ANNALS OF TRANSPLANTATION

ORIGINAL PAPER

e-ISSN 2329-0358
© Ann Transplant, 2024; 29: e944762
DOI: 10.12659/AOT.944762

Received: 2024.04.08
Accepted: 2024.06.28
Available online: 2024.07.17
Published: 2024.10.15

Urinary Chemokines CXCL9 and CXCL10 Are Non-Invasive Biomarkers of Kidney Transplant Rejection

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Financial support: Vilnius University
Conflict of interest: None declared

Background: Rejection is the main cause of kidney allograft failure, and kidney biopsy is the criterion standard method to diagnose it. However, non-invasive techniques to detect kidney transplant rejection are necessary. This study aimed to evaluate urinary chemokines CXCL9 and CXCL10 as potential biomarkers of kidney transplant rejection and to analyze chemokine association with allograft prognosis.

Material/Methods: We collected 117 urine samples from kidney transplant recipients undergoing allograft biopsy. CXCL9 and CXCL10 levels were measured by ELISA and the ratio to urine creatinine was calculated. Histology and other clinical data were collected from medical records.

Results: The diagnostic performance of urinary CXCL9/cre in discriminating rejection from all other histological groups showed an ROC AUC value of 0.728 (95% CI 0.632-0.824, $p<0.001$), and a cut-off value 0.11 ng/mmol had the best sensitivity (76.9%) and specificity (73.1%). The ability of CXCL10/cre to discriminate transplant rejection from all other histological groups had ROC AUC value 0.73 (95% CI 0.63-0.84, $P<0.001$), the cut-off value 0.42 ng/mmol with best sensitivity (71.4%) and specificity (84.6%). CXCL9 and CXCL10 levels were also increased in patients with polyoma BK virus, recurrent AA amyloidosis, and thrombotic microangiopathy. Patients with higher CXCL9/cre (≥0.11 ng/mmol) and CXCL10/cre (≥0.42 ng/mmol) levels were at increased risk of transplant progression to ESRD (HR 3.25, 95% CI=1.27-8.36, $P=0.01$), irrespective of serum creatinine at the time of biopsy.

Conclusions: Urinary CXCL9/cre and CXCL10/cre were able to distinguished between patients with transplant rejection and those without rejection. High levels of urinary CXCL9/cre and CXCL10/cre were associated with worse allograft survival.

Keywords: Biomarkers • Chemokines, CXC • Graft Rejection • Kidney Transplantation

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Indexed in: [Science Citation Index Expanded] [Index Medicus/MEDLINE] [Chemical Abstracts] [Scopus]

Introduction

The diagnosis of renal transplant rejection is evolving towards non-invasive diagnostic methods to early suspect or rule out renal transplant rejection, avoid unnecessary graft biopsy, or help monitor the level of immunosuppression to adjust the administration of immunosuppressive drugs accordingly. Much progress has already been made in the search for potential biomarkers of kidney transplant rejection in blood, urine, and kidney biopsy material, analyzing proteins, RNA, and gene expression.

Among the potential biomarkers are chemokines, which can be detected in both blood and urine. Chemokines are chemotactic cytokines involved in various biological processes, such as angiogenesis, hematopoiesis, and migration of leukocytes and other cells. The most promising chemokine biomarkers in kidney transplantation are chemokine 9 (CXCL9) and chemokine 10 (CXCL10).

CXCL9 (monokine induced by IFN-γ) and CXCL10 (IFN-γ-inducible protein 10, IP-10) act through their shared receptor CXCR3 [1,2] and can attract NK cells, mononuclear cells, specifically activated T cells, and B cells [3,4]. CXCL9 is a critical mediator of primed T cell trafficking in transplant models. In animal models, CXCL9 has also been shown to stimulate cytokine production by recruited T cells and promote Th1 cell proliferation through induction of the transcription factors T-bet and RORγT and production of type-1 cytokines (IL-2, TNF, and IFN-γ) [5].

CXCL10 is constitutively expressed in stromal cells of lymphoid organs (spleen, thymus, and lymph nodes), suggesting a potential role in T cell development and effector functions. CXCL10 is a biomarker for tubulointerstitial kidney allograft inflammation of any etiology [6], but CXCL10 is also increased in antibody-mediated rejection with higher "g", and "ptc" Banff scores [7]. In animal transplant models, CXCL9 and CXCL10 levels rapidly rise following reperfusion and during early rejection of the liver, kidney, and heart [8-10].

In kidney transplant recipients with T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR), the urinary chemokines CXCL9 and CXCL10 are increased compared with patients without rejection [11-13]. Treatment of allograft rejection reduces the level of urinary CXCL10 [14,15]. Subclinical allograft rejection correlates with CXCL10 but not CXCL9 levels [16]. It is also known that elevated urinary CXCL10 can predict kidney transplant rejection, but the ability of serum CXCL10 to assess the risk of allograft rejection remains controversial due to clinical confounding factors [17,18]. Notably, urine and serum CXCL10 are not specific for rejection because they can increase in patients with polyoma BK virus infection and urinary tract infection, while serum but not urinary CXCL10 increases in patients with cytomegalovirus infection [12,15,16,19,20].

We aimed to evaluate urinary CXCL9 and CXCL10 as potential biomarkers of kidney transplant rejection and to analyze their association with graft prognosis.

Material and Methods

Study Description and Collection of Samples

A total of 117 kidney transplant recipients undergoing surveillance or clinically-indicated kidney biopsy at Vilnius University Hospital Santaros Klinikos were included in the study in 2019-2022. Midstream urine samples were obtained before the biopsy and stored at -20°C to measure chemokines and creatinine, which were done regularly after collecting a group of samples. Patient clinical data were collected from a prospectively-completed electronic patient medical data system. The main patient characteristics are presented in **Table 1**.

Kidney biopsy cores were obtained using an 18-gauge needle under ultrasound guidance. Biopsies were evaluated in a single center by an experienced pathologist and reported using the Banff scheme, applying the most up-to-date criteria at the time of reporting [21,22]. For further analysis, biopsies were divided into 4 distinct groups: normal histology, rejection (ABMR), TCMR, mixed rejection), polyoma BK nephropathy, and other histology (global glomerulosclerosis, recurrent glomerulonephritis, thrombotic microangiopathy without rejection, calcineurin toxicity induced lesions, amyloidosis, interstitial nephritis).

Kidney transplant recipients received standard induction immunosuppressive therapy: basiliximab for moderate immunological risk and thymoglobulin for high immunological risk recipients. Maintenance immunosuppressive therapy mostly consisted of tacrolimus or cyclosporine (the latter used by earlier-transplanted patients), mofetil mycophenolate, and methylprednisolone.

