

## ORIGINAL ARTICLE

# High pretransplant serum levels of CXCL9 are associated with increased risk of acute rejection and graft failure in kidney graft recipients

Mario Rotondi,<sup>1,2\*</sup> Giuseppe Stefano Netti,<sup>1,3\*</sup> Elena Lazzeri,<sup>1</sup> Giovanni Stallone,<sup>3</sup> Elisabetta Bertoni,<sup>4</sup> Luca Chiovato,<sup>2</sup> Giuseppe Grandaliano,<sup>5</sup> Loreto Gesualdo,<sup>3</sup> Maurizio Salvadori,<sup>4</sup> Francesco Paolo Schena,<sup>5</sup> Paola Romagnani<sup>1</sup> and Mario Serio<sup>1</sup>

1 Excellence Center for Research, Transfer and High Education DENOthe, University of Florence, Florence, Italy

2 Unit of Internal Medicine and Endocrinology, Fondazione Maugeri, University of Pavia, Pavia, Italy

3 Department of Biomedical Science and BioAgroMed, University of Foggia, Foggia, Italy

4 Center for Nephrology, Dialysis and Transplantation, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy

5 Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy

## Keywords

acute rejection, chemokine receptor CXCR3, CXCL9, renal allograft survival, renal graft loss, renal graft outcome.

## Correspondence

Mario Rotondi MD, PhD, Unit of Internal Medicine and Endocrinology, Fondazione Maugeri, University of Pavia, Via S. Maugeri, 10, I-27100, Pavia, Italy. Tel.: 0039 0382 592 750; fax: 0039 0382 592 692; e-mail: mario.rotondi@fsm.it

\*Mario Rotondi and Giuseppe Stefano Netti equally contributed to this work.

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## Summary

Several clinical and experimental models have underlined the role of the CXCR3-binding chemokines in the immune-mediated kidney diseases. This study aimed to investigate the predictive value of measuring pretransplant CXCL9 levels for acute rejection (AR) onset and kidney transplantation outcome. Pretransplantation serum levels of CXCL9 were tested retrospectively in 252 kidney graft recipients, whose stratification in two groups according to CXCL9 levels (<272.1 pg/ml vs. >272.1 pg/ml) showed highly significant differences in 5-year survival rates (97.7% vs. 73.3%;  $P < 0.001$ ). Multivariate analysis demonstrated that among the analysed variables, CXCL9 [relative risk (RR) 11.708] and AR (RR 3.604) had the highest predictive power of graft loss. Accordingly, patients with AR ( $254.4 \pm 22.1$ ;  $P < 0.05$ ) and, even more, those with anti-thymoglobulin (ATG)-treated AR also showed increased pretransplant serum CXCL9 levels ( $319.3 \pm 28.1$ ,  $P < 0.001$ ). Moreover, CXCL9 expression and distribution were investigated in tissue specimens obtained from 10 patients affected by AR, and wide CXCL9 expression was detected not only in infiltrating inflammatory cells but also in vascular and tubular structures. Measurement of pretransplant serum CXCL9 levels might represent the tracking of a clinically useful parameter to identify subjects at high risk of AR and graft failure. These findings might be used for the individualization of immunosuppressive therapies.

## Introduction

Improvements in immunosuppressive drugs, surgical techniques and ancillary care have made transplantation a routine and preferred therapy for treatment of end-stage renal disease (ESRD) [1]. The incidence of ESRD is increasing at a faster rate than the availability of kidney donors [1], and the improvement in short-term graft survival rates has not been followed by substantial amelioration in long-term outcome [2]. Almost half of deceased

donor allografts will still be lost within 10 years after transplantation, chronic allograft nephropathy (CAN) being the most frequent cause of graft failure over a period of time [1–4]. In addition, transplant recipients show a markedly increased risk for cardiovascular disease, opportunistic infections and malignancy, which are the complications of prolonged immunosuppression [5–7]. As a result, 50% of graft loss are caused by death of patients with a functional graft [1,5–7]. Nowadays, the discovery of potential markers of graft function outcome

is a relevant clinical goal, and several clinical studies aimed at evaluating possible predictive markers of graft failure have been performed in order to identify patients at high risk for graft loss [8–12].

Both clinical and experimental evidences indicate that chemokines binding the CXC chemokine receptor 3 (CXCR3), may be particularly important for the immune response to graft. Indeed, CXCR3 ligands playing a pivotal role in the initiation and amplification of host alloresponses and also altering vascular cell functions, promote the development of acute rejection (AR), but also play a role in the pathogenesis of chronic allograft dysfunction, which finally leads to graft loss.

In previous studies, we have demonstrated that the pretransplant serum level of the CXCR3 ligand CXCL10 is a clinically useful parameter for the identification of subjects with a high risk of AR, chronic allograft nephropathy, and graft failure [11,12]. Studies showing the role of other CXCR3 binding chemokine have demonstrated that the measurement of urinary levels of CXCL9 is useful to predict AR onset [13]. Furthermore, a recent study demonstrated that CXCL9 is an even more powerful agent as compared with CXCL10 in promoting CXCR3-dependent immune-mediated kidney disease [14].

