Urinary CXCL9 and CXCL10 Levels and Acute Renal Graft Rejection

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ABSTRACT

Background: Monitoring of chemokines, CXCL9 and CXCL10, in serum may present a non-invasive detection method for rejection.

Objective: To investigate the relationship between urinary levels of CXCL9 and CXCL10 and graft function following renal transplantation.

Methods: 75 living-related donor renal transplant recipients were studied. Urinary levels of chemokines were collected pre-operatively, on post-operative 1st day, 7th day, 1st month, 3rd month, and at the time of rejection. Chemokines levels were assayed using and enzyme-linked immunosorbent assay.

Results: Clinical variables were monitored. 10 (15%) patients had biopsy-proven rejection during the follow-up period. The urinary CXCL9 level in those with rejection was significantly higher than that in those with non-rejection group at the 1st day ($p<0.001$), 7th day ($p<0.001$), and at the time of rejection ($p=0.002$). The urinary CXCL10 level was also significantly higher in those with rejection compared with non-rejection group at 1st day ($p<0.001$), 7th day ($p<0.001$), and at the time of rejection ($p=0.001$). Serum creatinine level was strongly correlated with the urinary CXCL9 and CXCL10 levels at the time of rejection ($r=0.615$, $p=0.002$; and $r=0.519$, $p=0.022$, respectively). Among those with T cell-mediated rejections the mean urinary CXCL10 level increased to as high as 258.12 ng/mL.

Conclusion: Urinary CXCL9 and CXCL10 levels might have a predictive value for T cell-mediated rejection in early post-transplantation period. Measurement of urinary CXCL9 and CXCL10 levels could provide an additional tool for the diagnosis of rejection.

KEYWORDS: Chemokines; Renal transplantation; Rejection; Biomarker; Graft function

INTRODUCTION

Renal transplantation is the treatment of choice for end-stage renal disease. However, since the development of immunosuppressive drugs and protocols, there has been no significant improvement in allograft survival. Acute rejection is one of the most important causes of early and long-term graft loss in kidney transplant recipients [1]. Acute rejection has been classified into 2 types—"acute cellular rejection," in which cytotoxic T lymphocytes and other inflammatory cells cause damage in the renal parenchyma; and "antibody-mediated rejection," which

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is triggered by the presence of donor-specific antibodies, morphologic evidence of acute injury and histological evidence of an antibody-mediated process [2-5]. An early diagnosis of acute rejection is critical for graft survival. Renal biopsy is currently the primary method to monitor the dynamic changes of graft rejection; however, this technique is invasive and graft damage is detected at a late stage. Although transplant biopsy is the most sensitive and specific means for the diagnosis and treatment of allograft rejection [6-8], many transplant centers do not consider it because of the associated risk of serious complications [9]. It has been a goal of clinicians to find a noninvasive method to monitor the alloreactivity after organ transplantation. This would be particularly advantageous in making or pre-empting the diagnosis of rejection, as at present the definitive way of rejection diagnosis is by biopsy of the renal allograft. Therefore, noninvasive markers may be helpful for early detection of acute allograft rejection [10].

Most studies have looked at gene expression levels within an allograft biopsy; however, relatively few have investigated gene expression levels in circulating inflammatory cells, which may be influenced by or reflect events within the allograft. Therefore, these inflammatory cells could potentially be useful in immunomonitoring [11]. There has been increasing evidence over the last decade on the role of chemokines in the immunological events around the time of allograft rejection [10, 11]. Chemokines and respective receptors are involved in the process of cell migration into grafts, which ultimately leads to allograft rejection. Clinical and animal studies have demonstrated the role of chemokines and their receptors in graft rejection [12, 13].

Chemokines are small inducible pro-inflammatory cytokines, which are normally expressed at low levels, and rapidly up-regulated at the onset of the immune response. These molecules and their receptors are involved in the process of cell inflammatory migration into grafts which trigger acute rejection [2-5].

