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Full Length Article

Advantages of plasmatic CXCL-10 as a prognostic and diagnostic biomarker for the risk of rejection and subclinical rejection in kidney transplantation

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ABSTRACT

This study evaluate the potential of plasmatic CXCL-10 (pCXCL-10) as a pre&post transplantation prognostic and diagnostic biomarker of T-cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR) and subclinical rejection (SCR) risk in adult kidney recipients considering BKV and CMV infections as possible clinical confounder factors.

Twenty-eight of 100 patients included experienced rejection (TCMR:14; ABMR:14); 8 SCR; 13 and 16 were diagnosed with BKV and CMV infection, respectively. Pre-transplantation pCXCL-10 was significantly increased in TCMR and ABMR and post-transplantation in TCMR, ABMR and SCR compared with nonrejectors. All CMV^+ patients showed pCXCL-10 levels above the cutoff values established for rejection whereas the 80% of BKV^+ patients showed pCXCL-10 concentration < 100 pg/mL.

pCXCL-10 could improve pre-transplantation patient stratification and immunosuppressive treatment selection according to rejection risk; and after kidney transplantation could be a potential early prognostic biomarker for rejection. Clinical confounding factor in BKV^+ and particularly in CMV^+ patients must be discarded.

1. Introduction

The identification of potential noninvasive prognostic biomarkers could be a critical step towards the efficient selection and adjustment of immunosuppressive (ISP) treatment and improvements in clinical graft outcome [1]. Knowledge of the individual immune status of patients, especially before transplantation, would enable patient stratification according to the risk of suffering a specific clinical event, such as rejection and/or infections and improved ISP treatment selection.

In this sense, the concentration of circulating IFN-γ-inducible protein (CXCL-10) has been proposed to be a promising biomarker of short- and long-term kidney graft function [1–3]. CXCL-10 is a potent chemoattractant for several immune cells, including CD4 and CD8 T cells, to the sites of inflammation. Before and after transplantation, increased concentrations of CXCL-10 in kidney and liver recipients have been associated with an increased risk of rejection [4–6]. With the current ISP

Abbreviations: AR, acute rejection; AUC, area under the curve; ABMR, antibody-mediated rejection; BKV, polyoma virus; BPAR, biopsy-proven acute rejection; CI, confidence interval; CMV, cytomegalovirus; Cr, creatinine; CXCL10, chemokine interferon-inducible protein 10; dnDSA, de novo donor-specific antigens; EVR, everolimus; GFR, glomerular filtration rate; IFN-γ, interferon-gamma; ISP, immunosuppressive; MMF, mycophenolate mofetil; MPA, mycophenolic acid; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; SCR, subclinical rejection; Tac, tacrolimus; TCMR, T cell-mediated rejection.

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regimens, the incidence of acute rejection (AR) after kidney transplantation is approximately 10–15%. AR is diagnosed by an increase in serum creatinine (Cr) or the development of new-onset proteinuria and is confirmed by biopsy, an invasive method that may favor some severe adverse events. Currently, there is a lack of biomarkers for subclinical rejection (SCR) apart from surveillance biopsy [7] being this clinical event of great relevance in function and graft lost [8,9].

Previous observational studies have demonstrated that increased urinary CXCL-10 levels can identify patients with an ongoing rejection episode several days before a biopsy is indicated by rising serum Cr levels. In a previous European multicenter study (*EudraCT number: 2013–001817-33*) on kidney transplant recipients [5] coordinated by our group, urinary CXCL-10 was identified as a prognostic and diagnostic biomarker of AR and graft outcome in the early post-transplant period [6,7,10]. For the first time, Rabant et al. [10] identified the association between urinary CXCL-10 levels and the diagnosis of ongoing antibody-mediated rejection (ABMR), a relevant cause of long-term kidney allograft failure [11]. In some transplant centers, de novo donor-specific antigen (dnDSA) monitoring has been implemented as a biomarker for the risk of ABMR, but its association is a subject of controversy. High within-patient variability of Tac blood concentrations was associated with an increase in the development rate of dnDSA, with a consequential increase in the risk of graft loss [12,13]. Importantly, CXCL-10 is also elevated in the urine of kidney recipients with polyomavirus (BKV) viremia. However, no association between cytomegalovirus (CMV) viremia and urinary CXCL-10 levels has been described [4,6,14].

Regarding serum CXCL-10, there are controversial results in terms of its utility as a potential biomarker for assessing the risk of rejection [15,16], mainly due to the presence of clinical confounding factors. A few studies reported that high pre-transplantation serum CXCL-10 concentrations were associated with long-term graft loss after kidney transplantation [16]. More recently, Xu et al. [17] showed that serum CXCL-10 levels measured on the 4th and 7th days after kidney transplantation were significantly higher in patients with AR than in patients without AR.

The aim of this European multicenter study was to evaluate the potential and clinical event specificity of the plasmatic CXCL-10 concentration measured before and after transplantation as a prognostic and diagnostic biomarker of the risk of TCMR, ABMR and SCR in a cohort of adult kidney transplant patients and to consider possible clinical confounding factors related to BKV and CMV infections. This prospective study could enlighten the benefits of CXCL-10 measurements in plasmatic samples pre- (reported here for the first time in kidney transplantation) and post-transplantation.

2. Methods

2.1. Study design and patients

In this prospective observational multicenter study, 100 adult kidney transplant patients were recruited from four European centers, two in Spain and two in Germany. Participants were de novo kidney transplant recipients from a deceased or living donor, with no other transplanted organs. Recipients older than 70 years and those positive for hepatitis B or C or human immunodeficiency virus were excluded. The study was approved by the Ethics Committees of the participating centers, and all patients provided their written informed consent (*EudraCT number: 2013–001817-33*).

Ninety percent of patients received ISP therapy consisting of tacrolimus (Tac), mycophenolate mofetil (MMF) and methylprednisolone or 10% Tac, everolimus (EVR) and methylprednisolone. Further details of ISP therapy are described in the **Supplementary Methods.**

Patients had a total of six visits during the study: pre-transplantation and during the 1st week and the 1st, 2nd, 3rd, and 6th months after transplantation. All of the participating centers used the same criteria for the diagnosis of rejection, which was always confirmed by histological evaluation of graft biopsies (biopsy-proven acute rejection, BPAR). The biopsy samples were read by the local pathologist and classified according to the current Banff classification [18,19]. Blood and urine samples were collected on the day of the biopsy in the morning and prior to the biopsy.

BKV infection was defined as detectable virus >1000 copies/mL [20,21], and CMV infection was defined as detectable CMV virus >1000 copies/mL [22].

2.2. Pharmacokinetic monitoring

For Tac and mycophenolic acid (MPA), the C_0 and completed AUC (0, 30 min, 1, 1.5, 2, 3, 4, 6, 8, and 12 h after drug administration) were measured in the 1st week post-transplantation, and the simplified AUCs (pre-dose, 2 h and 4 h post-dose) were determined in the 1st, 2nd, 3rd and 6th months post-transplantation. From the 1st month to the 6th month post-transplantation, the Tac and MPA complete AUC values were estimated [23].

Blood samples were collected in EDTA-K3 tubes. Whole-blood Tac and EVR concentrations were measured by liquid chromatography/tandem mass spectrometry, and MPA plasma concentrations were measured by high-performance liquid chromatography with an ultraviolet detector, as previously reported [24].

