Immunol Res (2016) 64:951–960
DOI 10.1007/s12026-016-8806-x

ORIGINAL ARTICLE

# Cytokines profile and its correlation with endothelial damage and oxidative stress in patients with type 1 diabetes mellitus and nephropathy

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Published online: 15 June 2016


**Abstract** The aim of the present study was to investigate the association between the presence of albuminuria and cytokines profile with biomarkers of endothelial damage and oxidative stress in patients with type 1 diabetes mellitus (DM1). The sample was composed by 35 healthy individuals, 63 DM1 patients with normoalbuminuria (<30 mg of albumin/g of creatinine) and 62 DM1 patients with micro- and macroalbuminuria (≥30 mg of albumin/g of creatinine). Plasma and urinary cytokines (TNF-α, IL-6 and IL-10) and thrombomodulin levels were determined by ELISA. Oxidative status was evaluated using the TBARS and MTT assays. Diabetic patients were characterized by elevated levels of urinary cytokines TNF-α, IL-6 and IL-10. Those with macroalbuminuria presented significantly higher TNF-α and IL-10 urinary levels when compared to other groups. Urinary and plasmatic levels of TNF-α were positively correlated with plasma levels of cystatin C, creatinine, urea and albuminuria, while they were negatively correlated with estimated glomerular filtration rate. Urinary IL-10 levels proved positive correlation with fasting glucose, HbA1c, thrombomodulin and TBARS, while IL-6 plasma levels were positively correlated with HbA1c and albuminuria. Only urinary TNF-α levels were associated with the presence and severity of macroalbuminuria, after logistic regression analysis. This finding suggests that measurement of urinary TNF-α level may be helpful to evaluate progression to nephropathy in DM1 patients.

**Keywords** Type 1 diabetes mellitus · Albuminuria · Cytokines · Oxidative stress · Endothelial damage

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## Introduction

Diabetic nephropathy (DN) is the main cause of kidney disease and affects about 40 % of type 1 and type 2 diabetic patients. DN presents a clinical diagnosis based on the finding of proteinuria or microalbuminuria. The diabetes mellitus is an independent risk factor for the chronic kidney disease (CKD) development and glomerular filtration rate (GFR) decrease, contributing to cardiovascular morbidity and mortality [1, 2].

DN is morphologically characterized by progressive thickening of glomerular basement membrane, accumulation of extracellular matrix proteins (collagen, fibronectin and laminins) in the glomerular mesangium and tubulointerstitial disease, which negatively correlates with glomerular filtration function [3].

Experimental and clinical evidences suggest that inflammation plays a role in the pathogenesis of DN, in addition to hemodynamic and metabolic changes [4]. Inflammation may contribute to the early risk of progression to microalbuminuria and evident nephropathy, even

when urinary albumin excretion is within normal limits. Factors that promote renal inflammation and the loss of protective pathways in the preclinical phase of DN are poorly understood, but may include endothelial shear stress associated with glomerular hyperfiltration [5]. Many studies showed an increase in macrophage infiltration and overproduction of leukocyte adhesion molecules in kidney from diabetic individuals as well as in experimental animal models of diabetes mellitus [6, 7]. In addition, long-term low-grade inflammation may be associated with the onset of several disturbances including insulin resistance, hyperglycemia, oxidative stress and endothelial dysfunction, which secondary play a critical role in perpetuating renal damage and progression [8].

Whereas inflammation, oxidative stress and endothelial damage are strongly associated with the risk of development of diabetic complications, a huge number of plasma and urine biomarkers have been investigated for early diagnosis of DN progression. In this context, the aim of this study was to evaluate the association and correlation between albuminuria, cytokines profile (TNF-α, IL-6 and IL-10) and markers of endothelial dysfunction (thrombomodulin) and oxidative stress (TBARS and MTT) in the development of DN. This study can contribute to expanding the understanding about the mechanisms associated with DN progression and potential laboratorial tools for prognosis and monitoring purposes.