Patients with biopsy-proven rejection were treated according to the histological phenotype and severity. Briefly, TCMR episodes were treated with steroids and severe clinical TCMR patients received thymoglobulin infusions. ABMR was mostly treated with plasmapheresis and intravenous immunoglobulins±rituximab. The study complies with all regulations, and informed consent was obtained from the participants. The experiments were conducted according to established ethics guidelines and the Declaration of Helsinki. This study was approved by the Bioethics Committee of the Vilnius Region (approval No. 158200-17-901-409).

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Table 1. Patient (n=117) characteristics.

Patient characteristics		Value
Gender	Male, %	63.2
	Female, %	36.8
Age	Average age, years	43±13
eGFR at biopsy	ml/min/1.73 m^2	39±18
Biopsy time after transplantation	Median months [IQR]	20.0 [6-96]
Transplant number	First transplant (% of patients)	76.1
	Second transplant (% of patients)	18.8
	Third transplant (% of patients)	4.3
Total life years on immunosuppression	Median [IQR]	4.3 [1.0-11.0]
Hemoglobin at biopsy	g/l	115.8±19.8
BMI	kg/m^2	24.7±4.8
Serum urea at biopsy	mmol/l	20.4±12.6
Biopsy result (% of cases)	Normal histology, %	22.2
	ABMR, %	21.4
	TCMR, %	7.7
	Mixed rejection, %	8.8
	BK virus nephropathy, %	4.4
	Other abnormalities, %	35.9
Immunosuppression	Tacrolimus, %	66.7
	Cyclosporine, %	25.6
	Mofetil mycophenolate, %	93.2
	Methylprednisolone, %	92.3
	Azathioprine, %	2.6
	Sirolimus, %	7.7
Immunosuppressant levels	Tacrolimus ng/ml	6.57±3.78
	Cyclosporine ng/ml	84.74±27.15
	Sirolimus, ng/ml	0.83±0.65

Chemokine Detection

Urinary CXCL9 and CXCL10 measurements were performed retrospectively on midstream urine samples (collected before kidney biopsy and stored at -20°C without any additives) with "Human MIG (CXCL9) Mini ABTS ELISA Development Kit" (Peprotech, Catalog #900-M87) and "Human IP-10 (CXCL10) Mini ABTS ELISA Development Kit" (Peprotech, Catalog #900-M39), respectively. All procedures were carried out as recommended by the manufacturer using ELISA reagents provided in the "ABTS ELISA Buffer Kit" (Peprotech, Catalog #900-K00). Briefly, for CXCL9 measurement, ELISA plate wells were coated overnight at room temperature with 1 µg/mL Rabbit Anti-Human MIG (CXCL9) antibody, and for the CXCL10 measurement, wells were coated overnight at room temperature with 0.5 µg/mL Rabbit Anti-Human IP-10 (CXCL10) antibody, washed, and then blocked. Each urine sample (100 µL per well) and ELISA kit standards were tested in triplicate by incubating for 2 h at room temperature. After washing, biotinylated Rabbit Anti-Human MIG (CXCL9) or IP-10 (CXCL10) secondary polyclonal antibody was added at 1 µg/mL or 0.25 µg/mL, respectively, and incubated for 2 h at room temperature. Plates were then washed, and incubated with avidin-horseradish peroxidase conjugate for 30 min at room temperature. After

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washing, the signal was developed with ABTS substrate by incubating for 25 min for CXCL9 detection and for 20 min for CXCL10 detection. Optical density (OD) was read at 405 nm with wavelength correction set at 650 nm using a "Multiskan GO" spectrophotometer (Thermo Scientific). CXCL9 and CXCL10 concentrations were calculated from standard curves using the 4-parameter logistic model curve fit in "OriginPro 8" (OriginLab) software. Detection ranges were 16-1000 pg/mL for both CXCL9 and CXCL10. Urine samples with measured ODs exceeding the OD of the 1000 pg/mL standards were diluted and retested. To correct for different urine dilutions, the excretion of urine proteins was normalized to urine creatinine (ie, ng protein/mmol creatinine).

Statistical Analysis

Continuous variables were presented as mean±standard deviation, or median (interquartile range) according to the type of data. The normality of quantitative data was tested by the Kolmogorov-Smirnov test. We used the *t* test and Mann-Whitney *U* test to compare continuous variables with normal and skewed distributions, respectively. The Spearman's rank correlation coefficient and unsupervised hierarchical clustering analysis with logarithmic values of CXCL9 concentration normalized to creatinine concentrations (CXCL9/cre) and CXCL10/cre were used to explore the relationship between urinary CXCL9/cre, CXCL10/cre and Banff scores.

The ability of urinary CXCL10/cre and CXCL9/cre to detect transplant rejection was analyzed by constructing receiver operating characteristic (ROC) curves. The Youden index was estimated from the ROC curve to calculate the optimal threshold value. These cut-off values were used to calculate the sensitivity and specificity of urinary CXCL10/cre for diagnosis of the rejection. A multivariate Cox proportional regression analysis was performed to analyze the association of CXCL/cre levels with graft survival. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).

Results

CXCL9/cre and CXCL10/cre Levels Are Increased in Patients with Rejection

The median of CXCL9/cre levels in the rejection group (5.55 [IQR 1.04-15.88] ng/mmol) was significantly higher than in the normal histology group (0.00 [IQR 0.00-0.89] ng/mmol) and the other histology group (1.83 [IQR 0.00-9.54] ng/mmol), $P<0.05$ (**Figure 1**).

The diagnostic performance of CXCL9/cre in discriminating transplant rejection from normal histology was estimated by receiver operating characteristic (ROC) curve analysis. The ROC area under the curve (AUC) value was 0.857 (95% CI 0.771-0.943, $P<0.001$). The cut-off value of CXCL9/cre that showed the best sensitivity (70.5%) and specificity (92.3%) was 2.45 ng/mmol. We further tested CXCL9/cre diagnostic performance in discriminating rejection from all other histological groups (including normal histology and other histology abnormalities like recurrent glomerulonephritis, and glomerulosclerosis). The ROC AUC value was 0.728 (95% CI 0.632-0.824, $P<0.001$), the cut-off value of 0.11 ng/mmol resulted in the best sensitivity (76.9%), and specificity (73.1%) (**Figure 2**). The negative predictive value was 82.5%, while the positive predictive value was 48.1%.

The median of CXCL10/cre levels in the rejection group (1.89 [IQR 0.57-5.89] ng/mmol) was significantly higher than in the normal histology group (0.18 [IQR 0.00-0.37] ng/mmol) and the other histology group (0.64 [IQR 0.22-1.47] ng/mmol), $P<0.05$. The ROC AUC value for CXCL10/cre in discriminating transplant rejection from normal histology was 0.827 (95% CI 0.729-0.925, $P<0.001$). The cut-off value of CXC10/cre with the best sensitivity (75.6%) and specificity (88.5%) was 0.65 ng/mmol. The ability of CXCL10/cre to discriminate transplant rejection from all other histological groups had an ROC AUC value 0.73 (95% CI 0.63-0.84, $P<0.001$). The cut-off value with the best sensitivity (71.4%) and specificity (84.6%) was 0.42 ng/mmol. The negative predictive value was 78.7%, while the positive predictive value was 48.6%.