Certain chemokines and chemokine receptor pathways have been shown to be critical in acute allograft rejection. Previous studies have demonstrated that chemokines CXCL9 and CXCL10 bind the G-protein-coupled receptor CXCR3, which is expressed on T cells and produce interferon-γ (IFN-γ) [12]. The IFN-γ-CXCR3-chemokine-dependent inflammatory loop is crucial in recruiting T lymphocytes during acute rejection following renal transplantation [14, 15]. Monokines (MIG, CXCL9), protein 10s (IP-10, CXCL10), and T-cell chemoattractants (I-TAC, CXCL11) are CXCR3-specific ligands that are induced by IFN-γ. It has been shown that CXCR3 is a marker for T helper cells type-1 associated with inflammatory processes, and that IP-10 and monokine-induced by interferon-γ (CXCL9) attract activated (but not resting) T cells [16, 17]. These ligands direct migration and stimulate the adhesion of activated Th1 cells and cytotoxic T lymphocytes via the IFN-γ CXCR3-chemokine loop [18]. CXCL10 has chemotactic properties for T helper cells (Th1), natural killer cells (NK cells), dendritic cells (DCs), γδ T cells, and macrophages. It is secreted by several immune (leukocytes, neutrophils) and non-immune (epithelial, endothelial) cells [14, 15].

Multiple chemokines act as pro-inflammatory cytokines and produce signals for the dynamic trafficking and recruitment of leukocytes, which leads to an inflammatory response. Consequently, they may be early predictors of graft dysfunction and in theory, they might provide information about the mechanisms underlying the immune attacks [16].

In the present study, urinary CXCL9 and CXCL10 protein levels were measured in a group of patients with biopsy-proven allograft rejection and biopsy-proven non-rejection. The objective of our study was to evaluate the prognostic value of urinary CXCL9 and CXCL10 levels before and after transplantation for predicting the onset of acute rejection episodes and graft outcome.

MATERIALS AND METHODS

Study Population and Procedures

This cohort study included 85 recipients aged between 18 and 70 years who underwent living-related kidney transplantation with well-functioning allografts from January 2014 to January 2017. The study protocol conformed to good medical and laboratory practice and the recommendations of the Declaration of Helsinki on Biomedical Research involving Human Subjects. All patients were followed for more than six months after kidney transplantation. Additional study visits occurred whenever a clinically-indicated biopsy was scheduled. Patients who had urinary tract infection or evidence of drug toxicity were excluded. The rejection cases were classified according to the Banff 97 classification and its updates [19, 20].

Biopsy Indications and Assessment

Indications for biopsy were 1.5 times increase in the basal serum creatinine levels and/or de novo occurrence of persistent proteinuria (>1 g/24 h) that prompted clinical suspicion of an acute allograft rejection. Biopsies were evaluated by the same nephropathologist. Kidney biopsy tissue was examined under light microscopy after being stained with hematoxylin/eosin, periodic acid Schiff (PAS), periodic acid methenamine silver (PAMS), and trichrome stains. Immunofluorescence staining with conjugated antibodies to IgG, IgM, IgA, C3, C1q, kappa, lambda and fibrinogen on frozen tissue was also performed. Light microscopic features of biopsies were scored by Banff criteria on all biopsies [19, 20]. C4d staining (anti-C4d antibody, polyclonal; Cell Marque, The Hague, the Netherlands) on paraffin-embedded tissue blocks was also done using immunohistochemistry with an automatic staining system (Ventana Bench Mark XT, IHC/ISH automated staining platforms, Roche diagnostics Ltd, Rotkreuz, Switzerland). Linear and circumferential staining in peritubular capillaries was considered positive according to the recent Banff scoring system (C4d > 0). Banff 2013 diagnostic categories and related criteria were used for the final pathologic diagnosis [19, 20].