AUC estimation methods and intrapatient variability in Tac-C_0 were estimated by means of the coefficient of variation (CV) (for details, see **Supplementary Methods).**

2.3. Plasmatic and urinary CXCL-10 measurements

For the analysis of plasmatic CXCL-10, whole blood was collected in EDTA-anticoagulant tubes before the morning dose of treatment and centrifuged, within the first 2 h post-extraction, at 3000 rpm for 10 min, and the plasma was stored at −70 °C for batch analysis. For the analysis of urinary CXCL-10, first morning urine samples were collected from all patients post-transplantation and centrifuged, within the first 2 h post-extraction, at 3000 rpm for 10 min, and the supernatant was stored at −70 °C. Both kinds of samples were shipped to the Pharmacology and Toxicology Laboratory (CDB), Hospital Clinic of Barcelona, for centralized CXCL-10 analysis in a blinded fashion. No samples (urine and plasma) remained frozen for more than a month until analysis, and they were only thawed once. CXCL-10 concentrations were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Further technical details are described in the **Supplementary Methods.**

2.4. Statistical analyses

Demographic data and molecular analysis results were collected in a unified database. All of the analyses were performed using SPSS software, version 23.0 (SPSS Inc., Chicago, IL, USA). The samples were adjusted to fit a nonparametric distribution. Statistically significant differences between groups were assessed with the Mann-Whitney test, and correlations between variables were assessed by Spearman's rho test. We used receiver operating characteristic (ROC) curve analysis to define the optimal biomarker cutoffs for distinguishing patients with and without TCMR, ABMR or SCR, and BKV or CMV infection was based on ROC curves and was calculated with the best Youden index (sensitivity + specificity – 1) [25,26]. All of the data are presented as the median ± standard deviation (SD). $P \leq 0.05$ was considered to indicate statistical significance. Further details of the statistical analysis are described **in the Supplementary Methods.**

3. Results

3.1. Patients

The main characteristics of the 100 kidney recipients are shown in Table 1. During the post-transplantation follow-up, 14 patients experienced TCMR, 14 ABMR and 8 were diagnosed with SCR. Of the patients who suffered TCMR, plasma and urine samples were obtained for CXCL-10 measurements. The diagnosis of rejection was based on clinical and laboratory findings and was confirmed by histological evaluation of graft biopsies. Table 1 shows the time post-transplantation of the clinical event. For all patients who rejected, plasma and urine were always collected for biomarker analysis before the ISP regimen was modified to resolve the rejection episode.

Thirteen patients were diagnosed with BKV infection and 16 with CMV infection (Table 1S) [27]. Of the 13 BKV-infected patients, 2, 1 and 3 patients experienced TCMR, ABMR and SCR, respectively. Two of the 16 CMV-infected patients also suffered for ABMR, but in no case were the two events coincident in time.

3.2. Pharmacokinetics

Table 2S summarizes the C_0 values for Tac, EVR and MPA, the $AUC_{0\text{-}12h}$ values and the $AUC_{0\text{-}12h}$/Cmin ratios for Tac and MPA, and the Tac, MPA, EVR and prednisone doses. During the 6 months post-transplantation, the Tac C_0 was 7–10 ng/mL. No statistically significant differences in Tac, MPA or EVR C_0 or prednisone dose were observed between rejectors (TCMR, ABMR or SCR), patients with CMV or BKV infection and nonrejectors during the evaluation period. Although the $AUC_{0\text{-}12h}$ values for Tac and MPA were similar among the analyzed groups, TCMR patients showed a clear tendency towards lower Tac $AUC_{0\text{-}12h}$ values than nonrejectors (median Tac $AUC_{0\text{-}12h}$ value from the 1st week to the 6th month post-transplantation: nonrejectors 177.2 ± 18.2 ngxh/mL vs. TCMR patients 133.04 ± 9.03 ngxh/mL); however, no differences were observed in the Tac $AUC_{0\text{-}12h}/C_0$ ratios. The results of the analysis of intrapatient variability in Tac C_0 are described in the **Supplementary Data.**

3.3. Plasmatic CXCL-10 concentration as a prognostic biomarker of rejection (TCMR, ABMR & SCR)

Pre-transplantation, there were significant differences in the plasmatic CXCL-10 concentration between nonrejectors ($n = 42$) and patients who experienced TCMR ($n = 14$; $P = 0.000$) or ABMR (n = 14; $P = 0.001$) (Fig. 1 & Table 2B); however, no significant differences were observed in CXCL-10 levels between those free of rejection and SCR patients ($P = 0.084$).

After transplantation, patients who experienced TCMR (n = 14) and ABMR (n = 14) and those diagnosed with SCR ($n = 8$) had significantly higher plasmatic CXCL-10 levels than nonrejectors (clinical event-free n = 42) throughout the study period ($P < 0.05$) (Fig. 1 & Table 2B). Plasmatic CXCL-10 median concentrations were similar in patients with BKV infection ($n = 13$) and in BKV-free patients during the entire study period (Fig. 1 and Table 2B), but high inter-patient variability was observed in the BKV^+ group since, in 3 patients, BKV+ showed plasmatic CXCL-10 levels >200 pg/mL, all of them at the 6th month post-transplantation (Table 1S-A). We noted that all of the patients with rejection had plasmatic CXCL-10 values greater than the specific cutoff established for each type of rejection, whereas for BKV infection, the 80% of patients showed CXCL-10 concentration values less than 100 pg/mL before and at the time of infection. Furthermore, in no case were the two events (rejection and BKV infection) coincident in time.

In addition, patients with active CMV infection ($n = 16$) had significantly higher plasmatic CXCL-10 concentrations than CMV-free patients (Fig. 1, Table 2B, Table 1S-B). Details about the time evolution pre- and post-transplantation of plasmatic CXCL-10 production in each rejector patient are described in **Supplementary Data and Figures.**

3.4. Pre- and post-transplantation prognostic values of plasmatic CXCL-10 concentration for the risk of rejection (TCMR, ABMR, and SCR) and possible confounding factors

Pre-transplantation cutoff values for predicting TCMR were determined based on AUC analysis of the ROC curve for plasmatic CXCL-10 levels, which were significantly higher in patients who experienced rejection (TCMR). ROC curve analysis showed that the plasmatic CXCL-

Table 1
Characteristics of 100 de novo kidney transplant recipients.

		Total N = 100	Free CE N = 42	TCMR N = 14	ABMR N = 14	SCR N = 8	BKV N = 13	CMV N = 16	p value
Recipient Sex (male)		65(65%)	23 (55%)	9 (64%)	10 (71%)	6 (75%)	11 (85%)	10 (63%)	0.426
Recipient Age (years)		51.5 ± 12.9	51.5 ± 13.7	57.0 ± 12.6	54.0 ± 13.6	55.0 ± 13.6	51.0 ± 8.9	45.0 ± 12.5	0.411
Donor Age (years)		56 ± 14.1	53.5 ± 12.1	59 ± 14.9	65.0 ± 12.7	63.5 ± 9.5	50.5 ± 9.8	47.0 ± 19.8	0.270
Time on Dialysis (months)		34 ± 55.6	34.2 ± 49.9	45.0 ± 41.9	22.0 ± 25.9	33.5 ± 31.1	35.5 ± 21.9	37.5 ± 12.6	0.241
Cold Ischemia Time (hours)		10.1 ± 7.9	10.3 ± 6.5	11.5 ± 7.1	10.8 ± 8.6	15.7 ± 10.9	13.1 ± 2.0	19.3 ± 3.4	0.147
Type of Donor	Cadaveric-Donor	55 (55%)	21 (50%)	11 (79%)	10 (71%)	5 (63%)	5 (38%)	7 (44%)	0.574
	Living-Donor	45 (45%)	21 (50%)	3 (21%)	4 (29%)	3 (37%)	8 (62%)	9 (56%)	
Immunosuppressive Regimen	Tac + MMF+ PDN	90 (90%)	42 (100%)	13 (93%)	8 (57%)	5 (63%)	11 (85%)	15 (94%)	0.065
	Tac + EVR + PDN	10 (10%)	0 (0%)	1 (7%)	6 (43%)	3 (37%)	2 (15%)	1 (6%)	
Induction Therapy	Yes	94 (94%	36 (86%)	14 (100%)	14 (100%)	8 (100%)	13 (100%)	16 (100%)	0.156
	No	6(6%)	6 (14%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Time Post-transplantation of the Clinical Event	1st week			3	5	2	0	0	
	1st month			2	2	0	1	0	
	2nd month			2	1	0	0	2	
	3rd month			2	0	2	3	4	
	6th month			5	6	4	9	10	

Quantitative variables are presented as the median and standard deviation. Count variables are presented as the raw and intragroup relative frequency. Free CE: free clinical event; TCMR: T-cell mediated rejection; ABMR: Antibody-mediated rejection; SCR: subclinical rejection; BKV: BK polyoma virus; CMV: Cytomegalovirus; Tac: Tacrolimus; PDN: Prednisone; MMF: Mycophenolate Mofetil; EVR: Everolimus. Kruskal-Wallis test groups. A value of $P \leq 0.05$ was considered significant.