CXCL/cre Ability to Discriminate between ABMR and TCMR

TCMR and mixed rejection cases were excluded from further analysis, leaving only cases with pure ABMR, normal histology, and other histology lesions (without rejection). CXCL9/cre ability to discriminate ABMR demonstrated an ROC AUC value of 0.73 (95% CI 0.62-0.84, $p<0.001$). The CXCL10/cre ROC AUC value was higher, at 0.75 (95% CI 0.63-0.87, $P<0.001$).

To further test the chemokine's ability to detect TCMR, we excluded cases with ABMR and mixed rejection from further analysis. There were 9 cases of TCMR and 73 cases of normal or other histology. ROC analysis of CXCL9/cre and CXCL10/cre showed AUC values of 0.67 (95% CI 0.47-0.86, $P>0.05$) and 0.61 (95% CI 0.35-0.86, $P>0.05$), respectively, revealing a poor ability to identify pure TCMR from normal or other histology lesions. We removed all polyoma BK cases (n=5) from further analysis, but this did not improve CXCL9/cre and CXCL10/cre values significantly (CXCL9/cre ROC AUC 0.68 and CXCL10/cre ROC AUC 0.63, but $P>0.05$).

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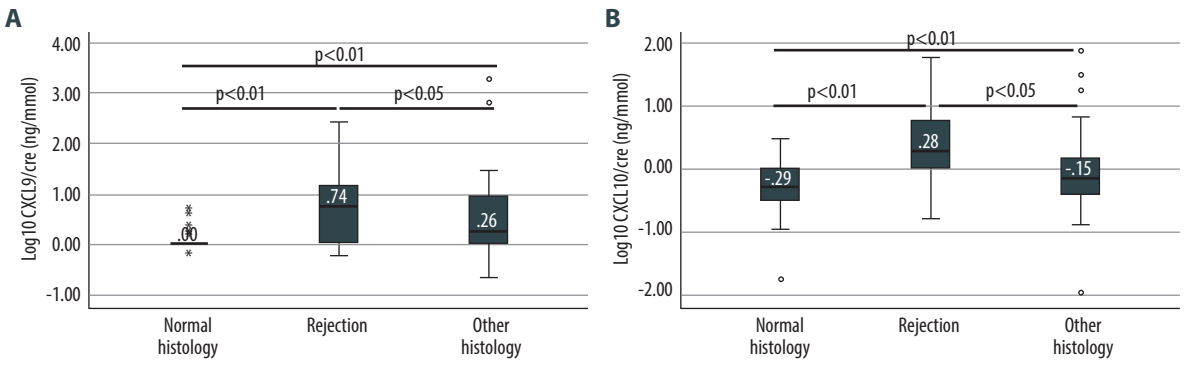

Figure 1. The logarythmic values of CXCL9/cre (**A**) and CXCL10/cre (**B**) levels in normal histology, rejection and other histology (eg, global glomerulosclerosis, recurrent glomerulonephritis, amyloidosis) cases. Both chemokines are significantly increased in rejection groups compared to other groups. *The figure was created with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).*

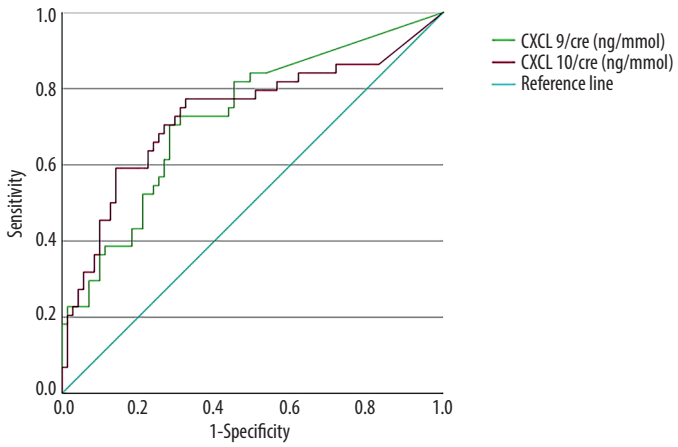

Figure 2. ROC curve for CXCL9/cre and CXCL10/cre at biopsy for discriminating rejection from all biopsies (eg, normal and with other histological abnormalities – global glomerulosclerosis, recurrent glomerulonephritis). *The figure was created with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).*

CXCL/cre in Polyoma BK Nephropathy Cases

From 117 kidney transplant recipients, 5 (4.3%) had biopsy-proven BK nephropathy. The median CXCL9/cre and CXCL10/cre levels were 9.91 [IQR 3.58-24.49] ng/mmol and 4.94 [IQR 1.10-18.88] ng/mmol, respectively. Levels of both chemokines were significantly higher in BK nephropathy compared to normal histology ($P<0.05$) but did not differ significantly from the rejection group.

Cases with Low CXCL/cre and Biopsy-Proven Transplant Rejection

A total of 7 patients with biopsy-proven kidney transplant rejection had CXCL9/cre levels <0.11 ng/mmol (subsequently referred to as "low CXCL9/cre"), while 10 patients with biopsy-proven kidney transplant rejection had CXCL10/cre levels lower than 0.42 ng/mmol (subsequently referred to as "low CXCL10/cre") – CXCL "false-negative" cases. However, none of those patients reached an ESRD of 11.5 during the median follow-up period [IQR 1.75-47.7], while in cases with increased CXCL9/cre and increased CXCL10/cre levels, 41.2% and 36.8% of patients reached ESKD, respectively.

In 40 cases with CXCL9/cre <0.11 ng/mmol (the cut-off value suggested by ROC), 82.5% did not have rejection on histology, but there were 3 cases of ABMR, 2 cases of T cell-mediated rejection, and 2 cases of mixed rejection, and those diagnoses may have been missed without a kidney biopsy.

In 47 cases with CXCL10/cre <0.42 ng/mmol (the cut-off value with best sensitivity and specificity), 78.7% of cases did not have rejection, but 5 cases of ABMR, 3 cases of TCMR, and 2 cases of mixed rejection may have been missed without a biopsy.

Using a combination of CXCL9/cre and CXCL10/cre with higher than cut-off values as an indication for kidney biopsy, 3 cases of ABMR, 2 cases of TCMR, and only 1 case of mixed rejection would have been missed without a biopsy.