Patient Groups

All patients were assigned to a diagnostic category based on allograft biopsy: recipients with acute rejection, or other causes for allograft dysfunction such as BK infection, calcineurin inhibitor toxicity or isolated interstitial fibrosis and tubular atrophy. Those with histopathological diagnoses such as interstitial fibrosis and tubular atrophy (IFTA), drug toxicity and bacterial or viral infection were excluded from the study. All patients were classified based on their graft function into "stable graft function" (non-rejection) and "rejection."

Immunosuppressive Protocol

The maintenance immunosuppressive therapy of renal transplant recipients included a combination of calcineurin inhibitor (tacrolimus) with mycophenolate mofetil or mycophenolate sodium (EC-MYF) and prednisolone (Pred). Acute cellular rejection episodes were treated with a high daily dose of intravenous methylprednisolone (500 mg each dose) for three days; in refractory cases, with ATG [(antithymocyte globulin (Merieux, France)] (2 mg/kg/day) for 10–14 days. Calcineurin inhibitor levels were not collected or analyzed as part of the study.

Study End-points

The primary outcome variable was biopsy-proven acute rejection during follow-up in kidney transplant recipients.

Clinical Outcomes

We analyzed demographic characteristic of patients, duration of surgery and anesthesia, cold and warm ischemia time. Serum creatinine (sCr) levels and estimated glomerular filtration rate (eGFR) were evaluated before the transplantation and at the 1st day, 7th day, 1st month, and 3rd month after transplantation. eGFR were calculated using the Modification Diet in Renal Disease Study equation. Pre- and post-operative immunologic data (number of HLA mismacth, and pre- and post-transplant HLA antibody status) were evaluated. The acute rejection attacks and graft loss within six months of transplantation were also recorded.

Table 1: Demographic details of the renal transplant recipients. Values are either mean±SD or n (%).

Parameters	Rejection (n=15)	Non-Rejection (n=70)	p value
Age, yrs	33.4±7.6	35.9±13.6	0.57
Female/Male	6 (40)/9 (60)	30 (42)/41 (59)	0.92
Follow-up period, m	23.2±11.8	24.1±13.4	0.84
Weight, kg	66.5±12.8	64.3±14.4	0.50
First/Second transplantation	12 (80)/3 (20)	60 (86)/10 (14)	0.45
Primary kidney disease			
Chronic glomerulonephritis	7 (47)	26 (37)	0.06
Tubulointerstitial nephritis	4 (27)	11 (16)	
Unknown	3 (20)	17 (24)	
Primary nephrosclerosis	1 (7)	4 (6)	
Amyloidosis	1 (7)	3 (4)	
Diabetic nephropathy	2 (13)	6 (9)	
Duration of aneasthesia, min	305.1±25.1	310.3±32.4	0.35
Duration of operation, min	296.6±41.0	282.8±40.0	0.21
Cold ischemia time, min	51.1±19.5	60.4±20.1	0.16
Warm ischemia time, min	5.8±2.2	6.4±2.5	0.44
Hospital stay, d	18.4±6.4	21.9±11.2	0.11

Collection of the Samples

Urine samples were collected before the transplantation and at the 1st day, 7th day, 1st month, 3rd month, at the time of rejection. Specimens were centrifuged at 2600 g for 10 min to remove sediment. Supernatants were separated and stored in 1-mL aliquots at -80 °C until used for quantification of CXCL9 and CXCL10 by an enzyme-linked immuno-sorbent assay (ELISA). Commercial ELISA (Abnova, Taiwan, Corporation) kits were used for measurements of urinary CXCL9 and CXCL10 levels. The optical density of the samples was determined at 450 nm/540 nm using the microplate reader (Synergy 2, BioTek® Instrument, Inc, USA). A standard curve was generated using the CXCL10 standards and the concentration of samples was calculated using the Gen5® software ver 1.08. Each sample was assayed in duplicate; the average of the CXCL10 protein levels was used for statistical analysis. The minimum detectable dose of CXCL9 and CXCL10 was 1.67 pg/mL.