The aim of our study was to evaluate the prognostic value of measuring the serum levels of CXCL9 before transplant for predicting the onset of AR episodes and the graft outcome.

## Subjects and methods

### Patients

Among patients with ESRD undergoing kidney transplantation between 1991 and 2002 at the Florence and Bari Transplant Centers, a final number of 252 caucasians subjects (166 male and 86 female patients) entered the study. Inclusion criteria were: (i) the availability of pretransplant frozen serum aliquots, (ii) a complete clinical record for at least 5 years since transplantation, (iii) a maintenance immunosuppression therapy with calcineurin inhibitors. Exclusion criteria were: (i) a Panel Reactive Antibody (PRA) >20%, (ii) a living donor, (iii) an elderly ( $\geq 60$  years) deceased donor (iv) induction therapy with any drug (basiliximab, rabbit anti-human thymocyte globulins or ATG, OKT3, etc).

All patients were on dialysis at the time of transplant and underwent measurements of main hematocemical parameters every 6 months. An aliquot was obtained from the serum specimen, which was used for final cytotoxic cross-match at the time of transplantation (within 6 months before the date of transplantation). Specimens were kept frozen at  $-70$  °C until the time of assay.

End-stage renal disease (ESRD) resulted from glomerulopathies (34.1%), interstitial nephritis (19.7%), autosomal dominant polycystic kidney disease (22.6%), nephroangiosclerosis (12.7%), and others (11.1%). Information on graft function, patient survival, and other clinical data were retrospectively collected for each patient at 1, 3, 6 months and at 1, 2, 3, 4, 5 years. The mean follow-up time after transplant, which included patients who experienced graft failure, was  $101.7 \pm 46.3$  months, while the observed 5-year overall survival rate was 90.5%. The maintenance immunosuppression consisted of a triple therapy including a calcineurin inhibitor (cyclosporine in 93% and tacrolimus in 7%) with either azathioprine or mycophenolate mofetil and steroids (prednisone).

All AR episodes were biopsy-proved, classified according to Banff classification [15] and treated with steroids. AR episodes showing vascular components at biopsy (grade II or III) and failure to reverse after a course of steroids were treated with ATG.

Renal biopsy specimens from a total number of 10 subjects suffering from AR were used throughout the study. As controls, normal kidney tissues were obtained from five patients who underwent nephrectomy for the treatment of primary localized renal tumors.

Gender- and age-matched adult healthy subjects ( $n = 50$ ) recruited from hospital staff and relatives were used as controls. An informed consent, concerning the future use of serum samples and clinical-pathologic data for research purposes, was obtained from all the subjects included in this study. This was in accordance with the Regional Ethics Committee on human experimentation.

### Serum assays

Serum CXCL9 was assayed by a quantitative sandwich immunoassay using a commercially available kit, following manufacturer's instructions (R&D Systems, Minneapolis, MN, USA), with a mean minimum detectable dose of 62.5 pg/ml and a maximum detectable dose of 4000 pg/ml. The intra- and interassay coefficients of variation were 2.1% and 6.3% respectively. Serum CXCL10 was assayed as previously described [10]. All serum samples that appeared above the maximum detectable levels of the assays were diluted and assessed again and only the final value was considered. Samples were assayed in duplicate. Quality control pools at low, normal, and high concentrations for all parameters were present in each assay.

### Immunohistochemistry

Immunohistochemical staining was performed as previously described [16]. For double label immunohistochemistry, the anti-CXCL9 (Peprotech, Rocky Hill, NJ, USA)

pAb was applied first, and aminoethylcarbazole (AEC) was used as peroxidase substrate. Sections were subsequently exposed to anti-CD68 (EBM11; Dako, Glostrup, Denmark) and Vector SG (Vector Laboratories, Burlingame, CA, USA) was used as a chromogen. No counterstain was applied.

### *In situ* hybridization

Cloning and sequencing of the CXCL9 probe and *in situ* hybridization were performed as previously described [16]. For combined *in situ* hybridization and immunohistochemistry, after hybridization with the CXCL9 probe, RNase digestion and appropriate washings, sections were stained with anti-alpha-smooth muscle actin ( $\alpha$ -SMA; 1A4; Sigma, Saint Louis, MO, USA) and then subjected to autoradiography, as reported earlier [16].