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Table 2. Spearman correlation between CXCL/cre levels and Banff scores.

		g	cg	mm	t	ptc	i	ci	ct	v	cv	ah
CXCL9/cre	rho	0.24	0.17	-0.07	0.08	0.39	0.31	0.13	0.08	0.19	0.02	-0.01
	p	0.01	0.08	0.45	0.42	<0.001	<0.001	0.19	0.42	0.05	0.84	0.94
CXCL10/cre	rho	0.32	0.18	0.02	0.24	0.41	0.36	0.05	0.06	0.22	-0.02	-0.01
	p	<0.001	0.048	0.82	0.01	<0.001	<0.001	0.58	0.53	0.02	0.84	0.89

CXCL/cre Levels Correlation to Banff Scores

The logarithm of urinary CXCL9/cre and CXCL10/cre levels were correlated to all Banff scores (**Table 2**). Glomerulitis (g), peritubular capillaritis (ptc), and inflammation (i) lesions were significantly correlated with CXCL9/cre and CXL10/cre levels, whereas chronic glomerulopathy (cg), tubulitis (t), and vascular lesions (v) had a significant correlation only with CXCL10/cre level. Unsupervised hierarchical cluster analysis (**Figure 3**) revealed that CXCL9/cre and CXCL10/cre were highly associated with Banff scores t, i, and v.

CXCL9/cre and CXCL10/cre levels were significantly higher in cases with higher scores of i and ptc (**Figure 4**). Tubulitis score t0 was defined as tubulitis-negative, while t1, t2, and t3 scores were defined as tubulitis-positive. CXCL9/cre and CXCL10/cre levels were higher in tubulitis-positive patients – the median CXCL9/cre level was 1.45 ng/mmol [IQR 0.00-6.00 ng/mmol] in t0 compared to 2.94 ng/mmol [IQR 0.00-11.85 ng/mmol] in the t1-3 group (P<0.01). The median CXCL10/cre level was 0.51 ng/mmol [IQR 0.17-1.82 ng/mmol] in t0 compared to 1.26 ng/mmol [IQR 0.17-3.93 ng/mmol] in the t1-3 group (P<0.05. There was a tendency towards higher levels of CXCL9/cre and CXCL10/cre in higher scores of g and v as well as higher CXCL9/cre in increased interstitial fibrosis (ci) cases, although the difference was not statistically significant.

CXCL in Other Histology Groups

Urinary CXCL9/cre and CXCL10/cre were measured in patients with other histological diagnoses – diffuse glomerulosclerosis (n=17), thrombotic microangiopathy without rejection (n=3), recurrent IgA nephropathy (n=3), interstitial nephritis (n=3), calcineurin inhibitor (CNI) toxicity induced lesions (n=8), and AA amyloidosis (n=1). CXCL9/cre and CXCL10/cre were increased in cases with thrombotic microangiopathy and cases with amyloidosis, and, to a lesser extent, in cases with interstitial nephritis, while in glomerulosclerosis, CXCL levels remained low in patients with recurrent IgA nephropathy and CNI toxicity (**Figure 5**).

CXCL/cre Levels and Time After Transplantation

To determine if CXCL/cre levels differ depending on the time to rejection after transplantation, patients with biopsy-proven transplant rejection were divided into 2 groups: patients with biopsy taken <12 months (n=29) and >12 months (n=13) after transplantation. CXCL9/cre and CXCL10/cre levels did not differ significantly between these groups.

CXCL/cre and Transplant Survival

The association of CXCL9/cre and CXCL10/cre with transplant survival was investigated using multivariate Cox proportional hazards regression. Patients with higher CXCL9/cre (≥0.11 ng/mmol) were significantly associated with transplant progression to ESRD (HR 5.16, 95%CI 1.85-14.42, P<0.01), irrespective of other covariates like patient age, sex, serum creatinine levels, and histological diagnosis of rejection. We found that 12.5% of patients with low CXCL9/cre started dialysis compared to 40.3% of patients with high CXCL9/cre (P<0.05) during the median follow-up of 49.5 months [IQR 15.5-67.0] in the low CXCL9/cre group and 11.0 months [IQR 3.0-23.5] in the high CXCL9/cre group.

Patients with higher CXCL10/cre (≥0.42 ng/mmol) were significantly associated with transplant progression to ESRD (HR 3.25, 95%CI 1.27-8.36, P=0.01), irrespective of the above-mentioned covariates. Dialysis was started by 12.8% of patients with low CXCL10/cre compared to 42.9% of patients with high CXCL10/cre (P<0.05). Kaplan-Meier curves of graft survival are presented in **Figure 6**.

Discussion

Despite significant achievements in immunosuppressive therapies and kidney transplant recipient care, allograft rejection remains the main cause of kidney graft failure [23]. Early diagnosis and therapy can improve transplant prognosis [24], and development of accurate non-invasive diagnostic tools is essential for better allograft care.

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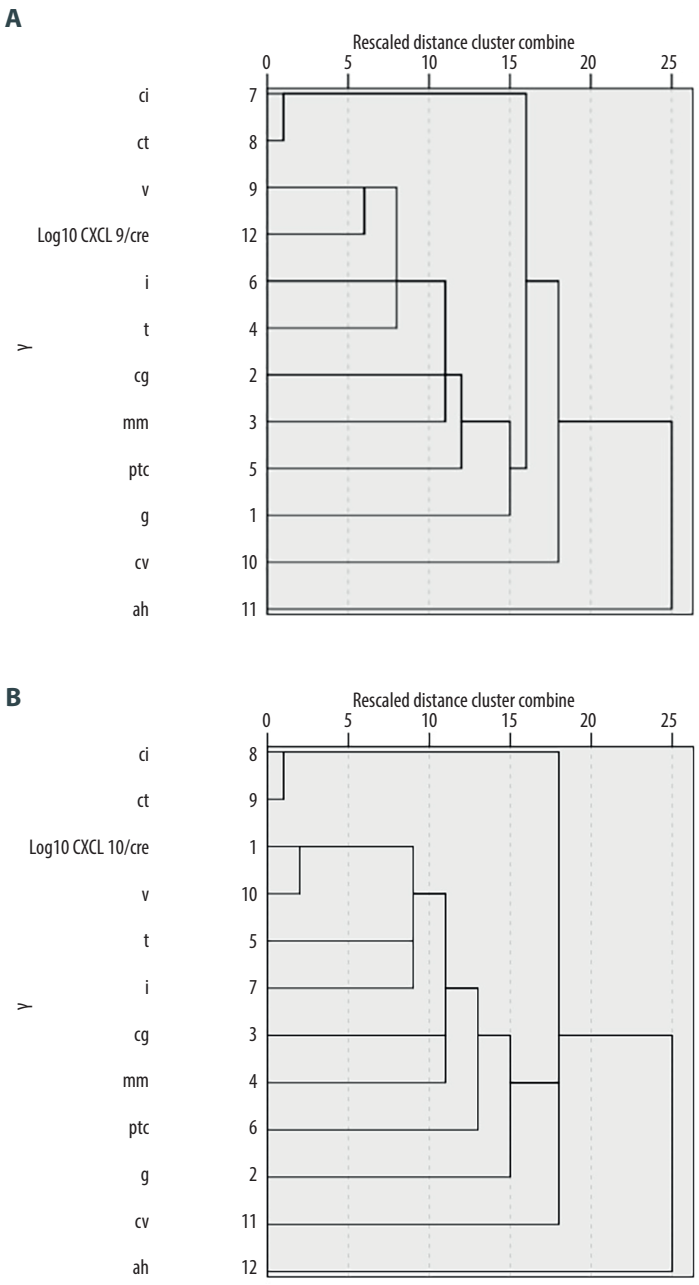

Figure 3. Dendrogram representation of unsupervised hierarchial cluster analysis of Banff scores and urinary CXCL9/cre (**A**) and CXCL10/cre (**B**). The figure was created with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).

