

Thrombomodulin and interleukin 6 as potential biomarkers of endothelial dysfunction and inflammation after renal transplant

Trombomodulina e interleucina 6 como potenciais biomarcadores da disfunção endotelial e da inflamação pós-transplante renal

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ABSTRACT

Introduction: Endothelial dysfunction may contribute to hypercoagulable and inflammation states presents in renal transplant, chronic kidney disease (CKD) and its causes. These disorders can be evaluated by markers, such as thrombomodulin (TM), von Willebrand factor (vWF) and interleukin 6 (IL-6). **Objectives:** The aim of this study was to assess TM, vWF and IL-6 in renal transplant recipients (RTR) and associate their plasma levels with primary cause of end-stage renal disease (ESRD) and allograft function. **Methods:** 160 RTR were grouped according to the primary cause of CKD (G1: glomerulopathy; G2: hypertensive nephrosclerosis; G3: diabetic nephropathy; and G4: other causes/unknown etiology); creatinine plasma levels (C1 < 1.4 and C2 ≥ 1.4 mg/dl); and the estimated glomerular filtration rate (eGFR) (R1 < 60 and R2 ≥ 60 ml/min/1.73 m²). TM and vWF were determined by the enzyme-linked immunosorbent assay (ELISA) and IL-6 by flow cytometry. The results were presented as median, minimum and maximum; *p*-value < 0.05 was considered statistically significant. **Results:** TM levels were significantly higher in the G1 group compared to the others (G1: 8.38; G2: 5.51; G3: 5.88; G4: 6.33 ng/ml, *p* < 0.0001), and in the R1 group compared to R2 (R1: 6.65; R2: 6.19 ng/ml, *p* = 0.02). The concentration of IL-6, measured by the mean fluorescence intensity, was higher in C2 group when compared to C1 (C1: 7.9; C2: 13.35, *p* = 0.03). There was no difference in vWF levels among groups. TM correlated positively with IL-6 and creatinine, and negatively with eGFR. IL-6 also correlated positively with vWF. **Conclusion:** TM and IL-6 can be identified as potential markers for evaluating renal graft function. TM was more related to the primary cause of CKD compared to vWF and IL-6.

Key words: kidney transplant; endothelium; thrombomodulin; interleukin 6; von Willebrand factor.

INTRODUCTION

Hypertension, diabetes *mellitus* (DM) and primary glomerulopathy are the main causes of chronic kidney disease (CKD) in Brazilian subjects⁽¹⁾. These diseases can also occur after renal transplantation and are related to endothelial dysfunction, vascular fragility and tissue hypoxia⁽²⁾. All these conditions strongly contributes to increase the cardiovascular mortality risk among renal transplant recipients (RTR)^(3,4).

In patients with hypertension and diabetes, an increased production of reactive oxygen species (ROS) and others

inflammatory markers are found, proving that endothelial dysfunction is a relevant mechanism of these diseases^(5, 6). It is important to report that immunosuppressive therapy and impaired vascular graft can be associated to hypertension and its complications in transplant patients⁽⁷⁻⁹⁾.

The primary glomerulopathy as a cause of CKD is also characterized by endothelial dysfunction, which is related to inflammatory response. The inflammatory glomerular lesions could be caused by circulating inflammatory cells, proliferating glomerular cells or binding of antibodies to the glomerular endothelial cells induced by complement activation⁽¹⁰⁾.

In order to better evaluate these primary causes of CKD in RTR, some biomarkers have been investigated, such as thrombomodulin (TM), von Willebrand factor (vWF) and interleukin 6 (IL-6). TM is a membrane glycoprotein, present on the surface of endothelial cells which participates in the physiological mechanism of natural anticoagulation mediated by proteins C and S^(11, 12). The damaged endothelial cell releases soluble TM that can be identified in plasma samples⁽¹³⁾. The vWF is a multimeric glycoprotein synthesized by endothelial cells and megakaryocytic and is essential to the platelet adhesion and aggregation^(14, 15). High levels of vWF are associated with thrombotic and atherosclerotic processes and endothelial function^(16, 17). IL-6 is a pro-inflammatory cytokine that play an important role in allograft function. Inflammatory lesions and extracellular matrix proteins deposition could be influenced by IL-6 production⁽¹⁸⁾. The precise role of these biomarkers in renal transplantation and endothelial dysfunction has not been fully investigated yet.