### Statistical analysis

Statistical analyses were performed using SPSS software (SPSS, Inc., Evanston, IL, USA). Comparisons of variables among different groups were performed by Student's *t*-test and Mann-Whitney *U*-test, as appropriate. Correlation between two variables was ascertained by Pearson and Spearman's correlation test, as appropriate. Frequencies were compared among groups by chi-squared test. Kaplan-Meier estimates were used to generate overall graft survival curves, censored for death with a functioning graft, and differences among groups were assessed by log-rank test. Graft failure was defined as the need to return to dialysis. To validate the effect of a variable on graft survival, a Receiver operator characteristic (ROC) curves analysis was performed to define an operational cut-off level in order to better differentiate between patient groups with and without graft loss with the highest specificity and sensitivity for the risk of graft loss. To test the independent effects of different variables on kidney allograft survival, Cox regression analysis was used and partial correlation coefficients were computed.

A *P*-value <0.05 was considered statistically significant. Results are expressed in the text as mean  $\pm$  standard error of the mean (SEM) unless otherwise stated.

## Results

### Pretransplant serum levels of CXCL9 in kidney graft recipients and their relation to graft outcome

The mean serum levels of CXCL9 were significantly higher in adult kidney graft recipients ( $n = 252$ ) as compared with healthy subjects ( $n = 50$ ) ( $221.1 \pm 9.4$  vs.  $37.3 \pm 7.9$  pg/ml respectively;  $P < 0.001$ ).

No differences were observed in serum pretransplant CXCL9 levels with regards to age, gender, year of transplantation, original disease, transplant number, type or duration of dialysis, PRA, or occurrence of delayed graft function (data not shown).

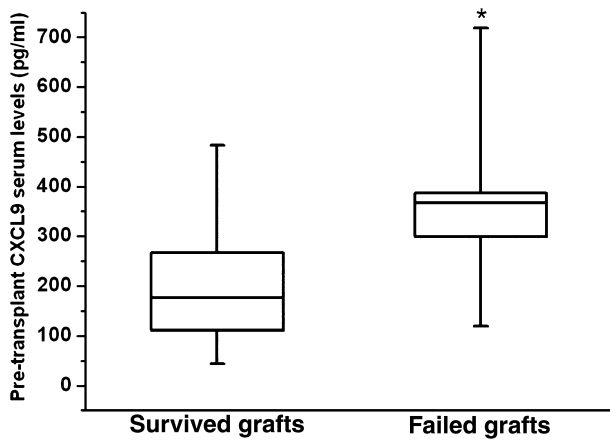
To evaluate the possible relation between the pretransplant variables and the kidney graft outcome, all patients were assigned to two groups in relation to their 5-year graft outcome. The pretransplant clinical characteristics did not differ significantly between the two groups (Table 1).

Patients with normally functioning grafts showed significantly lower pretransplant serum CXCL9 levels than those patients who experienced graft failure throughout a 5-year follow-up ( $205.6 \pm 9.2$  vs.  $367.8 \pm 31.8$  pg/ml,  $P < 0.001$ ) (Fig. 1). This finding was confirmed when the graft outcome was assessed at 12 ( $214.3 \pm 9.4$  vs.  $356.2 \pm 40.8$  pg/ml,  $P < 0.001$ ), 24 ( $212.1 \pm 9.5$  vs.  $353.4 \pm 33.4$  pg/ml,  $P < 0.001$ ), 36 ( $212.2 \pm 9.5$  vs.  $356.0 \pm 32.8$  pg/ml,  $P < 0.001$ ) and 48 months after transplantation ( $210.8 \pm 9.6$  vs.  $333.9 \pm 28.1$  pg/ml,  $P < 0.001$ ).

To further investigate the effect of CXCL9 on graft survival, ROC curve cut-off was constructed in order to define with the highest specificity and sensitivity the level of CXCL9 allowing differentiation between patient with and without graft loss. The cut-off value of pretransplant CXCL9 for increased risk of graft loss was identified at 272.1 pg/ml (specificity 83.3%, sensitivity 75.4%, Area Under the Curve 0.804, as shown in Fig. 2a;  $P < 0.001$ ).

**Table 1.** Clinical characteristics of the kidney transplant recipients and distribution of the analysed variables between patients with survived grafts and patients undergoing graft failure.

	Survived	Failed	<i>P</i> -value
No. patients	228	24	
Recipient gender (M/F)	149/79	17/7	0.590
Age at transplantation (years)	46.2 $\pm$ 0.8	50.0 $\pm$ 2.6	0.106
Time on dialysis (months)	36.8 $\pm$ 2.3	27.2 $\pm$ 3.0	0.411
Type of dialysis (Hemodialysis vs. Peritoneal dialysis)	200/28	18/6	0.083
Total mismatch (HLA-A, -B, -DR)	2.63 $\pm$ 0.06	2.79 $\pm$ 0.22	0.351
Donor gender (M/F)	138/90	12/12	0.318
Donor age (years)	42.2 $\pm$ 1.0	45.0 $\pm$ 2.3	0.557
Cold ischemia time (hours)	16.2 $\pm$ 0.3	17.9 $\pm$ 1.0	0.116
Transplant center (Bari versus Florence)	32/196	3/21	0.836