This study demonstrated that urinary CXCL9/cre and CXCL10/cre can distinguish between kidney transplant recipients with and without biopsy-proved graft rejection, which is consistent with previously published data [12,16,25,26].

There were slight differences in optimal chemokine cut-off values detected in our study compared to other reports. We used a CXCL10 cut-off value of 0.42 ng/mmol, while another study suggested the optimal cut-off of 1.535 ng/mmol for biopsy decision in subclinical pathologies and 2.586 ng/mmol for clinical pathologies, reducing surveillance and indication biopsy rate by 61% and 64%, respectively [6]. However, that study analyzed separate histology lesions (inflammation, tubulitis, vascular lesions), while our study evaluated CXCL diagnostic properties based on final histology entities according to the Banff classification system. Other authors found CXCL10 sensitivity 72% and specificity 71-73% in discriminating rejectors and non-rejectors with the optimal CXCL10/cre cut-off 0.43 ng/mmol [14,27]. These results are very similar to our study findings.

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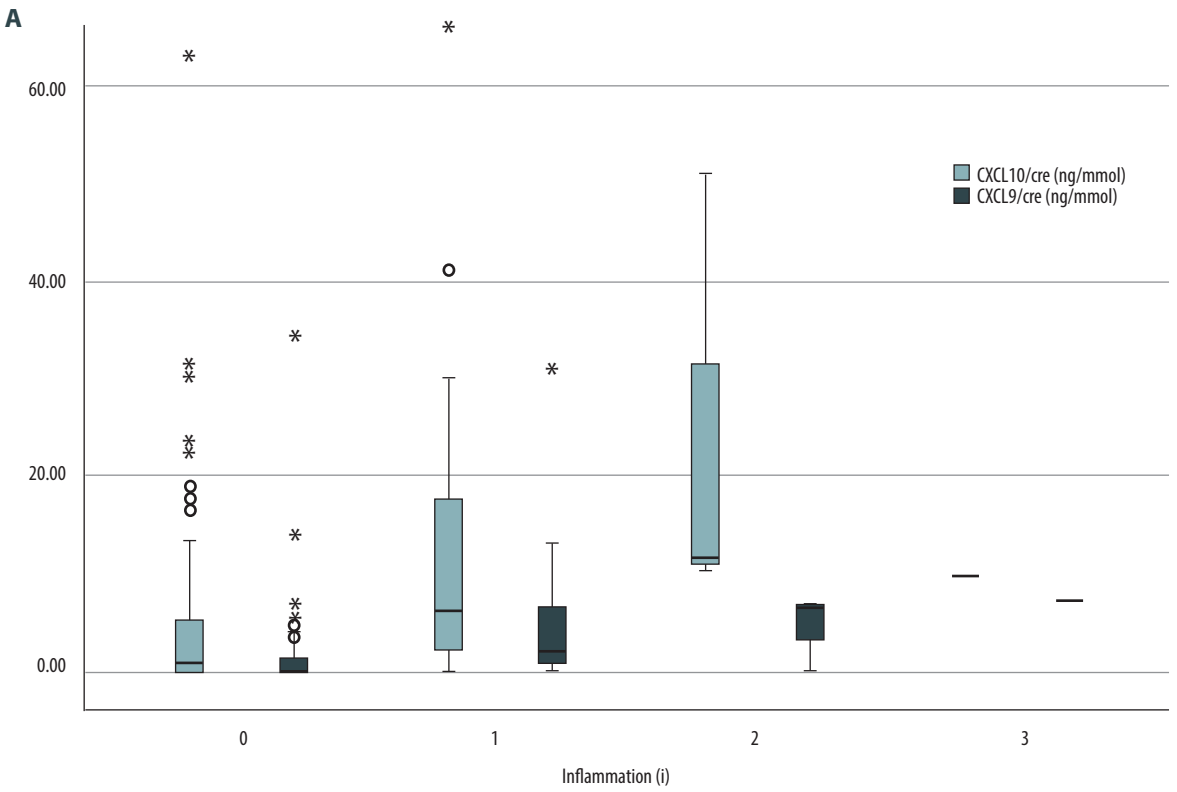