In this context, we aimed to evaluate TM, vWF and IL-6 in Brazilian RTR and correlate them to the primary cause of end-stage renal disease (ESRD), creatinine plasma levels and estimated glomerular filtration rate (eGFR). The evaluation of these unusual biomarkers can contribute to the understanding of endothelial dysfunction after renal transplantation. This research could provide relevant biomarkers as new laboratorial tools for prognostic and clinical monitoring.

METHODS

Ethical aspects

This study was approved by the Research Ethics Committee at the Universidade Federal de Minas Gerais (UFMG), Brazil (Protocol no. ETIC 387/09). All subjects signed the informed consent form. The research protocol did not interfere with any medical recommendation or prescription.

Study population

This cross-sectional pilot study included 160 RTR, selected in two Brazilian Renal Transplant Centers (Clinical Hospital of UFMG and Felício Rocho Hospital, Belo Horizonte, Minas Gerais, Brazil) from 2010 to 2011. The study population consisted of 103 men and 57 women aged from 19 to 73 years, the period after transplant ranged from 1 to 229 months and plasma creatinine level ranged between 0.59 and 2.98 mg/dl. Patients were allocated into four groups, according to the primary cause of ESRD, as follows: G1: glomerulopathy ($n = 27$), G2: hypertensive

nephrosclerosis ($n = 38$), G3: diabetic nephropathy ($n = 19$), and G4: other causes (urinary tract infection, vesicoureteral reflux and polycystic kidney) or unknown etiology ($n = 76$). The diagnosis of G1 and G2 groups were performed based on urine analysis, creatinine and plasma ureia concentration, proteinuria and renal biopsy. G3 and G4 groups were identified by the same tests and other complementary examinations (glucoses levels, urine culture and ultrasonography). These data were obtained from medical records of all patients. For a secondary analysis, all patients were allocated into two groups according to creatinine plasma levels, as follows: C1: creatinine < 1.4 mg/dl ($n = 82$) and C2: creatinine \geq 1.4 mg/dl ($n = 78$)⁽¹⁹⁾. Finally, all transplant patients were distributed into two groups according to eGFR, as follows: R1 < 60 ml/min/1.73 m² ($n = 48$) and R2 \geq 60 ml/min/1.73 m² ($n = 112$)⁽²⁰⁾. Main clinical and demographic characteristics of the study population are presented in **Table 1**.

TABLE 1 – Clinical characteristics of renal transplant recipients

Characteristics	Values
Age (years)	44 (19-73)
Sex	
Male	103 (64.4%)
Female	57 (35.6%)
BMI (kg/m ²)	24.8 (17.6-34.7)
Creatinine plasma levels (mg/dl)	1.38 (0.59-2.98)
eGFR (ml/min/1.73 m ²)	59.17 (18.24-103.97)
Post-transplant period (months)	59 (1-229)
Causes of CKD	
Hypertension	38 (23.8%)
Glomerulonephritis	27 (16.9%)
Diabetes <i>mellitus</i>	19 (11.9%)
Others	10 (6.2%)
Unknown	66 (41.2%)
Post-transplant comorbidities	
Urinary tract infection	11 (6.9%)
Dyslipidemia	6 (3.8%)
Hypertension	6 (3.8%)
Diabetes <i>mellitus</i>	4 (2.5%)
CMV infection	3 (1.9%)
Other comorbidities	35 (21.8%)
No comorbidities	95 (59.4%)
Immunosuppressive therapy	
Tacrolimus	96 (60%)
Cyclosporine	7 (3.4%)
Rapamycin	56 (35%)
Mycophenolate mofetil	158 (98.8%)
Prednisone	146 (91.3%)

Values are presented as median (range) or whole number (%). Other comorbidities: other virus infections (viral hepatitis, herpes simplex and BK-virus), hypothyroidism, hyperparathyroidism, other cardiovascular diseases, hypomagnesemia and depression. BMI: body mass index; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; CMV: cytomegalovirus infection; BK-virus: member of the polyomavirus family.