## Materials and methods

### Subjects

A total of 125 individuals with type 1 diabetes mellitus (DM1) (45 men, 80 women; aged 18–60 years ), consecutively selected in an outpatient diabetic clinic, were included in this study. After investigating the exclusion criteria, each patient was classified according to normoalbuminuria ($n = 63$, group 1A) or micro- and macroalbuminuria (diabetic kidney disease group) ($n = 62$, group 2A). The control was composed by 35 subjects (12 men, 23 women; aged 18–60 years), without diabetes mellitus, renal disease and hypertension.

The diagnosis of diabetes was performed according to the criteria of American Diabetes Association (ADA) [9, 10]. Normoalbuminuria was defined as <30 mg of albumin/g of creatinine and micro- and macroalbuminuria as ≥30 mg of albumin/g of creatinine. The presence of microalbuminuria or macroalbuminuria was confirmed in two out of three occasions, over a period between 3 and 6 months.

Data and samples were collected from patients assisted by the Endocrinology of the Clinic Hospital (Federal University of Minas Gerais) and Santa Casa of Belo Horizonte, in the period from November/2011 to September/2012. The individuals had their medical records analyzed for information such as the use of medications, comorbidities and complications of diabetes. Individuals (case and control group) with liver disease, alcoholics, coagulation disorder, cancer, infectious or inflammatory process in development, hemodialysis, renal transplantation history or pregnancy were excluded from this study. Individuals that presented C-reactive protein (CRP) >10 mg/dL were also excluded in order to avoid asymptomatic inflammatory process.

### Ethics

This study was approved by Ethics Committee at Federal University of Minas Gerais and Santa Casa of Belo Horizonte. Informed consent was obtained in all cases. The protocol was in accordance with the Declaration of Helsinki and the Convention on Human Rights and Biomedicine from the Council of Europe. The research protocol did not interfere with the medical recommendation.

### Biochemical determinations

Blood samples were drawn before breakfast after 8–12 h overnight fasting. Samples were collected in sterile tubes, centrifuged at 3000 *g* for 15 min at 4 °C and stored at −80 °C until assayed.

Fasting glucose, serum creatinine (Cr) and urea were determined by enzymatic method; serum albumin was assessed by a colorimetric method; and glycated hemoglobin (HbA1c) was determined by immunoturbidimetry using Johnson & Johnson kits—dry chemistry techniques (Ortho Clinical Diagnostics®) and analytical equipment VITROS 4600. Cystatin C was measured in plasma, collected in sodium citrate samples, by enzyme-linked immunosorbent assay (ELISA) using the kit Human Cystatin C (Biovendor®). The estimated glomerular filtration rate (eGFR) was calculated using the abbreviated modification of diet in renal disease formula [eGFR-MDRDa: 186 × plasma creatinine $(\text{mg/dL})^{-1.154}$ × age $(\text{years})^{-0.203}$ × 0.742 (if female) × 1.212 (if black)] [11].

First-morning urine samples (at least 4 h retention) were collected under sterile conditions, and 10 mL of urine was centrifuged at 3000 *g* for 10 min. The supernatant was stored at −80 °C until assay. The same specimen was used for urinary albumin excretion (UAE) determination. Urinary albumin was corrected by urine creatinine. Urinary albumin was evaluated by immunoturbidimetry and urinary creatinine was determined by enzymatic method, using Johnson & Johnson kits—dry chemistry technology (Ortho Clinical Diagnostics®) and analytical equipment VITROS 4600.

### Cytokines and thrombomodulin determinations

For determination of cytokines and thrombomodulin levels, plasma was collected using sodium citrate as anticoagulant and stored at −80 °C until assayed.

Thrombomodulin, which is used as a marker of endothelial damage, was measured by sandwich ELISA method (IMUBIND®, Sekisui Diagnostics). The plasma concentration of TNF-α was determined by a high-sensitivity quantitative sandwich enzyme immunoassay (R&D Systems®). The IL-6 levels were measured using a high-sensitivity quantitative sandwich enzyme immunoassay (R&D Systems®). For determination of plasma and urinary IL-10 concentrations and urinary levels of TNF-α, we used a solid-phase sandwich ELISA (R&D Systems®).

