et al.,*** also reported consistent observations confirming the implication of TLR4 in the pathogenesis of insulin resistance T2DM. [7,8] Similar results, but in type 1 DM (T1DM), were obtained by **Devaraj *et al.,*** who examined TLR2 and TLR4 expression in monocytes from 31 T1DM patients and 31 controls. They found that TLR2 and TLR4 surface expression and mRNA were significantly increased in T1DM monocytes compared with controls. [30]

In the current study, although the CD14 levels were elevated in normoalbuminuric T2DM patients compared to the control subjects, however this elevation was not statistically significant. **Fernández-Real *et al.,*** stated that systemic CD14 expression might play a role in obesity and inflammation-induced insulin resistance [31]. The presence of two forms of CD14 as well as the possible presence of other cofactors for TLR4 activation could be the reason why a significant elevation in T2DM patients was present in TLR4 but not in CD14 level. [16-19]

Although TLRs are increased in diabetic patients and have been suggested to play a role DN, the relation between TLR4 and the pathogenesis of DN has not been studied extensively. [32] In our study both CD14 and TLR4 expression was significantly elevated in patients with macroalbuminurea in comparison to normoalbuminuric and microalbuminuric T2DM patients as well as control subjects. Close findings, but *in vitro*, were observed by **Kaur and coworkers** who hypothesized that the expression of TLRs in the mesangium might be an important factor contributing to mesangial expansion and nephropathy. It is postulated that progression of DN involves altered mesangial cell (MC) function with an expansion of the mesangial matrix. So, they evaluated the effect of high glucose on TLR2 and

TLR4 expression in mouse mesangial cells (MMC) *in vitro*. They found that Exposure of MMC to 25 mM glucose for 24 h resulted in increased TLR4 mRNA and cell surface receptor expression compared with 5.5 mM glucose. They concluded that hyperglycemia activates TLR4 expression and activity in MMC and could contribute to DN.[32] Recently in 2014, concordant results were observed by **Verzola and colleagues.** They studied the TLR4 gene and protein expression and TLR4 downward signaling in kidney biopsies of 12 patients with T2DM and microalbuminuria, and compared them with 11 patients with overt DN, 10 with minimal change disease (MCD), and control kidneys from 13 patients undergoing surgery for a small renal mass. They found that both in microalbuminuria and in overt DN, TLR4 mRNA and protein were overexpressed 4- to 10-fold in glomeruli and tubules compared with the control kidney and in MCD. [33] Unlike our results, they observed significant differences in TLR4 levels in patients with both microalbuminurea and macroalbuminurea, while the presence of significant differences was only in patients with macroalbuminurea in our study. This could be explained by the fact that **Verzola and colleagues** measured TLR4 gene and protein expression in kidney biopsies, while we used Flowcytmetric analysis of TLRs expression on peripheral monocytes. The former is most probably more accurate in detecting minor changes at the level of the kidney tissues.

Furthermore, we found that both CD14 and TLR4 showed significant positive correlations with each of MAP, duration of DM, FBG, HbA1c, blood urea, serum creatinine and UACR and significant negative correlations with eGFR in T2DM patients whether with or without albuminurea. Consistent with our results, **Dasu *et al.,*** found that increased TLR2 and TLR4 expression correlated with BMI, fasting blood glucose and HbA1C in addition to homeostasis model assessment–insulin resistance (HOMA-IR), Nε-(carboxymethyl) lysine (CML), and free fatty acid (FFA). [29] On the other hand, **Lorenzen *et al.,*** found a significant positive correlation between MAP and both CD14 and TLR4 in 191 patients with chronic kidney disease (CKD) Stage V receiving hemodialysis therapy. [34]

Microalbuminuria is generally considered as the earliest non-invasive marker for the development of DN. [5] In our study, a multivariate regression analysis using all the parameters in the studied groups was done to detect the independent risk factors for the occurrence of microalbuminurea (i.e. in groups 3 and 4). It demonstrated that CD14 and TRL4 were independent risk factors for occurrence of microalbuminurea in T2DM patients.

Conclusion

In conclusion, we found that CD14 and TLR4 levels were higher in T2DM patients compared to normal subjects. These observations significantly add to the emerging role of TLRs in T2DM development. CD14 and TLR4 were also found to correlate well with the severity of albuminuria in T2DM and to be good predictors of microalbuminurea suggesting their possible role in pathogenesis and progression of DN.

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