Inclusion criteria

We have defined as inclusion criteria: patients clinically stable, according to medical evaluation at the time of blood collection; adults older than 18 years of age at time of recruitment; patients who had received kidney from living donors and were submitted to the same protocol of immunosuppression, which initially consisted of combined corticosteroid, calcineurin inhibitor (tacrolimus or cyclosporine) and mycophenolic acid, according to general guidelines on renal transplantation^(21, 22).

Exclusion criteria

Exclusion criteria were: acute or chronic allograft rejection defined by histological diagnosis, recent bone fracture and surgery (less than three months before blood collection), coagulopathy, thrombotic diseases, acute infection, recipients under hemodialysis treatment and acute allograft dysfunction. Acute allograft dysfunction was defined by an absolute enhance of 0.3 mg/dl or percentage increase ($\geq 50\%$) over baseline creatinine values, or oliguria (urine output of less than 0.5 ml/Kg/h for more than 6 h)^(20, 23-25).

Samples

Whole blood samples were drawn into sodium citrate 0.109 mol/l (Vacuette[®]) and EDTA-K2 1.8 mg/ml (Vacuette[®]) from all patients. The samples were processed within 1 hour after collection and centrifuged at 2.000 g for 20 min at 4°C to obtain plasma samples [platelet poor plasma (PPP)]. Plasma aliquots were separated immediately after centrifugation and stored at -80°C until the enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

TM and vWF measurements

Plasma TM and vWF levels were measured by ELISA, IMUNOBIND[®] Thrombomodulin ELISA and IMUNOBIND[®] vWF ELISA, respectively, following the manufacturer's instructions (American Diagnostica[®] Inc., USA). The TM reference range (provided by the manufacturer) was 4 to 5.35 ng/ml and vWF reference range was 683 to 1012 mU/ml (not provided by the manufacturer). We used the vWF reference range determined by our research group of a previous study⁽²⁶⁾.

IL-6 measurement

The determination of plasma IL-6 levels was performed by flow cytometry method/cytometric bead array (CBA) (BD[™] FACScalibur cytometer, California, USA) using the diagnosis

Human Inflammation kit (BD[®] Biosciences Pharmingen, USA). The CBA immunoassay was standardized by our workforce as described in a previous study⁽²⁷⁾ with minor modifications of the manufacturer's instructions. The results were expressed in mean fluorescence intensity (MFI). The expression of IL-6 results in MFI shows a better sensitivity to differentiate patients whose plasma cytokine levels are outside the linear range, especially when IL-6 are very low⁽²⁸⁾. Standard curves were performed daily to IL-6 as a quality control.

Creatinine plasma levels and eGFR

Plasma creatinine levels were measured by a specific enzymatic method (VITROS[®] 5.1 FS) according to the manufacturer's instructions and eGFR was calculated using the modification of diet in renal disease (MDRD) equation: $186 \times \text{plasma creatinine level}^{-1.154} \times \text{age}^{-0.203} \times 0.742$ (if female) $\times 1.212$ (if black)⁽²⁹⁾.

Statistical analysis

For statistical analysis, we used the GraphPad PRISM[®] software (version 5.0). The normal distribution was tested by Shapiro-Wilk. The comparison among groups was performed using Kruskal-Wallis followed by Dunn's test. The Spearman test was used for the correlation analysis (TM, vWF, IL-6, creatinine and eGFR). Results were expressed as median, minimum and maximum values. Statistically significance was defined as $p < 0.05$.

RESULTS

In this study, we compared different groups of RTR according to the primary cause of ESRD, plasma creatinine levels and eGFR. The demographic characteristics of the study population are showed in Table 1.

Median plasma TM concentration were significantly higher in G1 group (8.38 ng/ml; $p < 0.0001$) compared to the others (G2: 5.51 ng/ml; G3: 5.88 ng/ml; G4: 6.33 ng/ml). There were no differences between groups for plasma vWF ($p = 0.3$) and IL-6 ($p = 0.74$) concentrations regarding primary cause of ESRD (**Figure 1A**).