Urinary levels of cytokines (TNF-α, IL-6 and IL-10) were corrected by urinary Cr concentration and expressed as TNF-α/Cr (pg/g), IL-6/Cr (pg/g), IL-10/Cr (pg/g), respectively. The ratio between the plasma and urinary levels for each cytokine was also calculated.

### Oxidative stress determination

Lipid peroxidation was evaluated through plasma thiobarbituric acid reactive species (TBARS) concentration. Lipid peroxidation creates secondary products such as alkanes, aldehydes and isoprostanes that can be used to evaluate the oxidative status of biological materials [12]. The TBARS quantification was performed according to Duarte et al. [13] modified: 100 μL of plasma was added to 100 μL of ice cold solution of TBA 1 % (TBA 1 %, NaOH 0.05 mol/L and 0.1 mmol/L butylated hydroxytoluene—BHT) and 100 μL of phosphoric acid ($H_3PO_4$) concentrate. The solution was incubated in bath-dried for 25 min at 98 °C and then put in the freezer for 10 min. Thereafter, 375 μL of butanol was added, which was vortexed for 10 s and centrifuged for 5 min at 2000 *g*. The absorbance of the supernatant was measured in a spectrophotometer at 532 and 600 nm.

The antioxidant capacity of the plasma was determined with the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium (MTT) quantification, according to described by Medina et al. [14] modified. A solution containing 100 μL of plasma, 50 μL of PBS, and 12.5 μL of MTT solution was incubated at 37 °C for 60 min and protected from light. Then, 750 μL of a solution of isopropanol acid (0.04 M; HCl) was added and vortexed for 30 s. Subsequently, the tubes were centrifuged at 3000 *g* for 10 min. The absorbance of the supernatant was measured at 570 nm.

### Statistical analysis

Analyses were performed with SPSS package (version 13.0 for Windows; Chicago, Illinois, USA). Data were expressed as mean ± SD or median (interquartile range) as appropriate. The normality of the quantitative variables was assessed by the Shapiro–Wilk test. Student's *t* test and ANOVA or the Mann–Whitney and Kruskal–Wallis (followed by Bonferroni test) determined the differences between two and three groups in parametric and nonparametric variables, respectively. Differences in frequencies were evaluated with Chi-square test. Correlations between plasma and/or urinary cytokines and other parameters were performed using Spearman's correlation test. A forward stepwise multivariate logistic regression analysis was applied to identify the independent predictors of albuminuria (micro- and macroalbuminuria vs. normoalbuminuria) or macroalbuminuria (when compared to microalbuminuria) among the variables with a $p$ value <0.2 in the univariate analysis. Hosmer–Lemeshow test was applied to evaluate the adequacy of the multivariate model. Level of statistical significance was considered as $p$ value $< 0.05$.

## Results

Clinical and laboratory characteristics are described in Table 1. Gender, age and body mass index (BMI) were not different between the groups after Bonferroni test correction. Retinopathy was more prevalent on group 1A, while hypertension and dyslipidemia were more frequent in 2A group.

There were no significant differences between the groups 1A/2A regarding to glycemic control (fasting glucose and HbA1c) after Bonferroni test correction, but these levels were higher than the values presented by the control group. Duration of diabetes and neuropathy did not differentiate the 1A and 2A groups.

As expected, patients in the group 2A had higher levels of albuminuria, creatinine, urea, cystatin C and lower eGFR when compared to the control and 1A groups, whereas the 1A group presented higher levels of urea and albuminuria when compared to control group.

When compared to group 2A, individuals with normoalbuminuria (1A) presented significantly lower TNF-α and IL-6 urinary levels. The group 2A showed elevated urinary levels of TNF-α and IL-6, besides decreased plasma/urine ratio of IL-6 when compared with the control group. In addition, individuals with normoalbuminuria presented significantly higher levels of IL-10 urinary when compared to the control group, as well as lower plasma/urine ratio of IL-10 (Table 2).