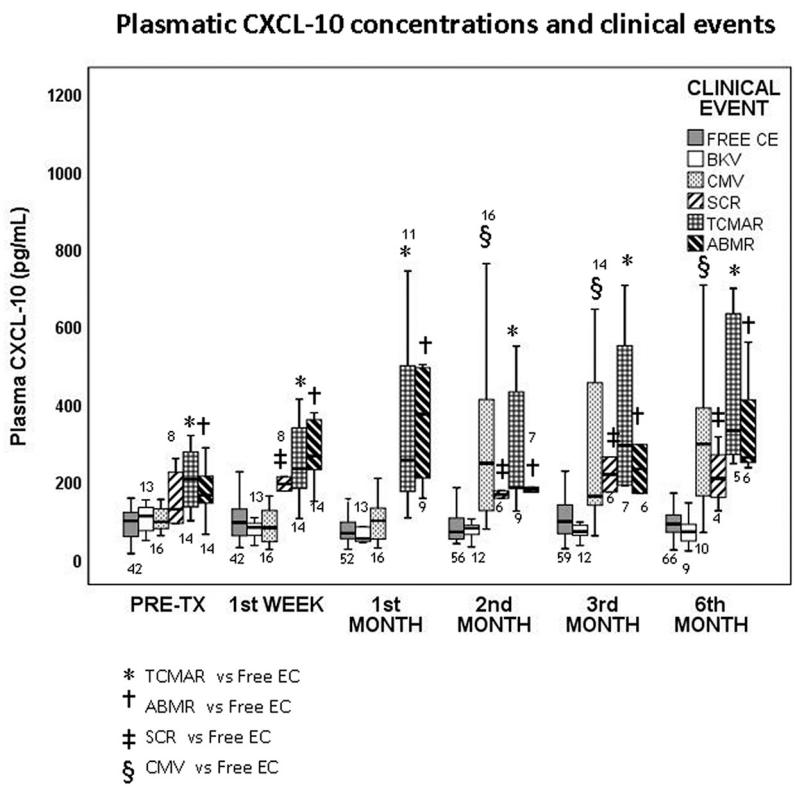

Fig. 1. Plasmatic CXCL-10 concentration and clinical events. Differences in the plasmatic CXCL-10 concentrations among patients free of clinical events (gray boxes), BKV^{+} patients (white boxes), CMV^{+} patients (dotted boxes) and patients diagnosed with SCR (left striped boxes), TCMR patients (hatched boxes) and ABMR patients (right striped boxes) during the first six months post-transplantation. Significant differences between groups were assessed with the Mann-Whitney test. A value of $P < 0.05$ was considered to indicate statistical significance: *patients free of clinical events vs. TCMR patients; †patients free of clinical events vs. ABMR patients; ‡patients free of clinical events vs. patients diagnosed with SCR and §patients free of clinical events vs. CMV^{+} patients).

10 concentration had an outstanding capacity to discriminate TCMR patients and nonrejectors (AUC = 0.920; 95% CI = 0.805–1.000) (Fig. 2A & B). The optimal cutoff value for predicting TCMR risk was 156.89 pg/mL, with 90% sensitivity, 80% specificity, 90% PPV and 95% NPV.

ROC curve analysis showed that post-transplantation plasma CXCL-10 levels also had an outstanding capacity to discriminate TCMR patients and nonrejectors: AUC = 0.944 (95% CI = 0.909–0.979), and the optimal cutoff value for predicting TCMR was 177.7 pg/mL with 84% sensitivity, 92% specificity, 80% PPV and 100% NPV (Fig. 2A & C).

When we evaluated the prognostic capacity of plasmatic CXCL-10 concentrations concerning ABMR risk, ROC curve analysis revealed an excellent capacity to discriminate ABMR patients and nonrejectors before and after transplantation: before transplantation AUC = 0.826; 95% CI = 0.713–0.939, optimal cutoff value 140.4 pg/mL, 71% sensitivity, 73% specificity, 82.3% PPV and 97.7% NPV (Fig. 3A & B); after transplantation AUC = 0.906; 95% CI = 0.874–0.938, optimal cutoff value 184.7 pg/mL, 82% sensitivity, 84% specificity, 84% PPV and 97.7% NPV (Fig. 3A & C).

Finally, the ability of pre-transplantation plasmatic CXCL-10 levels to predict the risk of SCR was evaluated; despite the results showing a tendency for higher levels in SCR patients than in nonrejectors, the ROC curve analysis showed a no ability to discriminate these groups (AUC = 0.693; 95% CI = 0.713–0.939) (Fig. 4A & B). However, additional ROC curve analysis showed that post-transplantation plasmatic CXCL-10 levels had an outstanding capacity to discriminate patients diagnosed with SCR and nonrejectors: AUC = 0.936 (95% CI = 0.893–0.979), and an optimal cutoff value for the prognosis of SCR of 131.0 pg/mL with a 91.7% sensitivity, 82.2% specificity, 88% PPV and 89% NPV (Fig. 4A & C).

We attempted to determine whether active CMV infection is a confounding factor for the clinical utility of plasmatic CXCL-10 concentrations as a prognostic and diagnostic biomarker of TCMR, ABMR and SCR. ROC curve analysis revealed that the plasmatic CXCL-10 level could discriminate patients with active CMV infection (from the 2nd month on, which is when there was active CMV infection) and patients free of both rejection and infection (AUC = 0.882; 95% CI = 0.801–0.961). The optimal cutoff value for predicting the risk of CMV infection based on AUC analysis of the ROC curve for plasmatic CXCL-10 levels was 142.1 pg/mL, with 70% sensitivity, 89% specificity, 78% PPV, and 92% NPV (Fig. 5A & B).

3.5. High urinary CXCL-10 concentration in kidney recipients with TCMR

Patients who experienced a TCMR episode ($n = 14$) had consistently higher urinary CXCL-10 levels than nonrejectors ($n = 42$) throughout the study period (Fig. 6 & Table 2A). Patients with BKV infection ($n = 13$) had significantly higher urinary CXCL-10 concentrations than BKV-free patients during the entire study follow-up period (Fig. 6 & Table 2A). There was no significant difference in the urinary CXCL-10 concentration between patients with TCMR and patients with BKV infection. However, urinary CXCL-10 concentrations were similar in patients with CMV infection ($n = 16$) and in patients free of rejection and CMV infection during the entire follow-up period (Fig. 6 & Table 2A). Details about the time evolution pre- and post-transplantation of urinary CXCL-10 production in each rejector patient are described in **Supplementary Data and Figures.**

3.6. Post-transplantation prognostic values of urinary CXCL-10 concentration for TCMR risk and possible confounding factors

We evaluated the capacity of the urinary CXCL-10 concentration to predict the risk of TCMR. ROC curve analysis showed that the urinary CXCL-10 levels had excellent capacity to discriminate TCMR patients and nonrejectors (AUC = 0.885; 95% CI = 0.825–0.944). The optimal cutoff value for predicting TCMR based on the AUC analysis of the ROC curve for urinary CXCL-10 levels was 87.85 pg/mL, with 87% sensitivity, 85% specificity, 92% PPV, and 86% NPV (Fig. 7A & C).

We sought to determine whether active BKV infection is a confounding factor for the clinical utility of the urinary CXCL-10

Table 2
Urinary and plasmatic CXCL-10 production.

2A	FREE CE (n = 42)	TCMR (n = 14)	BKV (n = 13)	CMV (n = 16)	FREE CE vs TCMR	FREE CE vs BKV	FREE CE vs CMV
	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week
uCXCL-10	37.1 ± 21.5	146.6 ± 48.4	146.5 ± 59.7	41.6 ± 29.9	P = 0.000	*P* = 0.000	*P* = 0.216
	FREE CE	TCMR	BKV	CMV	FREE CE vs TCMR	FREE CE vs BKV	FREE CE vs CMV
	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month
uCXCL-10	22.9 ± 17.7	152.9 ± 34.6	124.8 ± 103.5	32.6 ± 16.2	P = 0.000	*P* = 0.000	*P* = 0.164
	FREE CE	TCMR	BKV	CMV	FREE CE vs TCMR	FREE CE vs BKV	FREE CE vs CMV
	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month
uCXCL-10	23.9 ± 21.9	151.2 ± 32.1	120.4 ± 47.9	37.2 ± 19.8	P = 0.000	P = 0.000	*P* = 0.156
	FREE CE	TCMR	BKV	CMV	FREE CE vs TCMR	FREE CE vs BKV	FREE CE vs CMV
	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month
uCXCL-10	29.5 ± 20.6	178.7 ± 28.8	119.9 ± 54.2	38.3 ± 19.9	P = 0.000	P = 0.000	*P* = 0.405
	FREE CE	TCMR	BKV	CMV	FREE CE vs TCMR	FREE CE vs BKV	FREE CE vs CMV
	6th Month	6th Month	6th Month	6th Month	6th Month	P = 0.000	6th Month
uCXCL-10	24.8 ± 22.4	190.7 ± 31.5	139.2 ± 48.9	56.4 ± 26.3	*P* = 0.049		*P* = 0.069