Considering the patients distribution by plasma creatinine level, plasma IL-6 level were significantly higher in the C2 subgroup (MFI = 13.35; $p = 0.03$) compared to C1 (MFI = 7.9) (**Figure 1B**). On the other hand, no differences were found for TM ($p = 0.21$) and vWF ($p = 0.16$) values, between C1 and C2 groups.

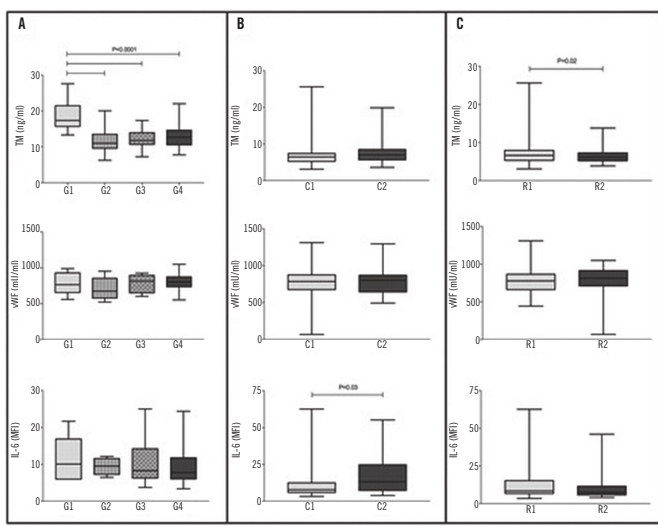


FIGURE 1 – Plasma concentration of TM, vWF and IL-6 from renal transplant recipients (RTR)

Plasma levels of TM, vWF and IL-6 according to primary cause of end-stage renal disease (ESRD) (A), creatinine plasma concentrations (B) and eGFR (C) in groups of RTR. All results are presented as median, minimum and maximum values. Data are expressed in ng/ml (TM), mU/ml (vWF) and MFI (IL-6); G1: glomerulopathy; G2: hypertensive nephrosclerosis; G3: diabetic nephropathy; G4: other causes or unknown etiology; C1: creatinine lower than 1.4 mg/dl; C2: creatinine lower or equal to 1.4 mg/dl; R1: eGFR lower than 60 ml/min/1.73 m²; R2: eGFR lower or equal 60 ml/min/1.73 m². Significant differences ($p < 0.05$) are highlighted by connecting lines.

TM: thrombomodulin; vWF: von Willebrand factor; IL-6: interleukin 6; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; RTR: renal transplant recipients; MFI: mean fluorescence intensity.

In the final analysis, the plasma eGFR, TM concentration were significantly higher in R1 (6.65 ng/ml; $p = 0.02$) when compared to R2 (6.19 ng/ml). Similarly to TM, IL-6 levels were higher in patients with low eGFR (R1), however not significantly ($p = 0.06$). No differences were observed for vWF ($p = 0.11$) levels comparing R1 and R2 (**Figure 1C**).

In correlation analysis, plasma TM concentration were positively correlated with concentrations of IL-6 ($r = 0.23$; $p = 0.01$) and creatinine ($r = 0.2$; $p = 0.01$) in plasma, and there was a negative correlation between TM and eGFR ($r = -0.17$; $p = 0.03$). However, all correlations were weak. Plasma vWF concentration were weakly correlated with IL-6 ($r = 0.23$; $p = 0.01$). The other correlations were not significant (**Table 2**).

DISCUSSION

Endothelial dysfunction is associated with systemic inflammation, insulin resistance and hypertension in patients with CKD. These patients have evidence of endothelial damage,

TABLE 2 – Correlation analysis between TM, vWF, IL-6 and plasma creatinine levels

Correlations	r coefficient	p-value
TM × IL-6	0.23	0.01
TM × creatinine	0.2	0.01
TM × eGFR	-0.17	0.03
TM × vWF	-0.09	0.2
vWF × IL-6	0.23	0.01
vWF × creatinine	-0.1	0.23
vWF × eGFR	0.1	0.23

TM: thrombomodulin; vWF: von Willebrand factor; IL-6: interleukin 6; eGFR: estimated glomerular filtration rate; $p < 0.05$ was significant.

measured by circulating markers, such as TM and IL-6⁽³⁰⁾. TM is a marker of endothelial function and it is present in cells along the vascular territory⁽³¹⁾. This marker is also an indicator of vascular damage⁽³²⁾.