Regarding the thrombomodulin, no difference was observed when compared the three groups, although higher mean levels in diabetic groups were obtained. Similarly, TBARS levels did not differentiate the three groups. However, the antioxidant capacity, measured through MTT

**Table 1** Clinical and laboratory variables in control and diabetic groups

| | Control group | Type 1 diabetes mellitus | | *P* value |
|---|---|---|---|---|
| | | 1A<br>UAE < 30 mg/g Cr | 2A<br>UAE ≥ 30 mg/g Cr | |
| Subjects *n* (%) | 35 (21.8) | 63 (39.4) | 62 (38.8) | |
| Age (years) | 32.80 ± 8.63 | 32.84 ± 9.54 | 34.56 ± 10.13 | 0.984[a] |
| | | | | 0.383[b] |
| | | | | 0.317[c] |
| Gender; male *n* (%) | 12 (21) | 25 (44) | 20 (35) | 0.675 |
| BMI (kg/m$^2$) | 24.1 (5.0) | 24.4 (4.5) | 22.9 (4.7) | 0.795[a] |
| | | | | 0.331[b] |
| | | | | 0.217[c] |
| Retinopathy *n* (%) | – | 45 (64.3) | 25 (35.7) | <0.001* |
| Neuropathy *n* (%) | – | 52 (49.5) | 53 (50.5) | 0.653 |
| Hypertension *n* (%) | 0 (0.0)[##] | 34 (41.0) | 49 (59.0)[#] | <0.001* |
| Dyslipidemia *n* (%) | 0 (0.0)[##] | 17 (34.7) | 32 (65.3)[#] | <0.001* |
| Duration of diabetes (years) | – | 18.44 ± 7.97 | 20.52 ± 7.97 | 0.154 |
| Fasting glucose (mg/dL) | 82.0 (8.0) | 166.0 (137.0) | 130.0 (152.0) | <0.001*,[a] |
| | | | | 0.001*,[b] |
| | | | | 0.030[c] |
| HbA1c (%) | 5.1 (0.4) | 8.3 (2.2) | 8.8 (2.3) | <0.001*,[a] |
| | | | | <0.001*,[b] |
| | | | | 0.037[c] |
| Creatinine (mg/dL) | 0.75 (0.19) | 0.80 (0.24) | 1.09 (0.88) | 0.173[a] |
| | | | | <0.001*,[b] |
| | | | | <0.001*,[c] |
| Urea (mg/dL) | 26.0 (9.0) | 29.0 (9.0) | 42.0 (25.0) | 0.020*,[a] |
| | | | | <0.001*,[b] |
| | | | | <0.001*,[c] |
| Cystatin C (ng/mL) | 754.9 (263.0) | 738.0 (196.0) | 1095.0 (1183.0) | 0.681[a] |
| | | | | <0.001*,[b] |
| | | | | <0.001*,[c] |
| Albuminuria (mg/g Cr) | 1.9 (3.4) | 6.6 (10.3) | 342.5 (1290.7) | <0.001*,[a] |
| | | | | <0.001*,[b] |
| | | | | <0.001*,[c] |
| eGFR (mL/min/1.73 m$^2$) | 108.4 ± 12.3 | 106.7 ± 28.2 | 72.2 ± 36.8 | 0.635[a] |
| | | | | <0.001*,[b] |
| | | | | <0.001*,[c] |

Normally distributed data were expressed as mean ± SD and compared by ANOVA and *t* test. Not normally distributed data were expressed as median (range) and compared by the Kruskal–Wallis or Mann–Whitney *U* test followed by Bonferroni correction. Categorical variables were expressed as frequencies *n* (%) and compared using the Chi-square test ($\chi^2$). * Significant. Adjusted residue: [#] more frequent; [##] less frequent

[a] Control group versus group 1A

[b] Control group versus group 2A

[c] Group 1A versus group 2A

levels, was higher in both groups with type 1 diabetes mellitus than in the control group (Table 3).