2B	FREE CE (n = 42)	TCMR (n = 14)	ABMR (n = 14)	SCR (n = 8)	BKV (n = 13)	CMV n = 16)	FREE CE vs TCMR	FREE CE vs ABMR	FREE CE vs SCR	FREE CE vs BKV	FREE CE vs CMV
	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX	PRE-TX
pCXCL-10	97.5 ± 39.8	201.6 ± 78.6	163.6 ± 79.4	127.5 ± 77.7	66.3 ± 35	94.8 ± 30.5	P = 0.000	P = 0.001	P = 0.084	*P* = 0.401	*P* = 0.395
	FREE CE	TCMR	ABMR	SCR	BKV	CMV	FREE CE vs TCMR	FREE CE vs ABMR	FREE CE vs SCR	FREE CE vs BKV	FREE CE vs CMV
	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week	1 st Week
pCXCL-10	93.3 ± 53.3	231.2 ± 90.6	264.8 ± 85.1	192.6 ± 26.1	71.2 ± 20.4	79.7 ± 53.6	P = 0.000	P = 0.000	*P* = 0.044	*P* = 0.203	*P* = 0.564
	FREE CE	TCMR	ABMR	SCR	BKV	CMV	FREE CE vs TCMR	FREE CE vs ABMR	FREE CE vs SCR	FREE CE vs BKV	FREE CE vs CMV
	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month	1 st Month
pCXCL-10	65.6 ± 47.3	254.6 ± 135.8	372.6 ± 169.7	–	51.6 ± 18.4	97.9 ± 62.4	P = 0.000	P = 0.000	P = 0.000	*P* = 0.311	*P* = 0.318
	FREE CE	TCMR	ABMR	SCR	BKV	CMV	FREE CE vs TCMR	FREE CE vs ABMR	FREE CE vs SCR	FREE CE vs BKV	FREE CE vs CMV
	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month	2 nd Month
pCXCL-10	69.1 ± 47.4	182.8 ± 155.4	177.9 ± 50.4	165.4 ± 13.4	78.5 ± 23.6	245.9 ± 151.1	P = 0.001	*P* = 0.032	*P* = 0.041	P = 0,844	P = 0.001
	FREE CE	TCMR	ABMR	SCR	BKV	CMV	FREE CE vs TCMR	FREE CE vs ABMR	FREE CE vs SCR	FREE CE vs BKV	FREE CE vs CMV
	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month	3rd Month
pCXCL-10	95.8 ± 55.6	292.3 ± 187.9	231.2 ± 88.4	217.2 ± 63.6	69.6 ± 32.2	160.7 ± 88.9	P = 0.001	*P* = 0.021	*P* = 0.028	*P* = 0.149	*P* = 0.004
	FREE CE	TCMR	ABMR	SCR	BKV	CMV	FREE CE vs TCMR	FREE CE vs ABMR	FREE CE vs SCR	FREE CE vs BKV	FREE CE vs CMV
	6th Month	6th Month	6th Month	6th Month	6th Month	6th Month	6th Month	6th Month	6th Month	6th Month	6th Month
pCXCL-10	89.1 ± 66.2	329.6 ± 145.1	261.1 ± 179.5	206.8 ± 78.9	69.4 ± 40.8	295.4 ± 162.5	P = 0.000	P = 0.001	*P* = 0.002	*P* = 0.157	P = 0.000

Data are expressed as the median and standard deviation. Mann-Whitney test groups. A value of $P < 0.05$ was considered significant. Free CE: free clinical event; TCMR: T-cell mediated rejection; ABMR: Antibody-mediated rejection; SCR: subclinical rejection; BKV: BK polyoma virus; CMV: Cytomegalovirus.
Data are expressed as the median and standard deviation. Mann-Whitney test groups. A value of $P < 0.05$ was considered significant. Free CE: free clinical event; TCMR: T-cell mediated rejection; ABMR: Antibody-mediated rejection; SCR: subclinical rejection; BKV: BK polyoma virus; CMV: Cytomegalovirus.

concentration as a prognostic and diagnostic biomarker of TCMR. The ROC curve analysis showed that the urinary CXCL-10 level had an excellent capacity to discriminate patients with active BKV infection and patients free of both rejection and infection (AUC = 0.955; 95% CI = 0.922–0.988). The optimal cutoff value for predicting the risk of BKV infection based on AUC analysis of the ROC curve for urinary CXCL-10 levels was 79.7 pg/mL, with 80% sensitivity, 93% specificity, 77% PPV, and 91% NPV (Fig. 7B & D).

4. Discussion

To the best of our knowledge, this European multicenter study is the first to demonstrate that the plasmatic CXCL-10 concentration could be a useful early prognostic and diagnostic biomarker for different types of rejection (TCMR and ABMR) before transplantation and during the immediate post-transplantation period (from the 1st week to the 6th month). Another novel contribution by our study is the finding that plasmatic CXCL-10 is a biomarker that can be sued to identify patients at high risk of SCR, thereby providing physicians with an opportunity for early intervention to modify these risk factors and improve graft and clinical outcomes.

Our results showed that there were significant pre-transplantation differences in the plasmatic CXCL-10 concentrations between non-rejectors and patients who experienced TCMR or ABMR. For both events, the NPV was >95%, and as a consequence, those patients with CXCL-10 levels below the established cut-off could be candidates for moderate ISP therapy, thus minimizing the adverse effects associated with treatment over immunosuppression. If this finding is confirmed in larger populations, it could be very useful since patients would be stratified before transplantation according to the risk of rejection (for both TCMR and ABMR), which could allow for individual tailoring of the immunosuppressive regimen.