Ethics

The study protocol was approved by the Research Ethics Committee of Istanbul University, Istanbul Faculty of Medicine, and informed consent was obtained from all participants.

Statistical Analysis

SPSS® for Windows® ver 21 (SPSS, Chicago, IL) was used for data analysis. Kolmogorov-Smirnov test was used to assess the normality of data distribution. Quantitative variables were summarized as mean and SD. For categorical data, Fisher's exact test was used. *Student's t* test and one-way ANOVA tests were used to compare means between two groups and three or more groups of normally distributed data, respectively. Multivariable regression analysis was used for demographic variables. The receiver operating characteristic (ROC) curve analysis was used to determine the diagnostic power—area under the curve (AUC), sensitivity and specificity—of CXCL9 and CXCL10 for those with rejection. A p value of <0.05 was considered statistically significant.

RESULTS

A total of 85 patients was included in this study.

Table 2: The immunologic characteristics of patients with and without rejection. Values are either mean±SD or n (%).

Parameters	Rejection (n=15)	Non-rejection (n=70)	p value
Immunologic Status			
Pre-Tx anti-HLA antibody status (Positive/Negative)	3 (20)/12 (80)	14 (20)/56 (80)	0.82
Post-Tx anti-HLA antibody status (Positive/Negative)	6 (40)/9 (60)	10 (14)/60 (86)	0.60
HLA mismatches	2.12±0.46	2.30±0.48	0.18
≥3 HLA mismatches	3 (20)	9 (12)	0.15
Maintenance IS Regimen			
FK+MMF+Pred/FK+EC-MYFNa+Pred	10 (67)/5 (33)	49 (70)/21 (30)	0.54
Graft loss	0 (0)	0 (0)	—
Induction therapy (Yes/No)	2 (13)/13 (87)	8 (11)/62 (87)	0.74
De novo DSA	1 (13)	2 (3)	0.02

Abbreviations: EC-MYFNa: enteric coated mycophenolate sodium, FK: tacrolimus, HLA: human leukocyte antigen; IVIG: intravenous immunoglobulin; IS: immunosuppressive; MMF: mycophenolate mofetil; Pred: prednisolone; Tx: transplantation

All parameters were analyzed in relation to allograft outcome. Non-rejection group consisted of 70 (82%) patients. Nineteen patients had biopsy after transplantation after a mean±SD period of 19±25 [IQR 5–90] days (Table 1). During the follow-up period, 15 (18%) recipients were complicated by biopsy-proven acute rejection—nine with T cell-mediated and six with antibody-mediated rejection. Three patients were diagnosed with acute tubular necrosis; one was diagnosed with recurrence focal segmental glomerulosclerosis. Renal transplant recipients with rejection included six men and four women with a mean±SD age of 33.4±7.6 years. The non-rejection patients included 38 (59%) men and 27 (42%) women with a mean±SD age of 35.9±13.6 years. No significant differences were observed in age, sex, and warm and cold ischemia time between the two groups.

All patients had negative flowcytometry crossmatches at the time of transplantation. There were no significant differences between the number of HLA mismatch, pre- and post-transplant HLA antibody status, *de novo* DSAs developed, and immunosuppressive regimen between the study groups (Table 2).

The function of the grafted kidneys up to the 3rd post-operative month is presented in Table 3. sCr levels had no significant difference between studied groups (Table 3), with the exception of the time of rejection and the 7th day (p=0.001 and p=0.04, respectively). eGFR was also not different between studied groups (Table 3), with the exception of the time of rejection and the 7th day (p=0.03 and p=0.001, respectively) (Table 3). sCr levels declined after surgery, remained under 2 mg/dL in patients with stable graft function. However, it started to rise at time of rejection (mean 19 [IQR 5–90] days) in those with rejection; after treatment, sCr significantly decreased. In contrast, eGFR levels decreased significantly at the time of rejection and rapidly normalized after the treatment.