In the group with diabetes, urinary TNF-α levels were positively correlated with plasma levels of cystatin C, creatinine, urea and albuminuria, while they were negatively correlated with eGFR. Similar correlations were found for plasma TNF-α levels to cystatin C, urea and albuminuria. IL-6 plasma levels were positively correlated

**Table 2** Plasma and urine cytokines levels in control and diabetic groups

| | Control group | Type 1 diabetes mellitus | | *p* value |
|---|---|---|---|---|
| | | 1A<br>UAE < 30 mg/g Cr | 2A<br>UAE ≥ 30 mg/g Cr | |
| Plasma TNF-α (pg/mL) | 5.98 (6.31) | 4.05 (5.61) | 6.20 (4.29) | 0.939[a] |
| | | | | 0.040[b] |
| | | | | 0.031[c] |
| Urine TNF-α (pg/g Cr) | 0.17 (0.27) | 0.23 (0.24) | 0.43 (0.59) | 0.931[a] |
| | | | | 0.007*,[b] |
| | | | | 0.003*,[c] |
| Ratio TNF-α (plasma/urine) | 12.52 (39.14) | 21.74 (31.90) | 14.28 (21.81) | 0.674[a] |
| | | | | 0.446[b] |
| | | | | 0.169[c] |
| Plasma IL-6 (pg/mL) | 1.90 (2.57) | 2.05 (1.83) | 1.47 (2.03) | 0.527[a] |
| | | | | 0.387[b] |
| | | | | 0.625[c] |
| Urine IL-6 (pg/g Cr) | 0.010 (0.01) | 0.017 (0.02) | 0.028 (0.07) | 0.573[a] |
| | | | | 0.014*,[b] |
| | | | | 0.011*,[c] |
| Ratio IL-6 (plasma/urine) | 214.3 (338.0) | 129.7 (334.2) | 76.6 (216.6) | 0.095[a] |
| | | | | 0.006*,[b] |
| | | | | 0.108[c] |
| Plasma IL-10 (pg/mL) | 8.33 (5.03) | 6.80 (9.18) | 7.07 (4.39) | 0.267[a] |
| | | | | 0.185[b] |
| | | | | 0.961[c] |
| Urine IL-10 (pg/g Cr) | 4.36 (7.60) | 6.89 (10.87) | 7.04 (16.49) | 0.006*,[a] |
| | | | | 0.038[b] |
| | | | | 0.953[c] |
| Ratio IL-10 (plasma/urine) | 2.13 (31.63) | 0.99 (2.78) | 1.27 (21.14) | 0.004*,[a] |
| | | | | 0.053[b] |
| | | | | 0.410[c] |

Not normally distributed data were expressed as median (range) and compared by the Kruskal–Wallis or Mann–Whitney *U* test followed by Bonferroni correction

* Significant

[a] Control group versus group 1A

[b] Control group versus group 2A

[c] Group 1A versus group 2A

with HbA1c and albuminuria. Finally, urinary IL-10 levels proved positive correlation with HbA1c, fasting glucose, thrombomodulin and TBARS (Fig. 1). No other significant correlation was observed between the cytokines levels and laboratorial parameters.

The logistic regression analysis showed that the only variable independently associated with macroalbuminuria (vs. microalbuminuria) in patients with diabetes was the urinary TNF-α levels, which showed an odds ratio of 8.96 (CI 2.19–36.68) and $p = 0.002$ (Hosmer and Lemeshow test = 0.657). Regarding the albuminuria (micro- and macroalbuminuria vs. normoalbuminuria), urinary TNF-α levels were also the only variable independently associated with this status, which showed an odds ratio of 5.47 (CI 1.24–24.14) and $p = 0.025$ (Hosmer and Lemeshow test = 0.497).