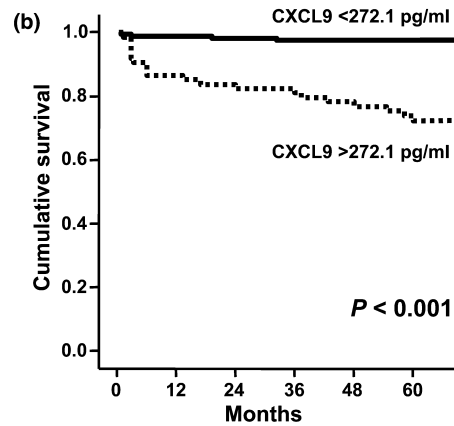
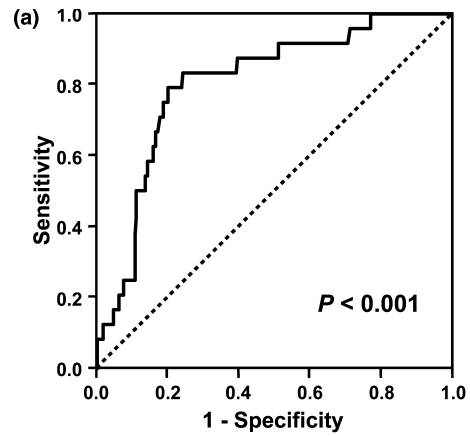
**Figure 1** Higher pretransplant serum CXCL9 levels in kidney recipients are associated with risk of graft failure. Pretransplant CXCL9 serum levels were significantly higher in patients who experienced graft failure ( $n = 24$ ) in comparison with patients with normally functioning grafts ( $n = 228$ ). ( $367.8 \pm 31.8$  vs.  $205.6 \pm 9.2$  pg/ml; Mann–Whitney  $U$ -test for unpaired data;  $*P < 0.001$ ). Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers while they are expressed as mean  $\pm$  standard error of the mean in the text.

Life-time analysis was performed after the assignment of all patients to two groups according to the operational cut-off of CXCL9. Recipients with pretransplant serum CXCL9 levels  $<272.1$  pg/ml ( $n = 173$ ) showed significantly higher 5-year rate of graft survival, as compared with patients with serum CXCL9 levels  $>272.1$  pg/ml ( $n = 79$ ) (97.7% vs. 73.3%;  $P < 0.001$ ) (Fig. 2b). When life-time analysis was performed to compare the rate of survived grafts after 1, 2, 3 and 4 years, differences between the two groups were significant at all time points (*data not shown*).

Specificity of pretransplant CXCL9 for predicting graft failure was further tested by comparing the CXCL9 serum levels between surviving kidney recipients and patients who died with functioning graft, but no influence of pretransplant serum CXCL9 on patient survival could be observed. In fact, the CXCL9 levels between the two groups were similar ( $219.4 \pm 9.8$  pg/ml and  $230.1 \pm 28.2$  for surviving and dead recipients after 5 years respectively;  $P = 0.882$ ).

**Combined measurement of both pretransplant serum CXCL9 and CXCL10 levels in kidney graft recipients and their relation to graft outcome**

We have measured the pretransplant levels of CXCL10 in our cohort of patients and we have related them to transplant outcome. To this aim, we have assessed a CXCL10 cut-off value (133.2 pg/ml) to better differentiate between



Patients at risk (n)						
CXCL9 <272.1	177	175	174	173	173	173
CXCL9 >272.1	75	65	62	61	58	55

**Figure 2** Association of pretransplant serum CXCL9 levels with renal allograft failure. (a) Prognostic value (ROC curve) of pretransplant CXCL9 serum levels  $>272.1$  pg/ml and the risk of allograft failure [specificity 83.3%; sensitivity 75.4%; positive predictive values (PPV) 25.6%; negative predictive values (NPV) 97.7%; Area Under the Curve (AUC) 0.804, Upper Limit 0.718, Lower Limit 0.890 for 95% CI;  $P < 0.001$ ] in a censored for death analysis of 252 patients. (b) Recipients with serum CXCL9 levels below 272.1 pg/ml ( $n = 173$ , black line) showed significantly higher 5 year-rate of graft survival in comparison with patients with serum CXCL9 levels above this cut-off ( $n = 79$ , dotted line) (97.7% vs. 73.3%;  $P < 0.001$ ; Kaplan–Meier life-time analysis and log-rank test). The outcome was ESRD requiring RRT. Patients at risk were the number of cases in observation at each time. The 5 years overall survival was 90.5%. In this analysis, death with a functioning graft was not counted as graft failure.

patient groups with and without graft loss with the highest specificity and sensitivity for the risk of graft failure (ROC AUC 0.753; Sensitivity 62.5%; Specificity 75.9%;  $P < 0.001$ ) and we have assigned the entire study cohort to two groups according to their pretransplant serum CXCL10 status: patients showing CXCL10  $< 133.2$  ( $n = 182$ ) and patients showing CXCL10  $> 133.2$

( $n = 70$ ). Life-time analysis has revealed 5-year graft survival rates of 95.0% in the first group and of 78.6% in the second group (Kaplan–Meier and log-rank test;  $P < 0.001$ ). No significant influence of pretransplant serum CXCL10 on patients' life survival was observed.