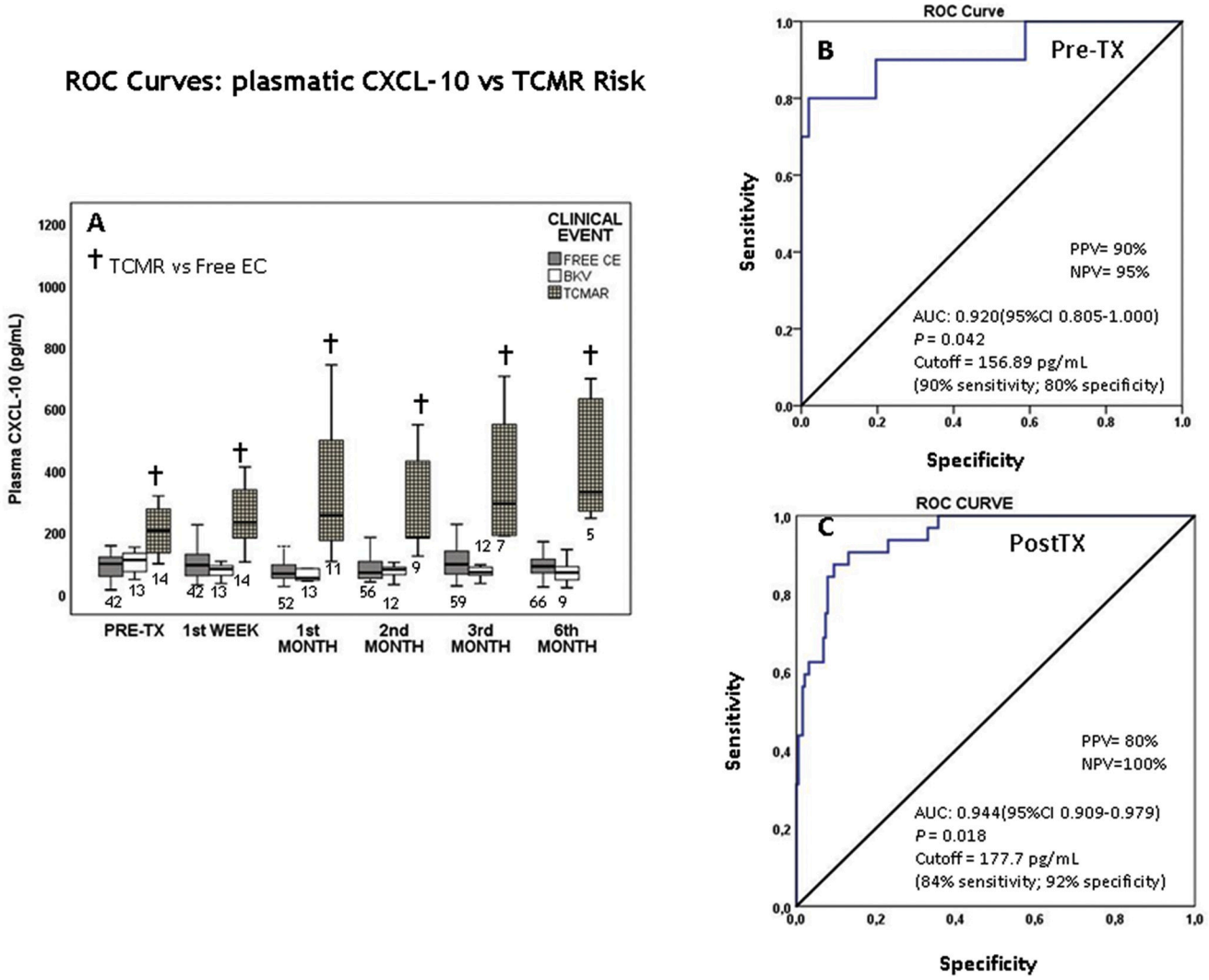

Fig. 2. Pre- and post-transplantation ROC curves: plasmatic CXCL-10 concentration vs. TCMR risk.
(A) Differences in the plasmatic CXCL-10 level between patients free of clinical events (gray boxes), BKV^{+} patients (white boxes) and TCMR patients (hatched boxes) during the first six months post-transplantation. Significant differences between groups were assessed with the Mann-Whitney test. $P < 0.05$ was considered to indicate statistical significance: †patients free of clinical events vs. TCMR patients.
ROC curve analysis for discriminating TCMR rejectors and nonrejectors based on plasmatic CXCL-10 levels before (B) and after transplantation (C). Before transplantation AUC = 0.920, 95% CI 0.805–1.000, cutoff value of 156.89 pg/mL, 90% sensitivity; 80% specificity, PPV = 90% and NPV = 95%; and after transplantation AUC = 0.944, 95% CI 0.909–0.979, cutoff value of 177.70 pg/mL, 84% sensitivity; 92% specificity, PPV = 80% and NPV = 100%.

A previous study showed a significant increase in the urinary CXCL-10 concentration in patients with ABMR [6], but this has not been reported to occur in plasma. A recent publication by Mülbacher et al. [28] reported that the diagnosis of ABMR was associated with significantly higher levels of urinary and serum CXCL-10 in donor-specific antibody-positive stable long-term kidney transplant recipients. Our results showed that the plasmatic CXCL-10 levels also have the capacity to distinguish ABMR patients from nonrejectors or BKV-infected patients, not only in the immediate post-transplantation period but also before transplantation. Similar to the study conducted by Rabant et al. [6], we obtained a high-value NPV (>97%) using plasma as a biological matrix, which would enable the accurate identification of patients with a decreased risk of experiencing ABMR and consequently avoid the need for biopsies in DSA-positive kidney recipients. The availability of non-invasive biomarkers, before transplantation, with predictive capacity for the risk of both TCMR and ABMR will allow both patient stratification according to the risk of rejection (TCMR/ABMR) and improved immunosuppressive treatment selection.

It is important to note that prognostic capacity of urinary CXCL-10 levels for assessing the SCR risk has been previously reported but that only one study evaluated plasmatic CXCL-10 production as a biomarker of subclinical (micro)vascular inflammation and did not find a robust association14. SCR detection is usually conducted via surveillance biopsies, but at present, there are very few centers that carry out protocol biopsies; therefore, having a noninvasive biomarker for SCR prediction would be very useful, taking into account that SCR impacts the long-term allograft outcome because it can lead to chronic tubulointerstitial damage, chronic graft dysfunction, chronic rejection and graft loss. Furthermore, identifying these patients by monitoring plasmatic CXCL-10 would allow them to be excluded from the minimization protocols of immunosuppressive therapy and for the immunosuppression to be adjusted accordingly. Several studies have shown that SCR impacts long-term allograft outcomes because SCR can lead to chronic tubulointerstitial damage, chronic graft dysfunction, chronic rejection and graft loss [29,30]. The prognostic capacity of urinary CXCL-10 levels for assessing the SCR risk has been previously reported [31,32]. Regarding SCR and potential clinical confounding factors, our study shows that early post-transplantation patients with SCR had significantly higher plasmatic

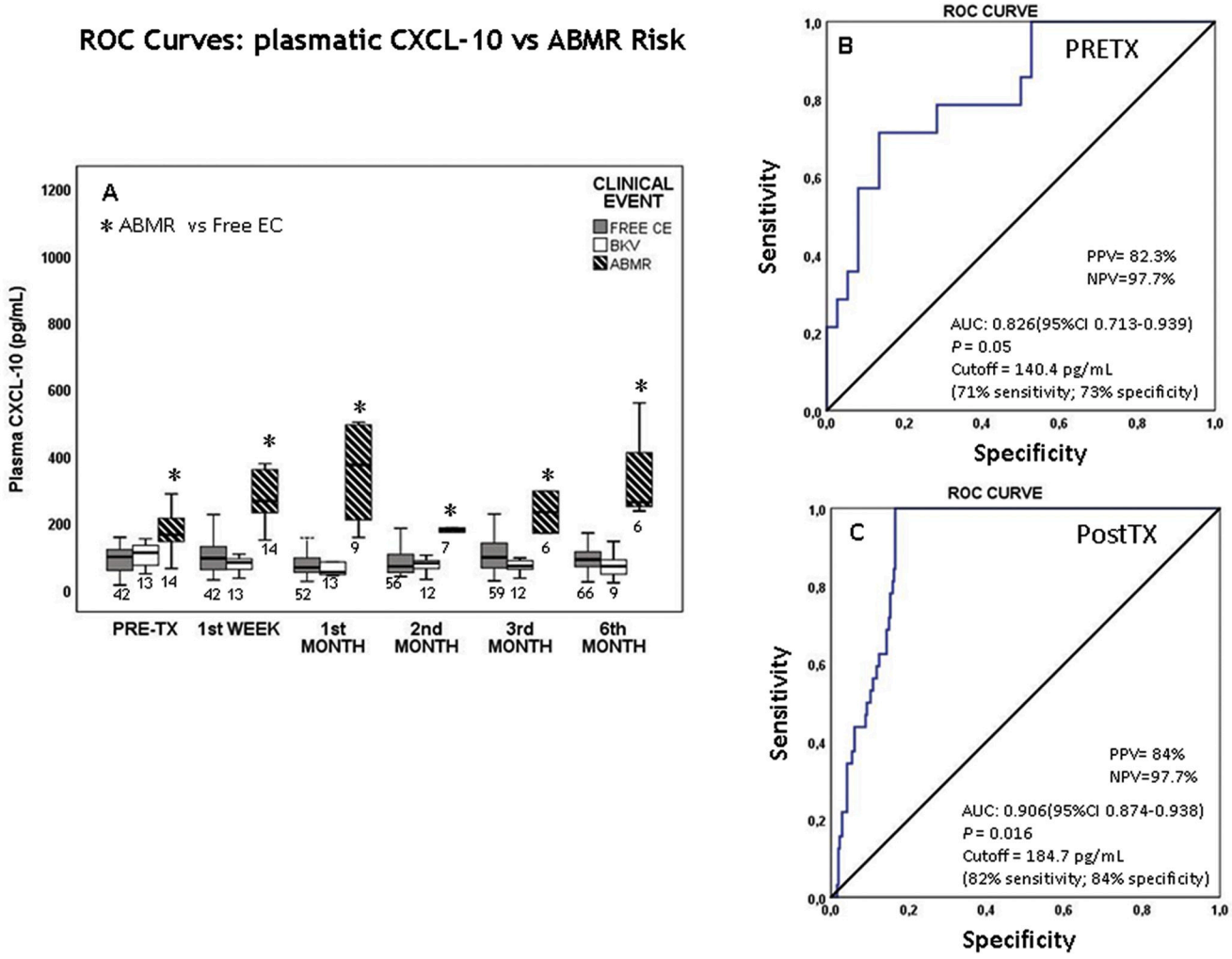

Fig. 3. Pre- and post-transplantation ROC curves: plasmatic CXCL-10 concentration vs. ABMR risk.
(A) Differences in the plasmatic CXCL-10 level between patients free of clinical events (gray boxes), BKV^+ patients (white boxes) and ABMR patients (hatched boxes) during the first six months post-transplantation. Significant differences between groups were assessed with the Mann-Whitney test. A value of $P < 0.05$ was considered to indicate statistical significance: *patients free of clinical events vs. ABMR patients.
ROC curve analysis for discriminating ABMR patients and nonrejectors based on plasmatic CXCL-10 levels before (B) and after transplantation (C). Before transplantation AUC = 0.826, 95% CI 0.713–0.939, cutoff value of 140.40 pg/mL, 71% sensitivity, 73% specificity, PPV = 82.3% and NPV = 97.7%; and after transplantation AUC = 0.906, 95% CI 0.874–0.938, cutoff value of 184.70 pg/mL, 82% sensitivity, 84% specificity, PPV = 84% and NPV = 97.7%.