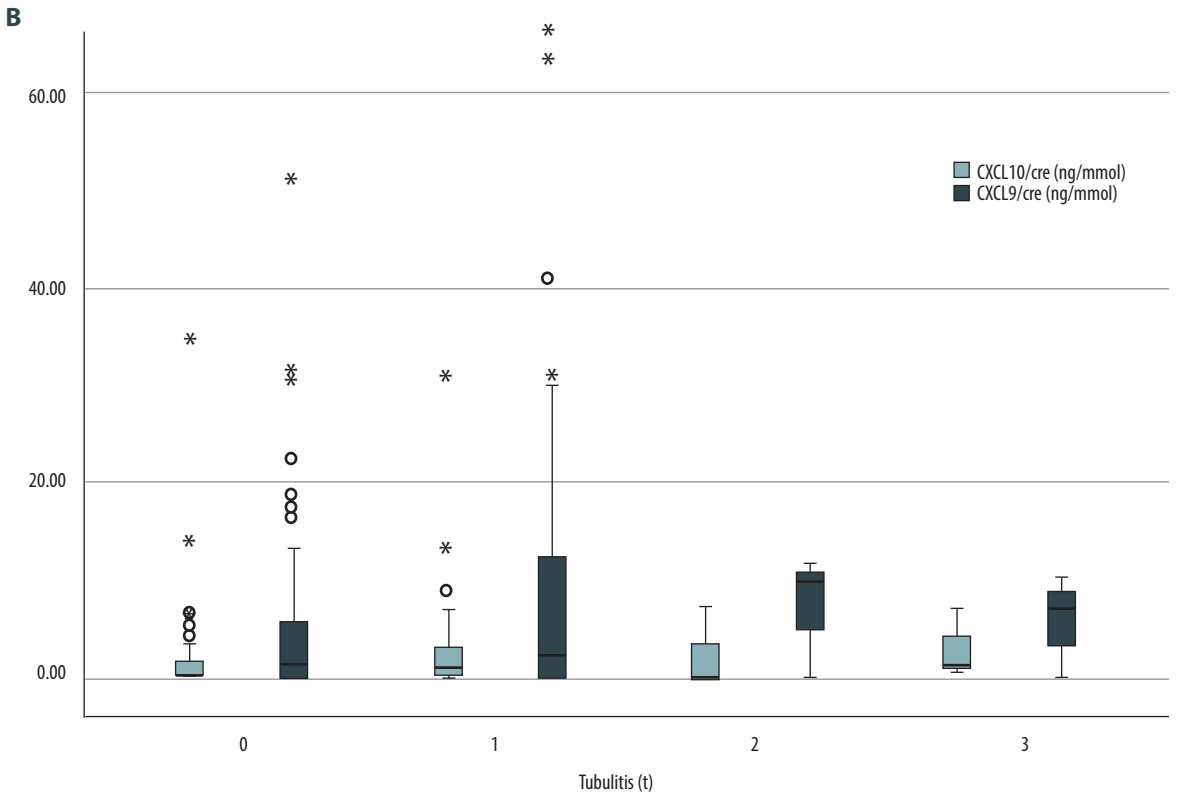

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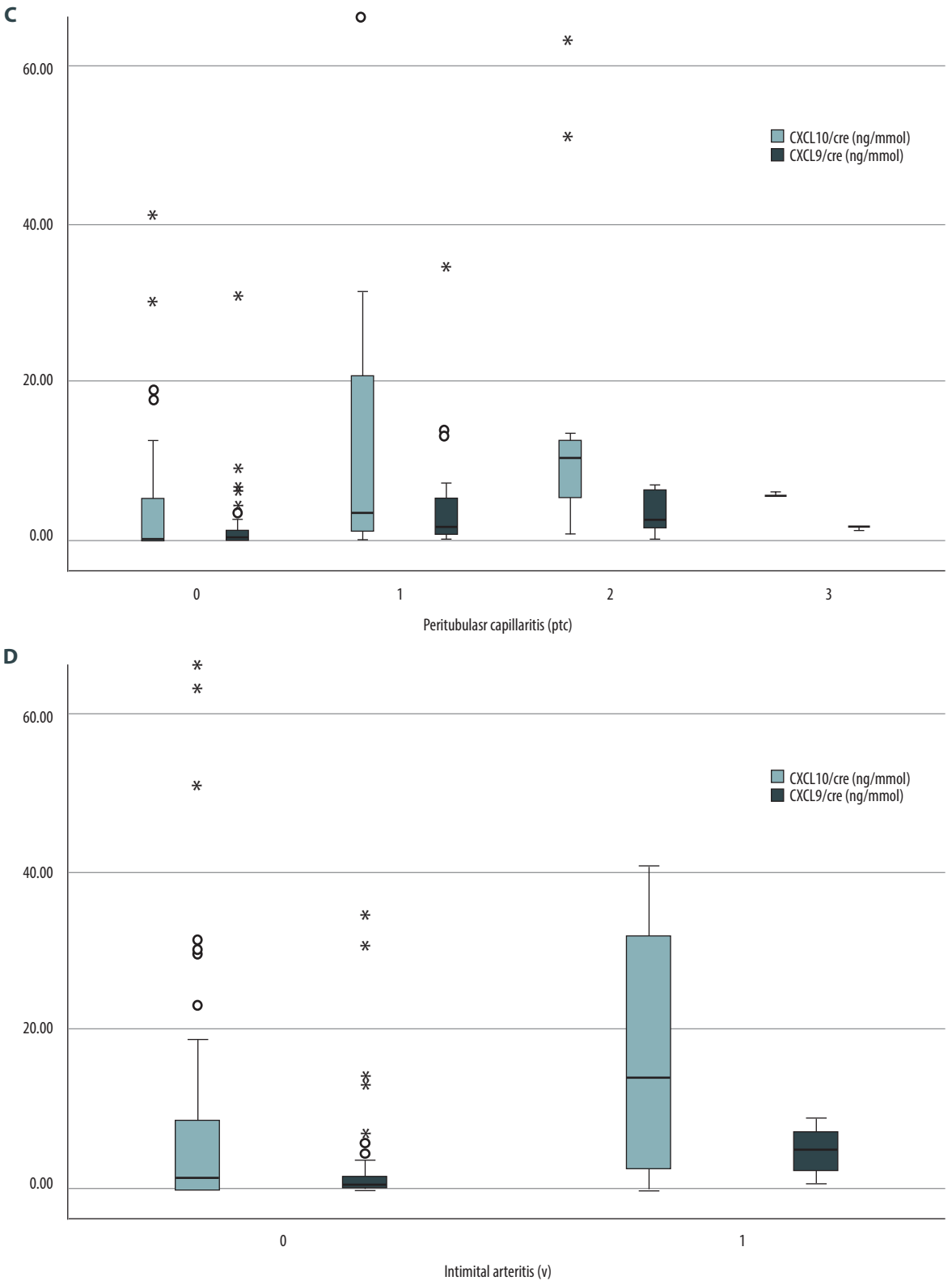

Figure 4. Boxplots representing increasing CXCL9/cre and CXCL10/cre levels in the higher Banff scores of inflammation (**A**), tubulitis (**B**), peritubular capillaritis (**C**) and intimal arteritis (**D**). *The figure was created with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).*