Moreover, in order to evaluate a possible combined role of both CXCL9 and CXCL10 chemokines onto graft survival, patients have been assigned to four groups according to their pretransplant serum chemokines status: Group I ( $n = 148$ ) patients showing both CXCL9 and CXCL10 levels  $<$ ROC cut-off (272.1 and 133.2 pg/ml respectively); Group II ( $n = 34$ ) patients showing only CXCL9 level  $>$ ROC cut-off; Group III ( $n = 29$ ) showing only CXCL10 level  $>$ ROC cut-off; Group IV ( $n = 41$ ) patients showing both CXCL9 and CXCL10 levels  $>$ ROC cut-off. Life-time analysis revealed 5-year survival rates of 99.3% in Group I, 76.5% in Group II, 89.7% in Group III and 70.7% in Group IV (Kaplan–Meier and log-rank test;  $P < 0.001$ ). In detail, recipients with both serum CXCL9 and CXCL10 levels  $<$ ROC cut-off showed significantly higher 5-year rate of graft survival in comparison to patients with only CXCL9 (99.3% vs. 76.5%;  $P < 0.001$ ), or with only CXCL10 (99.3% vs. 89.7%;  $P = 0.001$ ), or with both the two chemokines  $>$ ROC cut-off value (99.3% vs. 70.7%;  $P < 0.001$ ). Noteworthy, although no statistically significant differences have been observed between Group II and Group III (76.5% vs. 89.7%;  $P = 0.21$ ), between Group II and Group IV (76.5% vs. 70.7%;  $P = 0.54$ ), and between Group III and Group IV (89.7% vs. 70.7%;  $P = 0.07$ ), the percentages of graft loss were consistently increased in recipients with both serum CXCL9 and CXCL10 levels  $>$ ROC cut-off

value in comparison to patients with only CXCL9 or with only CXCL10  $>$  ROC cut-off value.

#### Predictive value of pretransplant serum CXCL9 levels and other factors for graft failure

To estimate the relative risk for graft failure in patients showing increased serum levels of CXCL9, Cox regression analysis was performed using graft failure as dependent variable, and pretransplant serum CXCL9, AR, recipient age, total number of HLA mismatches, primary disease, type of immunosuppression, cold ischemia time, donor age, delayed graft function, type and time of dialysis, and transplant center as covariates (Table 2). The only covariates significantly affecting graft survival were the pretransplant serum levels of CXCL9 and the occurrence of AR (RR 11.708, CI 3.905–35.104,  $P < 0.001$  and RR 3.604, CI 1.441–9.016,  $P < 0.01$  respectively).

#### CXCL9 pretransplant serum levels predict recipient's risk of severe acute rejection

Acute rejection episodes occurred within the first year in 63 out of 252 recipients (25%), while from the second to the fifth year the incidence of AR episodes increased by 5.2%, with a 5-year cumulative AR rate of 30.2% ( $n = 76$ ). Pretransplant serum levels of CXCL9 were compared between patients who developed AR and patients who did not, within the first year after transplantation. As shown in Fig. 3a, rejectors showed significantly higher median serum levels of pretransplant CXCL9 as compared with non rejector recipients ( $268.8 \pm 18.5$  vs.  $205.1 \pm 10.6$ ;  $P < 0.05$ ). When the patients were assigned

**Table 2.** Cox regression analysis showing the relative risk for allograft failure according to the selected risk factors.

	P-value	R.R.	95.0% CI	
			Lower	Upper
Pretransplant serum CXCL9 levels	<b>&lt;0.001</b>	<b>11.708</b>	<b>3.905</b>	<b>35.104</b>
Acute rejection	<b>0.006</b>	<b>3.604</b>	<b>1.441</b>	<b>9.016</b>
Type of immunosuppression*	0.640	1.361	0.374	4.945
Recipient age at transplantation	0.098	1.042	0.993	1.094
Time on dialysis	0.274	0.988	0.968	1.009
Donor age	0.628	0.991	0.957	1.027
Cold ischemia time	0.111	1.074	0.984	1.172
Total mismatch (HLA-A -B -DR)	0.747	0.920	0.553	1.530
Original disease	0.238	1.214	0.880	1.674
Delayed graft function	0.697	1.223	0.444	3.365
Type of dialysis†	0.227	1.874	0.677	5.182
Transplant center‡	0.213	0.363	0.074	1.786