CXCL-10 concentrations than patients without rejection or with BKV infection but significantly lower levels than patients with TCMR or ABMR. The AUC (=0.936), PPV (=88%) and NPV (=89%) indicated good discriminatory capacity for identifying patients at high risk of developing SCR. SCR detection is usually conducted via surveillance biopsies, but at present, there are very few centers that carry them out; therefore, having a noninvasive biomarker for SCR prediction would be very useful. Identifying these patients would allow early intervention in terms of personal immunosuppressive drug adjustments with the goal of controlling this maintained alloreactivity and preventing graft damage.

Nevertheless, plasmatic CXCL-10 concentrations were increased in patients with CMV infection to levels similar to those observed in rejectors. These results are in agreement with those previously published by Ho et al. [14], who reported that patients with normal histology and concomitant CMV infection had higher serum CXCL-10 levels than patients with normal histology and no infection. Therefore, when plasma is used as a biological matrix, active CMV infection is a potential clinical confounding factor and consequently, CMV viral load must be determined to identify patients with a decreased risk of rejection and those with increased CXCL-10 levels due to CMV infection. Few studies [14,33,34] have evaluated serum CXCL-10 as a biomarker for BKV^+ risk of infection in kidney recipients. All of them showed high inter-patient variability like we did, but despite this fact, in our cohort, plasmatic CXCL-10 concentrations were significantly different in BKV-infected patients compared with patients with TCMR, ABMR or SCR, and in no cases were the two events (rejection and BKV infection) coincident in time. We noted that all of the patients with rejection had plasmatic CXCL-10 values greater than the specific cutoff established for each type of rejection, whereas for BKV infection, 80% of patients had CXCL-10 concentration values below 100 pg/mL before and at the time of the infection. Considering that in all the studies [14,33,34] (including the present study), the number of patients evaluated with BKV infection is low, clearly, all of these findings must be confirmed in multicenter, randomized studies.

Our results confirm previous findings that the urinary CXCL-10 concentration may also be a useful prognostic and diagnostic biomarker of TCMR; however, BKV infection also increases the CXCL-10 concentration [4,35]. For this reason, when urine samples are used to evaluate the risk of TCMR, BKV viral load must be measured to identify patients with a decreased risk of rejection and those with increased

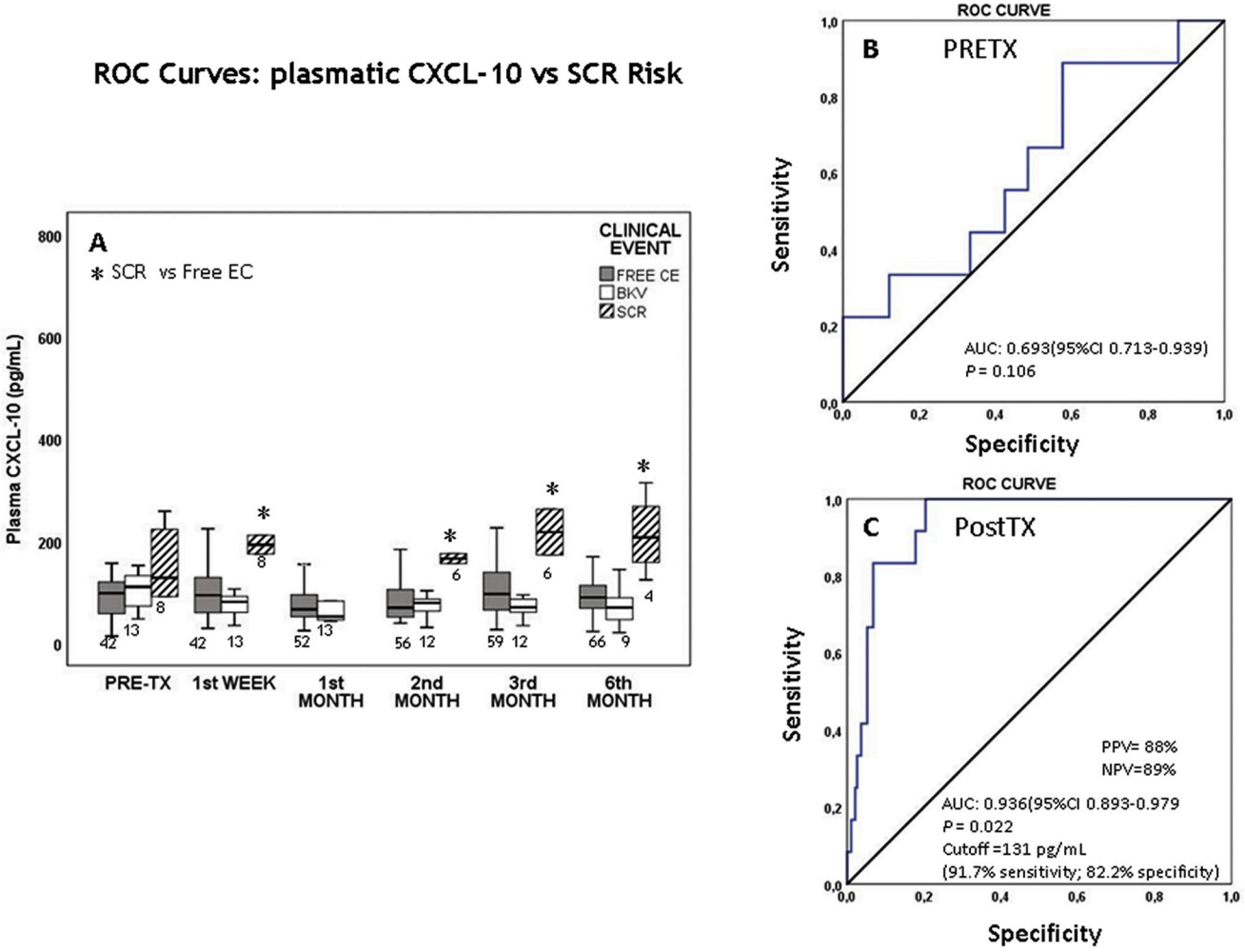

Fig. 4. Pre- and Post-transplantation ROC curves: plasmatic CXCL-10 concentration vs. SCR risk.
(A) Differences in the plasmatic CXCL-10 level between patients free of clinical events (gray boxes), BKV^{+} patients (white boxes) and patients with SCR (hatched boxes) during the first six months post-transplantation. Significant differences between groups were assessed with the Mann-Whitney test. $P < 0.05$ was considered to indicate statistical significance:*patients free of clinical events vs. SCR patients.
ROC curve analysis for discriminating patients with SCR and nonrejectors based on plasmatic CXCL-10 levels was evaluated before (B) and after transplantation (C). Before transplantation, no differences between patients free of clinical events and patients with SCR were observed: AUC = 0.693 (95% CI 0.713–0.939). After transplantation: AUC = 0.936, 95% CI 0.893–0.979, cutoff value of 131.0 pg/mL, 91.7% sensitivity, 82.2% specificity, PPV = 88% and NPV = 89%.

CXCL-10 levels due to BKV infection. However, the presence of CMV infection does not affect urinary CXCL-10 levels, which were similar in infected patients and in patients free of both rejection and infection.