## Discussion

While the mechanism responsible for the development of kidney injury is not completely understood, inflammation is critical in the development of early injury in preclinical DN [5]. Therefore, the evaluation of plasma and urine

**Table 3** Oxidative stress and endothelial damage markers in control and diabetic groups

| | Control group | Type 1 diabetics | | *p* value |
|---|---|---|---|---|
| | | 1A<br>UAE < 30 mg/g Cr | 2A<br>UAE ≥ 30 mg/g Cr | |
| Thrombomodulin (pg/mL) | 2.75 (5.27) | 3.74 (8.82) | 3.40 (4.27) | 0.154[a]<br>0.658[b]<br>0.391[c] |
| TBARS | 0.19 ± 0.33 | 0.17 ± 0.05 | 0.18 ± 0.04 | 0.095[a]<br>0.820[b]<br>0.089[c] |
| MTT | 0.23 (0.09) | 0.28 (0.21) | 0.29 (0.13) | 0.001*,[a]<br><0.001*,[b]<br>0.984[c] |

Normally distributed data were expressed as mean ± SD and compared by ANOVA and *t* test. Not normally distributed data were expressed as median (range) and compared by the Kruskal–Wallis or Mann–Whitney *U* test followed by Bonferroni correction

* Significant

[a] Control group versus group 1A

[b] Control group versus group 2A

[c] Group 1A versus group 2A

| VARIABLE | Plasma TNF-α (pg/mL) | Urinary TNF-α (pg/g Cr) | Plasma IL-6 (pg/mL) | Urinary IL-10 (pg/g Cr) |
|---|---|---|---|---|
| Cystatin C (ng/dL) | r=0.346<br>P<0.001 | r=0.434<br>P<0.001 | | |
| Creatinine (mg/dL) | | r=0.281<br>P=0.007 | | |
| Urea (mg/dL) | r=0.271<br>P=0.003 | r=0.379<br>P=0.007 | | |
| Albuminuria (mg/g Cr) | r=0.251<br>P=0.007 | r=0.410<br>P<0.001 | r=0.244<br>P=0.002 | |
| eGFR (mL/min/1.72m$^2$) | | r=-0.437<br>P<0.001 | | |
| HbA1c (%) | | | r=0.165<br>P=0.049 | r=0.166<br>P=0.036 |
| Fasting glucose (mg/dL) | | | | r=0.216<br>P=0.006 |
| Trombomodulin (pg/mL) | | | | r=0.211<br>P=0.021 |
| TBARS (arbitrary units) | | | | r=0.192<br>P=0.015 |

**Fig. 1** Significant correlation between cytokines levels and other variables in diabetic group

markers of inflammation that could predict the progression to macroalbuminuria in patients with diabetes is a focus in many studies.

The higher retinopathy frequency on 1A group, when compared to 2A, was unexpected, since it is not common the presence of nephropathy in the absence of retinal damage. However, retinopathy was information obtained from the medical records and we cannot exclude a possibility of missing information about this diabetes complication.

Increased TNF-α urinary levels were observed with the worsening of the nephropathy (UAE ≥ 30 mg/g Cr), since

higher values were observed for the 2A group when compared to 1A and controls. The TNF-α plasma levels were also higher in the 2A group; nevertheless, the significance was lost when the Bonferroni correction was applied. These results suggest that this cytokine could be potential markers of albuminuria progression (UEA $\geq$ 30 mg/g Cr) in diabetic patients.

These data are in agreement with Lampropolou et al. [15] and Ruotsalainen et al. [16]. In a previous study [17], we have also shown that inflammatory status is associated with nephropathy in DM1, since higher plasma levels of INF-γ, TNF-α, IL-6, IL-10 in patients with diabetes and CKD (albuminuria $\geq$30 mg/g and/or GFR <60 mL/min/1.73 $m^2$) were observed when compared to those without CKD. Interestingly, we suggested that, although IL-10 has been considered an anti-inflammatory cytokine, its increase could be associated with a compensatory mechanism, in consequence of proinflammatory cytokines levels increase.

Urinary TNF-α was a distinguishing factor associated with macroalbuminuria and albuminuria in patients with diabetes. This result can be strengthened by those found by Kalantarinia et al. [18] in an experimental animal model. Three days after the diabetes induction in mice promoted a significant increase in urinary and interstitial (renal) levels of TNF-α, which correlated with the increase in UAE. Similar results were reported by DiPetrillo et al. [19], who found that TNF-α urinary levels were still significantly higher than the interstitial levels, suggesting that there are other sections of the kidneys responsible for TNF-α production, especially in the renal cortex. In a logistic regression analyses, Ng et al. [20] found that the score of TNF-α system (plasma TNF-α and soluble forms of cellular receptors; sTNFR1 and sTNFR2) was independently associated with UAE. Likewise, Lampropoulou et al. [15] observed that urinary levels of TNF-α were the only independent contributor to UAE, although other factors had been included in the multivariate analysis: age, duration of diabetes, BMI, history of cardiovascular disease, presence of retinopathy, hypertension, and HbA1c levels.