A total of 490 urine samples were taken at different periods after transplantation from renal transplant recipients—115 samples from rejection and 325 from non-rejection cases. Urine CXCL9 and CXCL10 levels were assessed for each group. No significant difference was observed in pre-operative levels of urinary CXCL9 and CXCL10 between the studied groups (Table 4). The urinary CXCL9 levels were significantly higher in the rejection group compared with the non-rejection group at the 1st day, 7th day, and 1st month after transplantation (p<0.0001, p<0.0001, and p=0.002, respectively). The urinary CXCL10 levels were also significantly higher in the rejection group compared with the non-rejection

Table 3: Mean±SD serum creatinine and eGFR levels stratified by graft function

Parameter	Stable graft function (n=70)	Rejection (n=15)	p value
Creatinine (mg/dL)			
Pre-operative	8.64±1.92	9.12±1.66	0.62
1st day	1.72±1.07	1.86±0.88	0.12
7th day	1.37±0.34	1.78±1.25	0.04
1st month	1.27±0.39	1.29±1.65	0.06
3rd month	1.20±0.34	1.22±0.47	0.05
At the time of rejection	1.15±0.21	3.85±1.10	0.001
After treatment	1.15±0.21	1.39±0.66	0.08
eGFR (mL/min)			
Pre-operative	8.89±1.27	8.41±1.33	0.54
1st day	49.34±2.17	45.56±2.34	0.09
7th day	77.07±4.41	68.24±5.32	0.03
1st month	78.27±0.39	76.04±4.38	0.62
3rd month	88.34±6.28	86.22±5.39	0.43
At the time of rejection	76.12±5.94	59.46±5.47	0.001
After treatment	88.34±6.28	83.31±5.18	0.08

group on the 1st day, 7th day, and 1st month after transplantation (p<0.0001, p<0.0001, and p<0.001, respectively). Urinary CXCL9 and CXCL10 levels increased to 178.25 and 242.34 ng/mL in patients with rejection at the time of rejection (biopsy-proven) (Table 4). The levels in those with rejection was significantly higher at time of rejection than those in the stable graft function group (p=0.001, p=0.001 respectively). Within 2–3 weeks following treat-

Table 4: Mean±SD urinary CXCL9 and CXCL10 levels stratified by graft function

Parameter	Non-rejection (n=70)	Rejection n=15)	p value
CXCL9 (ng/mL)			
Pre-operative	54.93±8.15	59.84±9.82	0.28
1st day	64.78±23.34	137.24±45.27	<0.001
7th day	70.43±21.83	140.67±51.94	<0.001
1st Month	61.34±12.20	122.16±54.63	0.002
3rd Month	57.51±7.63	63.84±8.56	0.16
At the time of rejection	62.23±15.46	178.25±35.47	<0.001
After treatment	61.23±14.27	63.22±16.17	0.74
CXCL10 (ng/mL)			
Pre-operative	59.04±8.85	63.99±10.91	0.09
1st day	65.07±24.47	168.94±60.04	<0.001
7th day	71.02±25.02	191.53±41.59	<0.001
1st Month	61.85±13.60	136.24±67.26	0.001
3rd Month	62.13±9.51	69.18±8.36	0.56
At the time of rejection	64.25±10.21	242.34±59.42	<0.001
After treatment	62.18±11.17	89.12±9.65	0.07

Table 5: Mean±SD urinary levels of CXCL9 and CXCL10 stratified by histopathological groups and control. Groups were compared with one-way ANOVA.