Concerning the relationship between drug exposure and the risks of TCMR, ABMR and SCR, our results showed that although the differences between the evaluated groups were not statistically significant, patients who experienced rejection (TCMR) showed a clear tendency towards lower Tac $AUC_{0\text{-}12h}$ values than nonrejectors. Regarding the influence of drug exposure on CXCL-10 changes, no correlation between plasmatic or urinary CXCL-10 levels and Tac and MPA exposure (C_0) was observed.

High intrapatient variability in Tac exposure was recently shown to be associated with a high risk of dnDSA development [10] and to be a risk factor for late kidney transplant failure [36]. The negative impact of dnDSA on long-term outcome after kidney transplantation has been demonstrated in many studies [37,38]. A possible cause for high intrapatient variability Tac exposure is the nonadherence to ISP treatment, which contributes to ABMR as an independent risk factor for dnDSA development [39]. In previous studies, this intrapatient variability in Tac exposure was usually evaluated from 4 to 12 months after transplantation [12,40]. For the first the time, our study evaluated Tac exposure before and very close to the time of the TCMR or ABMR episode. Unlike the results of some previous studies [13], our results showed no association between within-patient variability in Tac C_0 and the risk of TCMR. This disagreement may be partially explained by the different post-transplantation periods in which this biomarker was evaluated. However, in patients with a %CV > 30%, we observed a higher risk of ABMR, which is not associated with dnDSA development since the 67% of ABMR rejectors did not develop dnDSA. In our cohort, all of the ABMR patients showed plasmatic CXCL-10 concentrations greater than the cutoff established for the risk of this clinical event. Therefore, plasmatic CXCL-10 measurements seem to be more effective in identifying those patients at risk for developing ABMR. Its implementation in clinical routine may provide a means for patient stratification to better prevent ABMR.

In our opinion, the analysis of plasmatic CXCL-10 as a biomarker for identifying kidney transplant patients at high risk of rejection, either TCMR or ABMR, has some advantages over the use of urinary CXCL-10. It is often difficult or impossible to obtain urine samples before transplantation, but collecting a pre-transplantation plasma sample permits both patient stratification according to rejection risk and improved ISP treatment selection. BKV and CMV infections are frequent complications after kidney transplantation, and current guidelines recommend

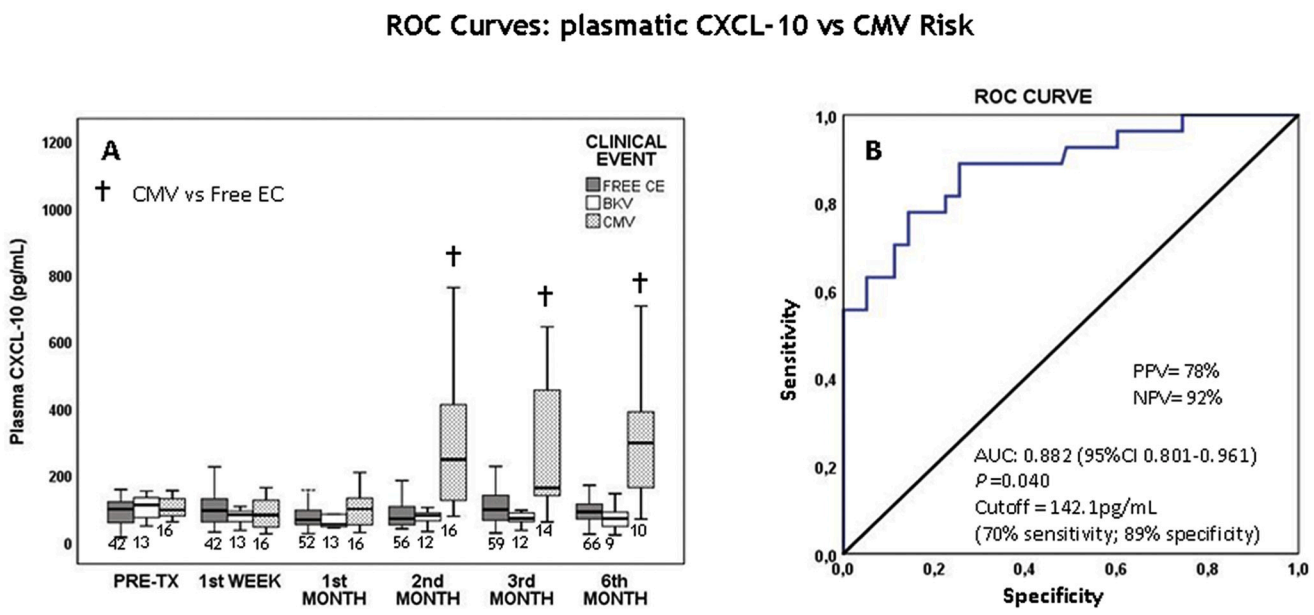

Fig. 5. Post-transplantation ROC curves: plasmatic CXCL-10 concentration vs. CMV risk.
(A) Differences in the plasmatic CXCL-10 concentration between patients free of clinical events (gray boxes), BKV^+ patients (white boxes) and CMV^+ patients (dotted boxes) during the first six months post-transplantation. Significant differences between groups were assessed with the Mann-Whitney test. $P < 0.05$ was considered to indicate statistical significance: †patients free of clinical events vs. CMV^+ patients.
(B) Post-transplantation ROC curve analysis for discriminating CMV^+ patients and nonrejectors based on plasmatic CXCL-10 concentration: AUC = 0.882, 95% CI 0.801–0.961, cutoff value of 142.1 pg/mL, 70% sensitivity, 89% specificity, PPV = 78% and NPV = 92%.

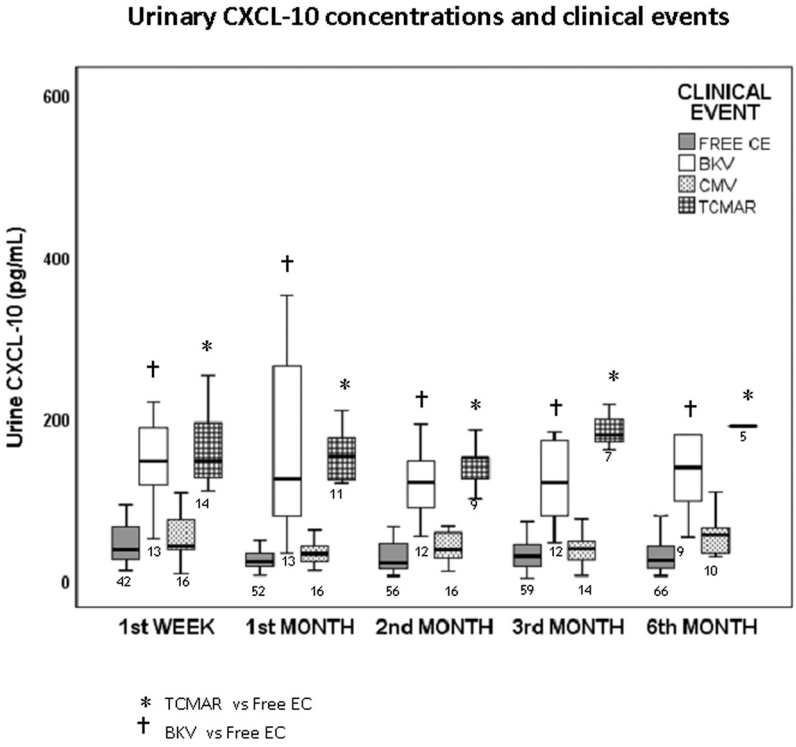

Fig. 6. Urinary CXCL-10 concentration and clinical events.
Differences in the urinary CXCL-10 concentration between patients free of clinical events (gray boxes), BKV^+ patients (white boxes), CMV^+ patients (dotted boxes) and TCMR patients (hatched boxes) during the first six months post-transplantation. Significant differences between groups were assessed with the Mann-Whitney test. $P < 0.05$ was considered to indicate statistical significance: *patients free of clinical events vs. TCMR patients; †patients free of clinical events vs. BKV^+ patients.

systematic quantitative monitoring of BKV and CMV-DNA in plasma. The effects of BKV infection on the graft outcome may be more severe than those of CMV infection. Therefore, it is very important to determine whether the elevated CXCL-10 levels are caused by rejection or reactivation of BKV infection; in contrast to the case with urine, both clinical events seem to be differentiated by the use of plasma, and only a small percentage of patients would present plasmatic CXCL-10 levels above the specific cutoff established for each type of rejection. However, in cases of CMV infection, all patients (100%) had plasmatic CXCL-10 levels above the cutoff values established for rejection; hence, CMV is a much more powerful confounding factor than BKV infection. Finally, the comparison of the performance analysis of plasma and urine in predicting the risk of rejection after transplantation revealed that the plasma matrix seems to be more robust. The 100% NPV indicated that