In the present study, TM levels were significantly increased in G1 group, which presented chronic glomerulopathy as the primary cause of CKD, when compared to other groups (G2, G3 and G4). It is important to mention that there were no differences between groups related to age and gender in this analysis. It should also be noted that the medians for the four groups were above the reference range for TM (4 to 5.35 ng/ml), showing rising level of this marker in plasma of RTR.

The evaluation of endothelial dysfunction in patients with CKD or RTR is complex due to its multiple nature. In glomerulopathy, many mechanisms as proteinuria, hypoalbuminemia, dyslipidemia and insulin resistance may result in endothelial damage and promote a procoagulant state in these patients⁽³³⁻³⁵⁾. In our study, patients with hypertension or DM did not show increased levels of TM compared to patients with chronic glomerulopathy. It seems that TM levels are more influenced by chronic glomerulopathy than by other comorbidities associated with CKD. An increase in plasma TM concentrations in RTR has been previously reported in other studies^(4, 31, 33, 34, 36).

In this study, high levels of TM have also been found in patients with reduced eGFR (< 60 ml/min/1.73 m²) compared to patients with eGFR ≥ 60 . Liu *et al.* (2014)⁽³³⁾, and we also noted high plasma TM levels in patients with low eGFR. Other previous studies have reported that the endothelial damage is more marked in transplant patients with low eGFR⁽³⁷⁻³⁹⁾. Levels of eGFR should be evaluated along with creatinine plasma levels since creatinine may be influenced by muscle mass, tubular secretion, diet and patient's hydration status^(40, 41).

Endothelial damage is one of the most important factors involved in the development of cardiovascular and thrombotic

complications. Additionally, patients with high thrombotic risk also have increased levels of inflammatory markers⁽³⁹⁾. There are many endothelial and inflammatory biomarkers, but in our study we chose to evaluate TM, IL-6 and vWF due to the previous experience of our research group^(26, 42).

On this background, we found a correlation between the concentrations of TM and IL-6 in plasma. Our findings showed a possible link between renal disease, inflammation and endothelial dysfunction in RTR. Indeed, pro-inflammatory cytokines such as IL-6 and tumor necrosis factor alpha (TNF- α) influence not only the mechanisms that lead to the development of CKD, but also the rejection process after renal transplantation. On the other hand, modulating cytokines, such as IL-4 and IL-5, are involved in mechanisms of immunological tolerance^(26, 37, 43-45). Correlations were also found between concentrations of TM and creatinine in plasma (positive correlation) and there was a negative correlation between TM and eGFR. Similarly, a previous study reported a correlation between concentrations of TM and creatinine in plasma⁽⁴⁶⁾.

Renal transplantation is a clinical intervention that causes an inflammatory response, regardless the primary cause of CKD. This may result in increase of cytokine synthesis in RTR after transplantation. In our data, an increase in plasma IL-6 concentrations in patients with creatinine ≥ 1.4 mg/dl was observed. We also found higher levels of IL-6 in the group of patients with lower eGFR (R2), which corroborates the results obtained for creatinine, although this finding was not significant. IL-6 was previously demonstrated as a sensitive inflammatory marker to monitor the post-renal transplant and whether the creatinine level rises, IL-6 levels increases in the same proportion. The decrease in filtration rate may also affect the clearance of pro-inflammatory substances directly due to high production or indirectly by renal retention of these markers^(4, 41).

Correlations were found between IL-6 and vWF levels (positive correlation). Bolton *et al.* (2001)⁽³⁷⁾ also reported a positive correlation between IL-6 and vWF levels. These results may be explained by the ability of cytokines to regulate vWF production in endothelial cells. Cytokines have effects on the endothelium and can reduce the clearance of endothelial products.