	CXCL9 (ng/mL)	CXCL10 (ng/mL)
Pre-operative		
T cell-mediated rejection (n=9)	56.83±9.86	67.95±12.50
Antibody-mediated rejection (n=6)	64.37±9.04	58.06±4.31
Non-rejection group (n=70)	54.93±8.15	59.04±8.85
	p=0.09	p=0.07
First day		
T cell-mediated rejection (n=9)	141.06±44.47$^{\&}$	171.10±59.60$^{\alpha}$
Antibody-mediated rejection (n=6)	131.51±31.73$^{\beta}$	167.70±77.83$^{\gamma}$
Non-rejection group (n=70)	64.78±23.34$^{\&,\beta}$	65.07±24.47$^{\alpha,\gamma}$
	p=0.886, $^{\&}$p<0.001, $^{\beta}$p<0.001	p=0.097, $^{\alpha}$p<0.001, $^{\gamma}$p<0.001
Seventh day		
T cell-mediated rejection (n=9)	149.15±55.46$^{\xi}$	222.51±62.25$^{*,\circ}$
Antibody-mediated rejection (n=6)	127.97±51.09$^{\epsilon}$	145.07±56.65$^{*,\mu}$
Non-rejection group (n=70)	71.02±25.02$^{\xi,\epsilon}$	71.02±25.02$^{\circ,\mu}$
	p=0.45, $^{\xi}$p<0.001, $^{\epsilon}$p<0.002	*p=0.001, $^{\circ}$p<0.001, $^{\mu}$p=0.002
First month		
T cell-mediated rejection (n=9)	128.70±64.31^{f}	155.65±80.35$^{\#,\$}$
Antibody-mediated rejection (n=6)	112.34±42.98$^{¥}$	107.13 830.50$^{\#,\dagger}$
Non-rejection group (n=70)	61.34 812.20$^{f,¥}$	61.85±13.60$^{\$,\dagger}$
	p=0.363, ^{f}p=0.02, $^{¥}$p=0.03	$^{\#}$p=0.002, $^{\$}$p<0.001, †p=0.04
Third month		
T cell-mediated rejection (n=9)	62.88±9.21	69.31±10.65
Antibody-mediated rejection (n=6)	65.28±8.61	69.00±4.56
Non-rejection group (n=70)	57.51±7.63	62.13±9.51
	p=0.88	p=0.89
At the time of rejection		
T cell-mediated rejection (n=9)	175.12±8.73$^{\$,\circ}$	258.12±11.47$^{*,\circ}$
Antibody-mediated rejection (n=6)	119.64±9.09^{S}	134.41±6.92$^{*,\mu}$
Non-rejection group (n=70)	59.24±6.97$^{\circ,S}$	60.07±10.01$^{\circ,\mu}$
	$^{\$}$p=0.02, $^{\circ}$p<0.001, ^{S}p<0.001	*p=0.002, $^{\circ}$p<0.001, $^{\mu}$p<0.001

ment for the rejection, CXCL9 and CXCL10 levels significantly decreased in patients with rejection (Table 4).

Analysis of CXCL9 and CXCL10 among different categories of sub-classification of rejection group with non-rejection cases showed significant differences between T cell-mediated rejection and antibody-mediated rejection (p<0.0001, p<0.0001 respectively) at the time of rejection. Among the sub-classification of rejection group, a higher median value of CXCL9 was detected in patients suffered from T cell-mediated rejection (175.12 ng/mL) followed by antibody-mediated rejection (119.64 ng/mL). At the same time, the highest median value of CXCL10 for T cell-mediated rejections was 258.12 ng/mL (Table 5).

ROC analysis was performed to assess the diagnostic value of urine CXCL9 and CXCL10 in acute allograft rejection. We generated ROC curves for urinary CXCL9 and CXCL10 cut-off values for the 1st day, 7th day, 1st month,

Table 6: Test characteristics of urinary CXCL9 and CXCL10 levels as a test for rejection