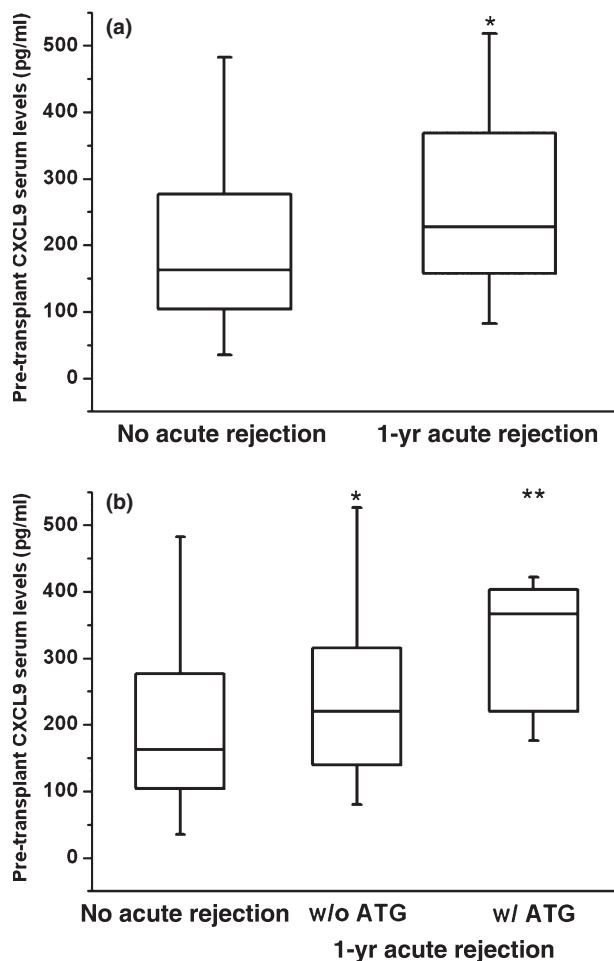
\*Cyclosporine-based versus Tacrolimus-based maintenance immunosuppression.

†Hemodialysis versus Peritoneal Dialysis.

‡Bari versus Florence Transplant Center.

The parameters showing significant influences on graft survival are shown in bold characters.





**Figure 3** Association of pretransplant serum CXCL9 levels with acute rejection (AR). (a) Pretransplant serum mean levels of CXCL9 were significantly higher in transplant recipients who experienced AR within the first year after transplantation ( $n = 63$ ) in comparison with patients with normally functioning grafts that did not experience AR episodes ( $n = 189$ ) ( $268.8 \pm 18.5$  vs.  $205.1 \pm 10.6$  pg/ml; Mann-Whitney  $U$ -test for unpaired data;  $*P < 0.001$ ). (b) Among graft recipients, patients with normally functioning grafts that did not experience AR episodes ( $n = 189$ ) exhibited lower pretransplant serum levels of CXCL9 in comparison with both patients with early AR ( $n = 49$ ) and patients who experienced severe (antithymocyte globulin-treated) AR episodes ( $n = 14$ ) ( $205.1 \pm 10.6$  vs.  $254.4 \pm 22.1$  vs.  $319.3 \pm 28.1$  pg/ml, Kruskal-Wallis  $H$ -test for unpaired data:  $P < 0.001$ ; Mann-Whitney  $U$ -test for unpaired data:  $*P < 0.05$ ;  $**P < 0.001$ ; \* vs.  $**P < 0.05$ ). Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers while they are expressed as mean  $\pm$  standard error of the mean in the text.

to two groups according to their pretransplant CXCL9 serum level, graft recipients with CXCL9  $> 272.1$  pg/ml showed a significantly higher incidence of first-year AR episodes than those with CXCL9  $< 272.1$  pg/ml (33.3% vs. 21.5%,  $P < 0.05$ ).

The same analysis was performed periodically during the entire period of the study, which showed a 5-year AR rate of 30.2% ( $n = 76$ ), while the serum levels of pretransplant CXCL9 between rejectors and not rejectors were also significantly different ( $259.9 \pm 18.7$  vs.  $204.3 \pm 10.5$ ;  $P < 0.05$ ).

Moreover, during the first year follow-up, the 14 patients who required anti-thymoglobulin (ATG) treatment because of severe rejection, showing vascular components at biopsy (grade II or III) and failure to reverse after a course of steroids pulse, were characterized by even higher pretransplant mean serum CXCL9 levels in comparison with both patients who experienced AR episodes only treated with steroids ( $n = 49$ ) and patient with normally functioning grafts that did not experience AR episodes ( $n = 189$ ) during the 1-year follow-up ( $319.3 \pm 28.1$  vs.  $254.4 \pm 22.1$  vs.  $205.1 \pm 10.6$  pg/ml,  $P < 0.001$ ) (Fig. 3b).