The main mechanism proposed indicates that high glucose and advanced glycation end products (AGEs) induce expression of monocyte chemoattractant protein-1 (MCP-1), colony-stimulating factor-1 (CSF-1) and cell adhesion molecules (ICAM-1 and VCAM-1) in many renal cellular compartments, which enhance recruitment and maturation of monocytes/macrophages in the kidney under high-glucose environment. AGEs are also involved on polarization toward a proinflammatory M1 phenotype and release considerable amounts of proinflammatory factors, as TNF-α, which consequentially stimulate production of reactive oxygen species (ROS), leading to subclinical chronic inflammation, glomerular and tubular damage, preceding the albuminuria [21–25]. This increased local production of TNF-α could result in urinary release, which confirms our data. The importance of this cytokine in the progression of DN was also demonstrated experimentally by Awad et al. [26], who showed that TNF-α-neutralizing antibody prevents kidney hypertrophy, albuminuria and preserves kidney function in a murine model of DM1. Furthermore, we observed a correlation between TNF-α and the markers currently used in the DN evaluation, suggesting that the use of all these markers together could enhance the sensibility of laboratorial diagnosis of DN progression.

IL-10 is an anti-inflammatory cytokine produced by macrophages, lymphocytes and locally at mesangial cell. IL-10 plays an important role in renal physiology, as well as during acute kidney injury and progression to chronic renal failure. The degree of proteinuria correlates with progression of glomerulosclerosis and tubulointerstitial fibrosis, and IL-10 can promote the deposition of mesangial immune complexes, which contributes for the progression of glomerular injury [27–30].

Curiously, we observed an increase of IL-10 in urinary samples from patients with type 1 diabetes mellitus (with normoalbuminuria and micro-macroalbuminuria), but significant between 1A and control groups, besides a lower plasma/urine ratio. These data suggest an increased IL-10 kidney production previously of the albuminuria progression in type 1 diabetic patients or increased IL-10 renal excretion due to hyperfiltration commonly observed in early kidney disease. A renal production of this cytokine could be a counter-regulatory inflammation marker, acting in order to reduce the inflammatory process observed in the DN. In agreement with our results, Wong et al. [31] showed that IL-10 plasma levels present a significant positive correlation with the UAE in patients with DN, and Mysliwska et al. [32] found strong evidence that IL-10 may influence the degree of albuminuria. Moreover, urinary IL-10 levels were correlated (Fig. 1) with fasting glucose, HbA1c, TBARS and thrombomodulin. Evidence has indicated that high-glucose conditions can modulate thrombomodulin expression in human aortic endothelial cells and mouse glomerular capillaries, as well as oxidative stress [33, 34]. These studies support our findings, in which we demonstrated a positive correlation between urinary levels of IL-10 with glycemic control markers, suggesting that inefficient glycemic control may induce an increase in urinary production of IL-10, endothelial injury and lipid peroxidation.

Urinary IL-6 levels were elevated in 2A group when compared to 1A and controls groups; besides, plasma/urine ratio was decreased in 2A when compared to control group. Moreover, its plasmatic levels were positively correlated with HbA1c and albuminuria. IL-6 has multiple stimulatory effects on cell growth and inflammation and it is involved in initiation and sustaining of the acute phase

inflammatory response in immune regulation and in non-immune events in many cell types and tissues outside the immune system. Also, it affects the homeostasis of glucose by the action in skeletal muscle cells, adipocytes, hepatocytes, pancreatic β cells and neuroendocrine cells [35–37].