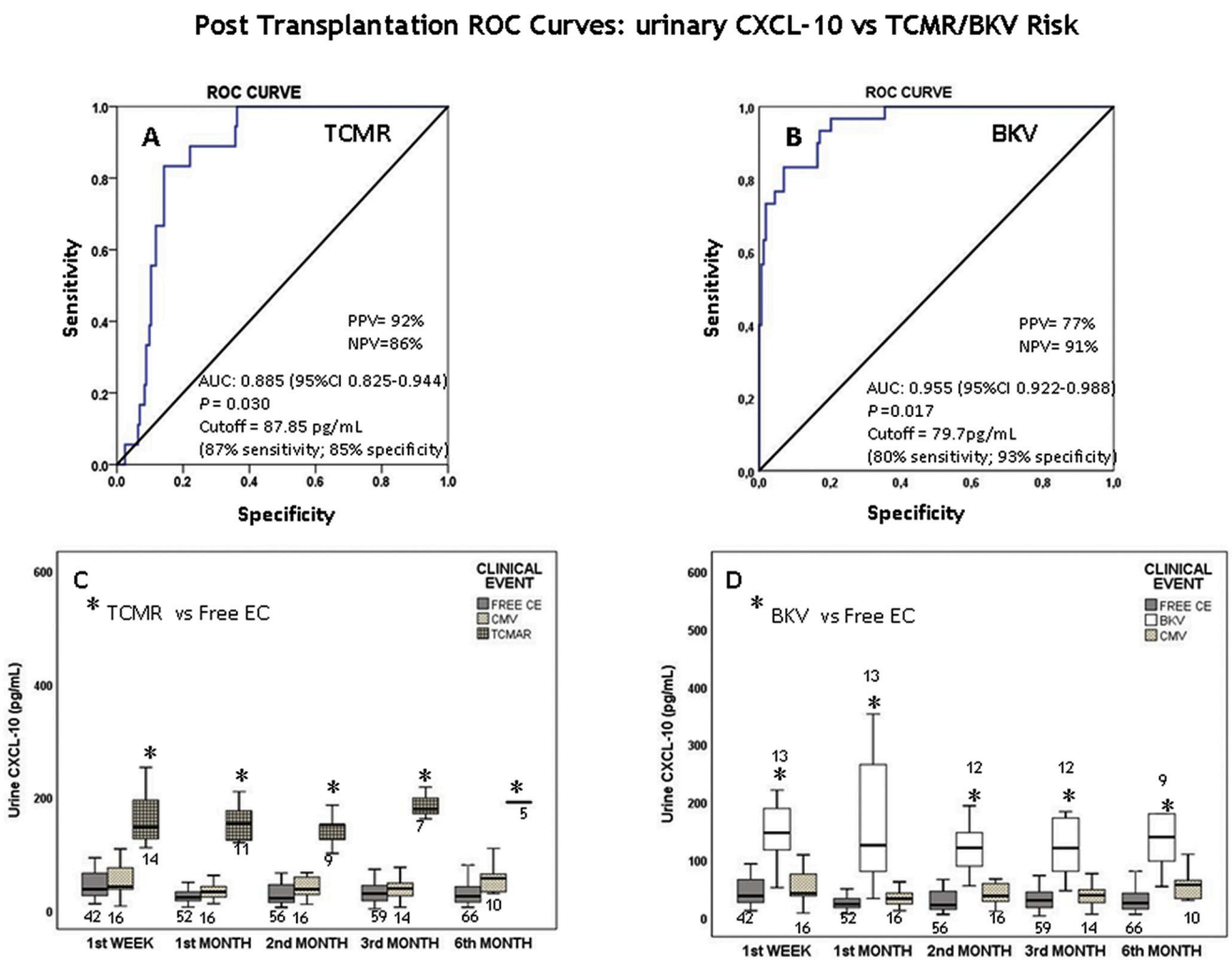

Fig. 7. Post-transplantation ROC curves: urinary CXCL-10 concentration vs. TCMR/BKV risk.
ROC curve analysis for discriminating TCMR patients (A) and BKV+ patients (B) from nonrejectors based on urinary CXCL-10 concentration. The capacity of post-transplantation urinary CXCL-10 levels to discriminate TCMR patients and BKV$^+$ patients from nonrejectors was reported as follows: (A) AUC = 0.885, 95% CI 0.825–0.944, cutoff value 87.85 pg/mL, 87% sensitivity, 85% specificity, PPV = 92% and NPV = 86%; (B) AUC = 0.955, 95% CI 0.922–0.988, cutoff value 79.70 pg/mL, 80% sensitivity; 93% specificity, PPV = 77% and NPV = 91%.
Differences in the urinary CXCL-10 concentration between patients free of clinical events (gray boxes), CMV$^+$ patients (dotted boxes) and TCMR patients (hatched boxes) during the first six months post-transplantation (C). Significant differences between groups were assessed with the Mann-Whitney test. $P < 0.05$ was considered to indicate statistical significance: *patients free of clinical events vs. TCMR patients.
Differences in the urinary CXCL-10 concentration between patients free of clinical events (gray boxes), CMV$^+$ patients (dotted boxes) and BKV$^+$ patients (white boxes) during the first six months post-transplantation (D). Significant differences between groups were assessed with the Mann-Whitney test. $P < 0.05$ was considered to indicate statistical significance: *patients free of clinical events vs. BKV$^+$ patients.

plasma matrix analysis is capable of accurately identifying patients with decreased risk of experiencing TCMR, and as a consequence, patient candidates for more moderate ISP therapy, thus minimizing the adverse effects associated with treatment.

A possible explanation for the discrepancies in CXCL-10 levels in patients with BKV or CMV depending on biological matrix (plasma or urine) is that the specific inflammatory response to these viruses occurs in different compartments. BKV infection targets tubular epithelial cells with subsequent hematogenic spreading, thereby increasing urinary CXCL-10 levels, whereas CMV infection can affect circulating hematopoietic cells and potentially vascular endothelial cells, leading to an increased plasmatic CXCL-10 concentration.

Our study has some limitations, including the relatively low number of events; the evaluation of the prognostic capacity of both biological matrices (plasma and urine) for the risk of only TCMR; and the inclusion of a Caucasian population only; our findings should be validated in other ethnic populations.

In summary, chemokines are normally expressed at low levels and rapidly up-regulated at the onset of the immune response. During the dynamic process of the interaction between the graft and immune system, changes in CXCL-10 concentrations allow the identification of patients at high risk of TCMR, ABMR and SCR and enable early intervention to modify these risks factors and provide personalized immunosuppressive treatment accordingly, thus improving the graft and patient outcomes. It is well known that CXCL-10 modulates chemotaxis during several immune inflammatory processes and influences cytokine secretion, thus regulating the immune response against the implanted graft. An intrinsic limitation of the use of urinary CXCL-10 levels to noninvasively detect rejection is that the level of this chemokine is also increased in other inflammatory conditions. In addition, as the results show that this biomarker seems to have predictive capacity for the assessment of the risk of TCMR and ABMR before transplantation and as it is often difficult or impossible to obtain urine samples pre-transplantation, in this situation, plasma samples have a

clear advantage over urine samples, allowing both patient stratification according to rejection risk and improved immunosuppressive treatment selection. Furthermore, plasmatic CXCL-10 can be used to identify patients at high risk of SCR.

5. Conclusions

The results of this European prospective, observational multicenter study suggest that the plasmatic CXCL-10 concentration could be a potential early, noninvasive prognostic biomarker for rejection (TCMR and ABMR) in kidney transplant recipients. In addition, plasmatic CXCL-10 is a good biomarker for identifying patients at high risk of SCR, providing an opportunity for physicians to initiate early interventions to modify these risk factors and improve the graft and clinical outcomes. As this biomarker can be evaluated before transplantation, its implementation in clinical practice would allow better patient stratification according to rejection risk and improved decision-making regarding ISP treatment. There is a need for prospective data from large, independent cohorts of kidney transplant patients in randomized, multicenter clinical trials to refine the cutoff values identified in the present study and to evaluate the clinical usefulness of this biomarker.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *Clinical Immunology*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2021.108792.

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