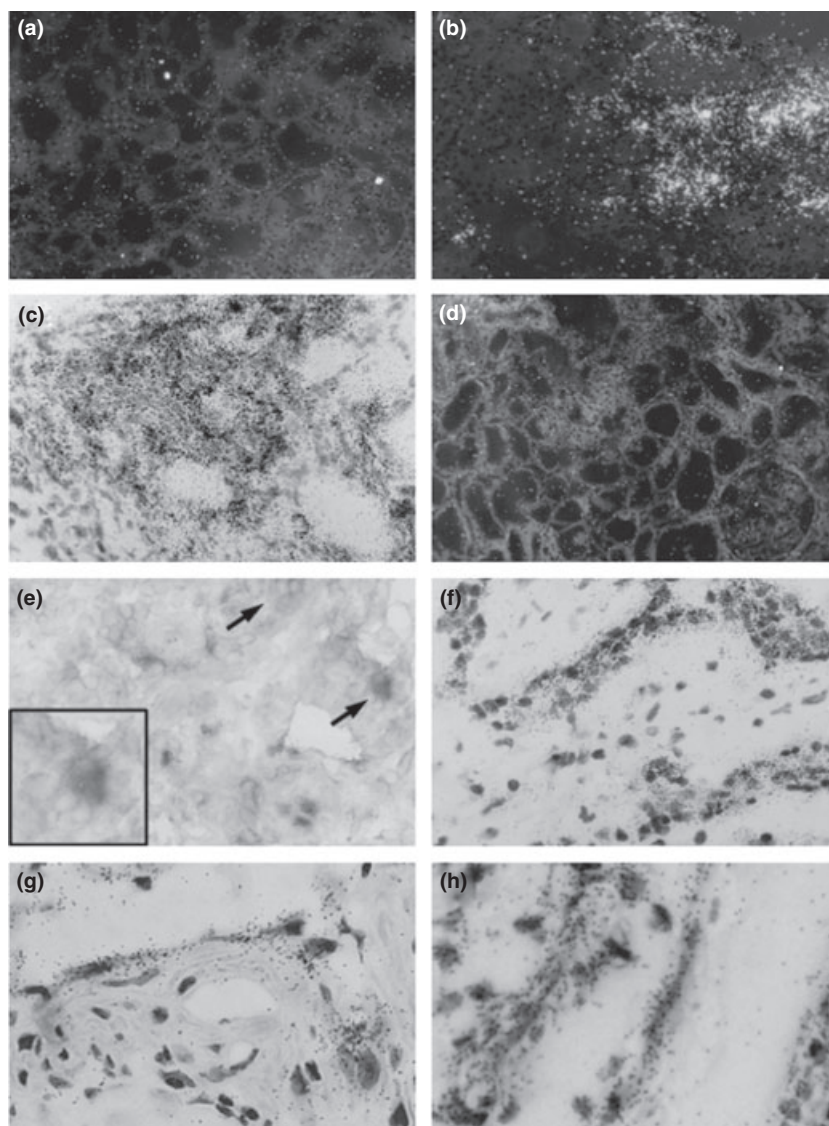
#### CXCL9 is highly expressed in kidney biopsies of subjects with AR

To further investigate the role of CXCL9 in the onset of AR, CXCL9 levels and distribution were assessed by *in situ* hybridization and immunohistochemistry on kidney tissue specimens from 10 subjects affected by acute allograft rejection.

CXC chemokine ligand 9 (CXCL9) mRNA was absent in normal kidney tissues (Fig. 4a), while it appeared to be highly expressed in biopsy specimens from kidneys of patients affected by AR (Fig. 4b and c), as assessed by *in situ* hybridization. Sections hybridized with a sense CXCL9 RNA probe showed virtually no signal (Fig. 4d). Double immunostaining for CXCL9 and CD68 confirmed that in AR, monocytes/macrophages were the main source of this chemokine (Fig. 4e). Moreover, in kidneys from patients with AR, positive cells were identifiable in vascular and tubular structures (Fig. 4f and g). Combined *in situ* hybridization and immunohistochemistry for CXCL9 and  $\alpha$ -SMA provided direct evidence that vascular smooth muscle cells (SMC) (Fig. 4h), in addition to other resident and infiltrating cells, were sources of CXCL9.

#### Discussion

Given the rapidly increasing number of patients with ESRD, and the well-known problem of organ shortage, successful renal transplantation with a high long-term allograft survival rate has become essential. Despite the continuous improvements in the immunosuppressive regimes, the allograft AR remains one of the most relevant clinical event in the post-transplant period. AR mainly occurs in the early post-transplant period and, despite the fact that