Period	Predictors, ng/mL	AUC (95% CI)	PPV	NPV	Se	Sp	p value
Rejection *vs* non–rejection							
First day	CXCL9	0.924 (0.852–0.996)	0.71	0.83	0.85	0.80	<0.001
First day	CXCL10	0.937 (0.878–0.995)	0.59	0.87	0.78	0.84	<0.001
Seventh day	CXCL9	0.946 (0.885–1.000)	0.60	0.81	0.83	0.79	<0.001
Seventh day	CXCL10	0.965 (0.920–1.000)	0.70	0.84	0.81	0.85	<0.001
First Month	CXCL9	0.946 (0.932–1.000)	0.69	0.90	0.79	0.88	<0.001
First Month	CXCL10	0.973 (0.881–1.000)	0.73	0.86	0.82	0.85	<0.001
Third Month	CXCL9	0.716 (0.549–0.884)	0.68	0.71	0.70	0.37	0.03
Third Month	CXCL10	0.751 (0.586–0.916)	0.69	0.74	0.80	0.58	0.01
T cell *vs* antibody rejection							
First day	CXCL9	0.998 (0.811–0.986)	0.58	0.81	0.73	0.79	0.001
First day	CXCL10	0.844 (0.686–1.000)	0.61	0.83	0.76	0.84	0.01
Seventh day	CXCL9	0.931 (0.928–1.000)	0.55	0.79	0.73	0.80	<0.001
Seventh day	CXCL10	0.969 (0.789–1.000)	0.65	0.86	0.79	0.85	<0.001
First Month	CXCL9	0.948 (0.871–1.000)	0.53	0.69	0.69	0.79	<0.001
First Month	CXCL10	0.802 (0.524–1.000)	0.61	0.72	0.71	0.75	0.02
Third Month	CXCL9	0.653 (0.420–0.886)	0.60	0.58	0.72	0.49	0.22
Third Month	CXCL10	0.596 (0.277–0.915)	0.62	0.64	0.68	0.55	0.45

NPV: negative predictive value, PPV: positive predictive value, Se: sensitivity, Sp: specificity

and 3rd month after transplantation in those with rejection. ROC analysis confirmed a positive predictive value of 73% and a negative predictive value of 84% for CXCL10 at the 1st month. The analysis also confirmed a positive predictive value of 65% and a negative predictive value of 86% for CXCL10 at the 1st month on sub-classification of rejection (Table 6).

DISCUSSION

Transplantation across HLA-specific antibody barriers is now increasingly routine but limited by poorer long-term outcomes that may, in part, be due to the effects of rejection. Early diagnosis and selective immunosuppression of acute rejection are essential for long-term outcomes. The development of new non-invasive biomarkers after transplantation remains essential for a more individualized therapy to optimize benefit/risk ratios [20].

This study has demonstrated that significant changes in chemokine levels in urine, especially CXCL9 and CXCL10, in the early post-renal-transplantation period can be detected and that some of these changes correlate with allograft rejection. We analyzed 75 renal transplant recipients with a mean follow-up of 23 months. Ten patients had biopsy-proven rejection during the follow-up. The urinary CXCL9 and CXCL10 levels were significantly higher in the rejection group compared with the non-rejection group at the 1st day, 7th day and 1st month after transplantation; our results also showed that there was no significant difference in urinary levels of CXCL9 between non-rejection and rejection group before transplantation.

It was previously shown that CXCL10 levels did not predict graft survival [8, 21]. We could also not find any relationship between chemokine levels and graft survival. However, Heidt, *et al* [22], reported that high pre-transplantation serum CXCL9 and CXCL10 levels were associated with long-term graft loss. This finding suggests that pre-transplantation CXCR3-binding chemokine assessment may identify patients at risk of acute rejection and graft loss. Current available data show

that CXCL9 and CXCL10 levels are insufficient to predict the short-term graft survival, but more work is needed to predict a long-term survival. In a recent study, Field, *et al* [21], demonstrated that there was no difference in pre-transplantation serum IP-10 levels in the rejection group. Also, Lazzeri, *et al* [8], showed that high pre-transplantation levels of serum IP-10 in immunologically uncomplicated transplants are correlated with worse graft outcomes and rejection within the first 30 days.

In our study, pre-operative levels of CXCL9 and CXCL10 were not related with graft outcomes after follow-up—similar to Field, *et al* [21] and Lazzeri, *et al* [8].