Navarro-Gonzalez et al. [38] reported an IL-6 overexpression in the kidneys of diabetic rats directly associated with increased kidney size. Likewise, IL-6 overexpression was associated with increased UAE [38]. It is plausible that IL-6 affects extracellular matrix dynamics at the mesangial cell and podocyte levels, contributing to mesangial expansion and glomerular basement membranes [39].

Although we did not observed different thrombomodulin levels in the three groups, several studies have demonstrated that thrombomodulin plasma levels are significantly increased in diabetic patients with nephropathy [40–44]. In a cohort study, Von Scholten et al. [45] showed higher levels of thrombomodulin and TNF-α in diabetic patients with microalbuminuria, associated with cardiovascular disease and mortality. Bao et al. [46] showed an elevated plasma level of soluble thrombomodulin in CKD patients compared with the healthy subjects and a significant correlation with disease severity.

The loss of podocytes is a clear event in the progression of DN, which results of apoptosis, induced by a high concentration of glucose in several renal compartments. Yang et al. [47] have demonstrated that the recombinant thrombomodulin had a significant inhibitory effect on apoptosis in podocytes, in vitro. Some mechanisms have been proposed to explain the anti-inflammatory potential of thrombomodulin, at the renal setting, including: (1) interference with the binding of lipopolysaccharide to its receptor and inhibition of inflammatory mediator production by macrophages; (2) suppression of adhesion molecule expression by neutrophils acting via the NF-κB and MAPK pathways; (3) prevention of leukocyte activation; and (4) interference with complement activation (C3a and C5a) via the classical and lectin pathways [47–49]. Such hypotheses can be raised as an adaptive event to inflammatory state *per se* present in DN or by direct endothelial injury.

The role of oxidative stress in the pathophysiology of diabetes and nephropathy is evident and can be demonstrated in various experimental and clinical studies that shown a significant increase in lipid peroxidation, which correlates with glycemic control and may be consequent of nonenzymatic glycation activation of the sorbitol pathway and metabolic stress, resulting in increased production of free radicals and the decrease in the antioxidant capacity [50, 51]. Lipid peroxidation and free radicals generation can induce nephropathy by different means. A probable mechanism may result from the increased activity of phospholipase A2 (PLA2) that produces thromboxane A2 (TXA2) and the vasodilator prostacyclin (PGI2). A balance between PGI2 and TXA2 preserves natural vascular tune, but in diabetes, levels of TXA2 are increased while PGI2 levels are decreased and this imbalance may lead to a decrease in blood flow, associated with nephropathy [52].

The results observed in our study are in agreement with those reported by Medina et al. [14] who demonstrated that the antioxidant status of patients with type 2 diabetes mellitus is lower than in healthy subjects. In our study, this difference was observed among patients with type 1 diabetes mellitus (1A and 2A group) when compared with control group. In studies of Medina-Navarro et al. [53, 54], the results demonstrated that albumin isolated and purified from diabetic patients with advanced stages of renal damage and lower GFR presented a higher antioxidant response to stress than albumin from normal patients or patients in earlier stages of DN.

Some limitations may be raised in this study, as the small sample size and the transversal design. However, our results can contribute to better understand the relationship between inflammation and DN development. Moreover, to the best of our knowledge, this is the first study that investigated the plasma/urine ratio of cytokines levels in order to establish the association with UAE.

## Conclusion

The DM1 patients were characterized by elevated levels of urinary cytokines (TNF-α, IL-6 and IL-10). However, only urinary TNF-α levels were independently associated with the presence of micro- or macroalbuminuria, indicating that its intra-renal production may be involved in the pathogenesis and progression of DN. While further longitudinal studies are needed to elucidate the exact role of this cytokine in DN, this finding suggests that measurement of urinary TNF-α levels may be helpful, in association with other marker, to evaluate progression to nephropathy in DM1 patients.

**Acknowledgments** The authors thank FAPEMIG, CAPES and CNPq/Brazil. LPS, APF and KBG are grateful to CNPq Research Fellowship (PQ).

**Compliance with ethical standards**

**Conflict of interest** The authors report no declarations of interest.

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