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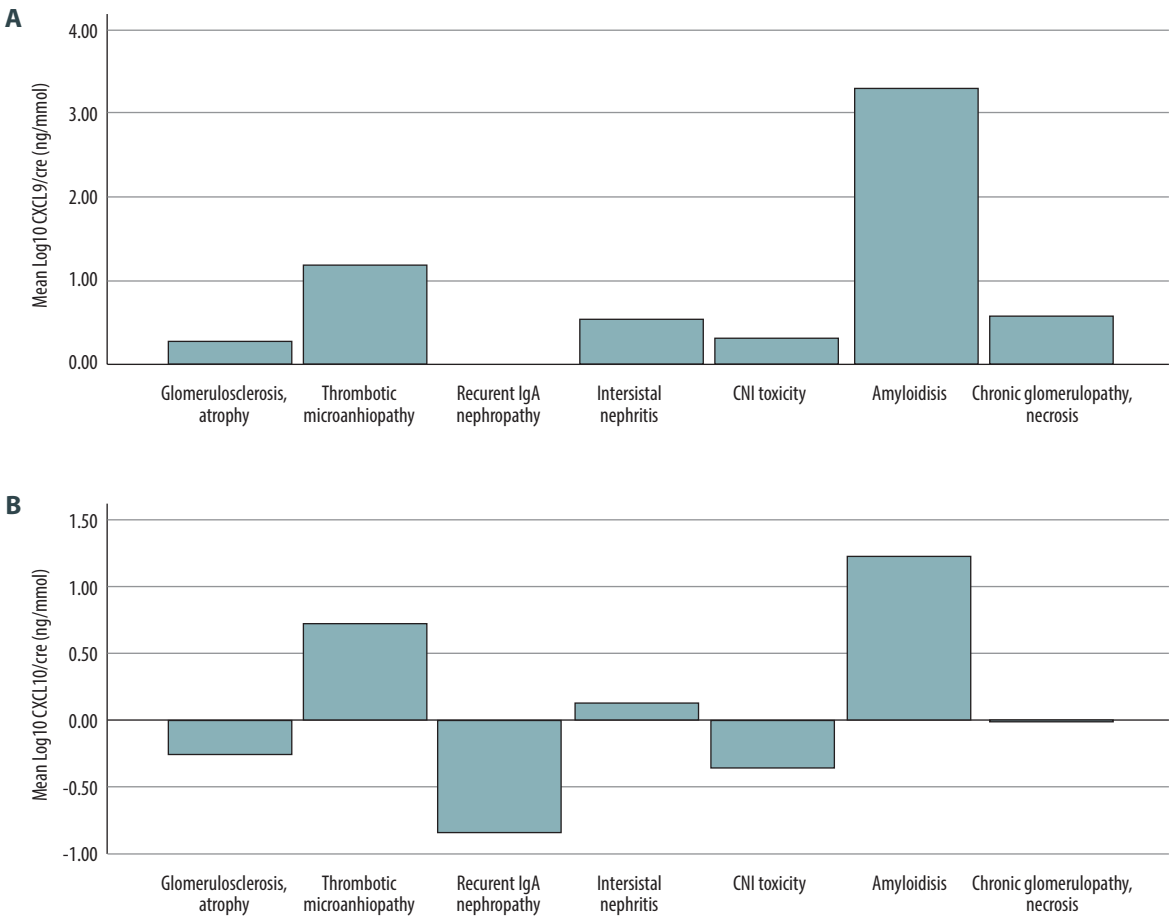

Figure 5. The means of Log10CXCL9/cre (**A**) and Log10CXCL10.cre (**B**) levels in cases with other histology abnormalities, no rejection. *The figure was created with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).*

There were some discrepancies between our study results and other publications about the association of CXCL with different Banff scores. Our data showed that both chemokines were highly associated with Banff scores t and i. This agrees with other studies [11,28] and can be explained by the fact that chemokines reflect inflammatory damage of the tubular compartment, irrespective of etiology [29]. Arnau et al found ptc, g, and cg scores to be associated with CXCL10/cre, while Ho et al found that CXCL10 was elevated with patients with higher ptc but not g scores [16]. Our Spearman correlation analysis showed both chemokines were associated with ptc and i, representing acute inflammatory lesions within a graft. CXCL10/cre was also associated with v lesion, while Ho et al showed that CXCL10 was increased in microvascular compartment injury but not in cases of isolated vascular lesions [16]. We found no association between chemokines and chronic graft lesions such as cv, but CXCL10/cre was significantly correlated with cg, which is consistent with the findings of Arnau et al [7].

Treatment of allograft rejection is different in cases with TCMR and ABMR; therefore, it is necessary to discriminate those types of rejection [30]. However, current data show that chemokines are unable to discriminate between these 2 types of rejection but distinguish well both types of rejection from normal histology patients. Ho et al found CXCL10 had an AUC of 0.81 (95% CI, 0.74-0.88) for detecting TCMR [31], and Ciftci et al showed that early posttransplant increase of CXCL9 and CXCL10 predicts TCMR and the time of rejection [26]. However, our data showed a poor ability of CXCL9/cre and CXCL10/cre to detect TCMR, possibly due to the small sample size in the TCMR group (we had only 9 cases of TCMR, while Ho et al analyzed 71 cases of mild to severe TCMR).

Another study showed that urinary CXCL10 had good discrimination ability for AbMR (AUC-ROC 0.760, *P*=0.001) [7]. Our data showed quite similar results – the CXCL10/cre AUC-ROC was 0.75.

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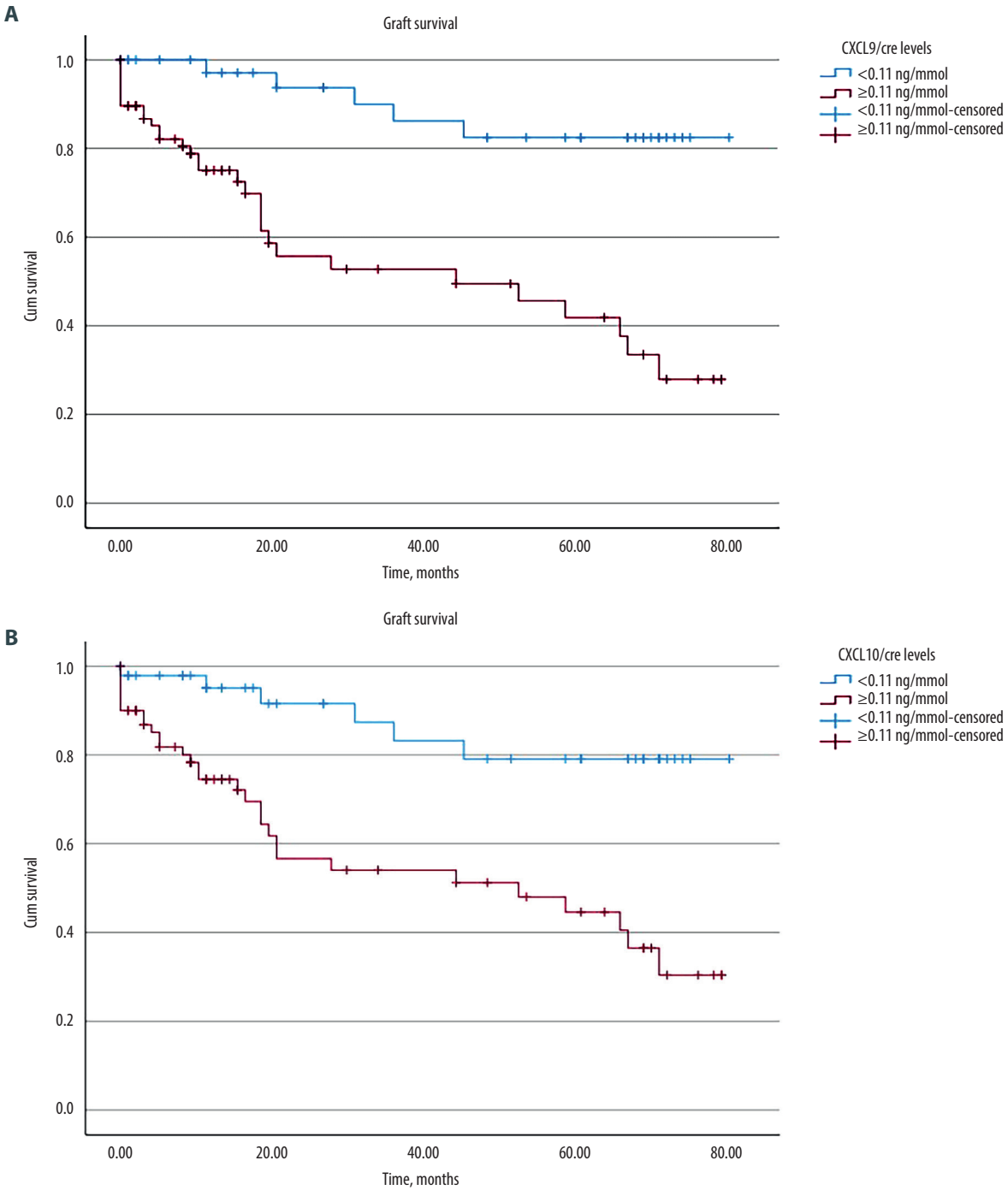