In our study, no significant differences were found in vWF levels according to primary cause of CKD, creatinine plasma levels and eGFR. It is known that endothelial damage causes an increase in vWF secretion, which considerably increases the circulating levels of this factor. Damage of microvascular endothelial cells is a condition present in CKD and acute vascular rejection⁽⁴⁷⁾. In agreement, other studies showed high

levels of vWF in patients with CKD⁽³⁷⁾ and RTR^(4, 36). However, another study demonstrated that treatment with corticosteroids or mycophenolate might reduce vWF levels⁽⁴⁸⁾. In our research, 91.3% of patients have used corticosteroids and 98.8% have used mycophenolate. In fact, the immunosuppressive therapy can be a negative influence on immune response and inflammation, since it prevents cellular immune defense avoiding the activation of T-cells, which could also reduce endothelial dysfunction^(26, 49, 50). However, these immunosuppressive drugs could be associated with adverse effects related to endothelial cells damage and toxicity⁽⁵⁰⁾. Once the effects of immunosuppressive regimen appear to be unpredictable, the absence of significance for plasma vWF levels could be explained in part by the type immunosuppressive therapy used. Thus, we have as perspective of study to evaluate the influence of immunosuppressive drugs in TM, vWF and IL-6 levels.

We observed that the endothelial damage markers, such as TM and inflammatory markers, as IL-6, could play an important role in the evaluation of endothelial function in RTR. The results suggest that TM and IL-6 are associated with eGFR and creatinine plasma levels in these patients.

It is important to mention that the relatively low number of subjects and heterogeneity of the cohort, with time post-transplant ranging from 1 month to more than 20 years, limited this study. Despite the influence of the period post-transplant on TM, vWF and IL-6 levels has not been considered as a variable of interest in this study, this analysis was performed and showed no difference. The cross-sectional design of the study may also have interfered in the establishment of the influence of other accumulated comorbidities by patients on the primary cause of CKD.

CONCLUSION

These results indicate that TM and IL-6 could be used as biomarkers of allograft function in RTR. TM was also correlated to primary cause of CKD, vWF was not associated with any study variables. Further analysis is required to elucidate the role of these biomarkers in renal transplantation.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

RESUMO

Introdução: A disfunção endotelial pode contribuir para estados de hipercoagulabilidade e inflamação presentes no transplante renal e na doença renal crônica (DRC) e suas causas, podendo ser avaliada por marcadores como trombosmodulina (TM), fator de von Willebrand (FvW) e interleucina 6 (IL-6). **Objetivos:** Avaliar TM, FvW e IL-6 em receptores do transplante renal (RTR) e associar seus níveis com a causa primária de DRC pré-transplante e função do enxerto. **Métodos:** Foram alocados 160 RTR em grupos de acordo com a causa primária da DRC (G1: glomerulopatias; G2: nefrosclerose hipertensiva; G3: nefropatia diabética; e G4: outras causas/etiologia desconhecida), os níveis plasmáticos de creatinina ($C1 < 1.4$ e $C2 \geq 1.4$ mg/dl) e o ritmo de filtração glomerular estimado (eRFG) ($R1 < 60$ e $R2 \geq 60$ ml/min/1.73 m²). A TM e o FvW foram determinados pelo ensaio de imunoabsorção enzimática (ELISA) e a IL-6, por citometria de fluxo. Os resultados foram apresentados como mediana, mínimo e máximo; $p < 0,05$ foi considerado significativo. **Resultados:** Níveis de TM foram significativamente maiores no grupo G1 em comparação com os demais (G1: 8,38; G2: 5,51; G3: 5,88; G4: 6,33 ng/ml, $p < 0,0001$), e no grupo R1 comparado com o R2 (R1: 6,65; R2: 6,19 ng/ml, $p = 0,02$). A concentração de IL-6, avaliada pela intensidade média de fluorescência, foi maior no grupo C2 quando comparada com o C1 (C1: 7,9; C2: 13,35, $p = 0,03$). Não houve diferença entre os grupos para o FvW. TM correlacionou-se positivamente com IL-6 e creatinina e negativamente com eRFG. A IL-6 foi positivamente correlacionada com o FvW. **Conclusão:** TM e IL-6 podem ser apontadas como potenciais marcadores para avaliar a função do enxerto renal. A TM relacionou-se mais com a causa primária da DRC, se comparada com FvW e IL-6.

Unitermos: transplante de rim; endotélio; trombosmodulina; interleucina 6; fator de von Willebrand.

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