Raza, *et al* [23], reported that CXCL10 concentration increased to 228 pg/mL in those with rejection, 60 in non-rejection, and 10.5 in the control group. A similar pattern for CXCL10 levels has also been reported in other studies for the rejection and non-rejection cases [24].

The authors analyzed urine CXCL9 protein in samples obtained prior to the diagnostic biopsies. They observed elevated CXCL9 concentrations in those with histologically-diagnosed acute rejection up to 30 days prior to the clinical diagnosis of graft dysfunction. Urinary CXCL9 values fell within 30 days after treatment [24]. However, in their study, the preoperative CXCL10 levels increased and remained high during the time of rejection. Our data showed that patients with rejection had higher levels of CXCL9 and CXCL10 compared with the non-rejection group at the time of rejection. In contrast, in our study, CXCL9 and CXCL10 levels decreased after treatment. As such, CXCL9 and CXCL10 levels may be used to determine the efficacy of surgery and recovery in long-term post-operative follow-up.

Several studies report that CXCL10 may be a useful marker for rejection [25, 26] and that elevation in urine levels of CXCL9 and CXCL10 may be associated with acute rejection [27, 28]. Jackson, et al [7], demonstrated that urine CXCL9 value of 37.8 ng/mL has 86% sensitivity and 80% specificity, and that urine CXCL10 value of 28 ng/mL has a sensitivity of 80% and specificity of 76% for the diagnosis of acute rejection. Jackson, et al [7], have shown that the CXCL10 chemokine in urine identifies early renal allograft inflammation and renal injury with better sensitivity and predictability than serum CXCL10. Hu, et al [26], also reported higher sensitivity (86.4%) and specificity (91.3%) with rejection at >100 pg/mL.

In our study, a cut-off value of 65.36 ng/mL for urinary CXCL9 was associated with 85% sensitivity and 80% specificity and a cut-off value of 66.46 ng/mL for CXCL10 had a sensitivity of 78% and specificity of 84% for the diagnosis of rejection. We found results similar to previous studies indicating that urinary CXCL9 and CXCL10 levels have a high sensitivity in identifying patients with rejection. The variance in the results of various studies with levels of CXCL9 and CXCL10 are probably due to differences in the measurement methods used.

Regarding rejection subtypes, our study showed that CXCL9 and CXCL10 levels were significantly higher in those with T cell-mediated rejection compared with those with antibody-mediated rejection at the 7th day and 1st month. A previous study showed that urinary CXCL10:serum creatinine ratio has a sensitivity of 81% and specificity of 37% in detecting patients with T cell-mediated rejection [29]. Raza, *et al* [23], reports that when sub-classes of rejection groups acute cellular rejection, acute vascular rejection, and borderline rejection, were compared with the non-rejection group, acute cellular rejection *vs* non-rejection, and acute vascular rejection *vs* non-rejection had statistically significant differences. No significant differences were observed in the borderline rejection *vs* non-rejection groups. In contrast to the report of Hirt-Minkowski, *et al* [30], the number of acute cellular rejection was low in the present study.

According to our data urinary CXCL9 and CXCL10 levels were more useful markers for detection of acute T cell-mediated rejection so

that they could be used to determine the cause of early graft dysfunction.

The strengths of the current study included measurements of chemokine levels and assessment of allograft biopsy in a single center that made data more valid and accurate. Nonetheless, this would be considered a limitation too—the results might be hard to apply to other centers. Other limitations of the study included lack of data on protocol biopsies, small sample size and short follow-up period.

In conclusion, this study demonstrated that the potential biomarkers CXCL9 and CXCL10 had good ability at predicting rejection in living-related kidney transplant patients. Urinary chemokine levels may be useful in patients whose biopsy reveals T cell-mediated rejection. If the level of these biomarkers accurately determine graft function in the early post-transplantation period, it might be beneficial for the individualized therapy of patients undergoing renal transplantation.

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