Figure 6. Kidney graft survival (time from kidney biopsy to initiation of dialysis) of patients with low and high levels of CXCL9/cre (**A**) and CXCL10/cre (**B**). *The figure was created with SPSS 29.0 (SPSS, Inc, Chicago, IL, USA).*

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The CTOT-01 consortium determined urinary CXCL9 protein positive predictive value for acute rejection as 67%, whereas the negative predictive value was 92%, and found that low 6-month urinary CXCL9 protein identified patients without subclinical allograft injury and who were most likely to maintain stable kidney function [25]. Our data showed lower CXCL9/cre positive and negative predictive values – 48.1% and 82.5%, respectively.

With the use of urinary CXCL9 and CXCL10 biomarkers, both types of rejection are well distinguished from patients without rejection; therefore, these cytokines can be utilized to monitor kidney transplant recipients in a non-invasive manner. Minkowski demonstrated that in asymptomatic kidney transplant recipients, a urinary CXCL10-guided strategy would have reduced surveillance biopsies at 3 and 6 months by 61% [6]. In patients with graft dysfunction, the CXCL10-guided strategy would have reduced the number of indication biopsies by 64%. However, kidney biopsy should always be considered when allograft dysfunction with a high probability of rejection is present.

However, CXCL9 and CXCL10 are insufficient as the sole means of diagnosing kidney transplant rejection as they lack specificity and their levels can be higher in cases of viral or bacterial infections. Levels of CXCL9 and CXCL10 in urine were found to be substantially higher in patients with BKPyVAN compared to control subjects, including those with ABMR [32]. Our data confirmed that BK nephropathy cases had higher CXCL9/cre and CXCL10/cre levels compared to normal histology cases, but did not differ from CXCL levels in rejection cases.

To the best of our knowledge, little data is available about urinary CXCL9 and CXCL10 levels in transplant recipients with various recurrent native kidney diseases. Although our group of patients with recurrent kidney disease was very small, we found that both chemokines were much higher in patients with AA amyloidosis and thrombotic microangiopathy, which was mainly atypical hemolytic-uremic syndrome. Both diseases are associated with increased inflammatory response and cytokine production [33,34]. In patients with marked nephrosclerotic lesions, recurrent IgA nephropathy, and CNI toxicity, CXCL9 and CXCL10 levels remained low, demonstrating a different mechanism of kidney injury. These findings are interesting and more research should be done in the area of recurrent glomerulonephritis and chemokines.

An important advantage of CXCL9 and CXCL10 is that they can show rejection earlier than other currently used biomarkers. Observational studies demonstrate that CXCL10 rises prior to serum creatinine and decreases after treatment of rejection [12,35,36]. Moreover, chemokines can predict graft prognosis and stratify patients into a high and low risk of rejection. Our study evaluated dialysis-free graft survival and revealed better graft survival in patients with lower CXCL9/cre and CXCL10/cre levels irrespective of serum creatinine at the time of biopsy. Interestingly, low CXCL/cre levels predicted better graft survival irrespective of the presence of acute rejection in kidney biopsy. Patients with low CXCL or CXCL10 levels and biopsy-proven kidney transplant rejection had a better prognosis than patients with rejection and high levels of chemokines – none of the "rejectors" with low levels of chemokines reached end-stage renal disease during the follow-up period. It has been reported that low 6-month CXCL10 (<0.70 ng/mmol) is associated with a 95% endpoint-free (no rejection, no GFR reduction >20%, no graft loss) 5-year survival compared to 78% with high 6-month CXCL10 [37]. Rabant et al showed that CXCL10/cre at 3 months after transplantation predicted acute rejection independent of concomitant protocol biopsy results, but they used a CXCL10/cre cut-off value of 2.79 ng/mmol [15].

Our study has some limitations. First, we had donor-specific antibody data only for a limited number of patients; therefore, all kidney transplant ABMR diagnoses were based only on allograft biopsy histology lesions. To calculate the accuracy of CXCL9 and CXCL10 levels as biomarkers of transplant rejection, we regarded kidney biopsy as the criterion standard, but some of the Banff scores (eg, pct) have inter-pathologist agreement of 0.52. ptc [38]. Therefore, it would be rational to employ digital image analysis for a more objective and reproducible quantitative evaluation of Banff scores [39]. The second limitation is the relatively small sample size in different types of graft rejection and BK nephropathy, limiting the ability to detect significant differences in CXCL level profile in particular types of rejection.

Despite the above-mentioned limitations, this study provides valuable insights into the potential of both chemokines, CXCL9 and CXCL10, to detect kidney transplant rejection, confirming the results of previous studies and providing additional information about CXCL9 and CXCL10 levels in patients with non-rejection histology lesions. Our study also adds substantial information about the relationship of CXCL/cre levels with overall graft survival, showing CXCL9/cre and CXCL10/cre are independent predictors of graft loss.

Conclusions

This study confirmed previous findings that urinary CXCL9/cre and CXCL10/cre levels distinguish patients with transplant rejection from those without it. Low levels of urinary CXCL9/cre and CXCL10/cre were associated with better allograft prognosis. Our data supplement findings about CXCL9 and CXCL10 in relation to Banff scores—both chemokines were significantly increased in higher scores of inflammation, tubulitis, and

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peritubular capillaritis. Moreover, we found that CXCL9 and CXCL10 were significantly higher in patients with polyoma BK virus infection and in those with AA amyloidosis and thrombotic microangiopathy.

Declaration of Figure's Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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