**Figure 4** CXCL9 expression and distribution in kidney biopsy specimens from patients affected by acute rejection (AR). (a) Absence of CXCL9 mRNA expression in a section of normal human kidney (x100). (b–c) CXCL9 mRNA expression (white grains in A, black grain in B at the inflammatory infiltrating cells level of a kidney specimen from a subject affected by acute allograft rejection (dark field in A, bright field in B, x100). (d) Absence of signal in a section hybridized with a sense mRNA probe (x100). (e) High CXCL9 protein expression at level of infiltrating monocytes/macrophages, as demonstrated by double-label immunohistochemistry for CXCL9 (red color) and CD68 (blue color) (arrows, x400; selected area x800). (f) High CXCL9 mRNA expression (black grains) on tubular structures (x250). (g) High CXCL9 mRNA expression (black grains) lining the endothelial cells of a vessel (x400). (h) Combined *in situ* hybridization for CXCL9 mRNA (black grains) and immunohistochemistry for  $\alpha$ -smooth muscle actin (red color), demonstrating CXCL9 expression not only by vascular smooth muscle cells (red cells), but also by other resident and infiltrating cells (x1000).

nowadays it causes graft loss only in a minority of cases, it remains an important cause of comorbidity and hospitalization in kidney graft recipient population leading to the development of chronic allograft dysfunction, which in turn strongly affects the long-term outcome [17].

It has been a primary goal of transplant immunologists to develop tests for assessing the pretransplant risk of patients for immunologic rejection and graft failure. Various risk factors, such as recipient age, number of HLA-A, -B, and -DR mismatches, original disease, type of immunosuppression, donor age and delayed graft function, have all been proposed as means for differentiating between patients with a good chance of long-term survival of a renal allograft and those with a poor chance.

In previous studies, we have evaluated the association between elevated pretransplant serum levels of CXCL10

and the onset of AR, CAN and graft failure [11,12]. However, based on the recent finding demonstrating the leading role of CXCL9, another CXCR3 ligand, as mediator of immune-mediated renal diseases [14], we aimed to investigate the pretransplant serum content in CXCL9 in a cohort of kidney graft recipients. In this study, pretransplant levels of CXCL9 were measured in 252 patients affected by ESRD who underwent kidney transplantation. The predictive role of CXCL9 in relation to AR episodes onset throughout a 1-year follow-up and kidney graft failure throughout a 5-year follow-up was evaluated. Patients who experienced graft failure showed higher pretransplant CXCL9 levels than patients with functioning graft. Moreover, in a multiple regression model including a number of variables previously proposed as possible predictors of transplant failure, only a pretransplant level of circulating

CXCL9 greater than 272.1 pg/ml and the occurrence of AR had a significant predictive power for allograft loss. Accordingly, the pretransplant serum levels of CXCL9 were also able to predict the recipient's risk for severe AR, underlining the close correlation between the pretransplant immunologic status and the onset of posttransplant immune-mediated events.

Furthermore, CXCL9 was found to be highly expressed in kidney biopsy specimens obtained from patients affected by acute allograft rejection, such expressions related to infiltrating inflammatory cells, particularly macrophages, the main sources of CXCL9.

More importantly, during AR, CXCL9 is expressed not only by infiltrating mononuclear cells but also by vascular and tubular structures. Indeed, the presence of CXCL9 mRNA on vascular smooth muscle cell, as demonstrated by colocalization with  $\alpha$ -SMA, suggest a possible link between AR and development of chronic vascular damage, leading to renal fibrosis and graft loss. This would be in line with the notion that  $\alpha$ -SMA is a specific marker of activated fibroblast also known as myofibroblast [18], which play a role in the pathogenesis of renal fibrosis regardless of the initial cause [19,20]. Taken together, these findings are suggestive of a role for CXCL9 not only in pathogenesis of AR but also in the onset of chronic allograft dysfunction and, finally, graft failure.

The chemokines CXCL10 and CXCL9 are members of the C-X-C subfamily. They induce chemotaxis of stimulated human T lymphocytes and are believed to be more important in the Th1-type immune responses [21,22]. CXCL9 and CXCL10 are able to mediate pleiotropic biologic functions [23–25], and are involved in transplant tolerance through CXCR3 receptor interaction [26–28]. These chemokines are potent chemoattractants for activated lymphocytes and NK cells [29], mediate vascular pericyte proliferation [25,26,30,31], act as powerful angiostatic agents [24–27] and, therefore, they are critically involved in AR and CAN. Indeed, several studies demonstrated increased expression of these chemokines in biopsies from subjects affected by AR or CAN [11,32,33]. Furthermore, neutralization of CXCL10 or CXCL9 prolongs the allograft survival [34–37].

However, targeting of a single chemokine is only partially effective in promoting graft survival for long periods of time [34–37], while treatment directed to block the effects of all CXCR3-binding chemokines leads to permanent engraftment in experimental animal models [26–28,38].

Consistent with these studies, we have recently demonstrated that measurement of pretransplant serum CXCL10 levels, but not of other unrelated chemokines, might represent the tracking of a clinically useful parameter to identify subjects who are at higher risk for early, severe,

AR and subsequent chronic allograft nephropathy, finally resulting in renal allograft failure [11,12]. These results were confirmed by other studies [13,39], which suggested that the measurement of the urinary levels of CXCL9, is also useful to predict AR [13]. In this study we demonstrate for the first time that higher pretransplant CXCL9 levels are associated with AR onset and severity, and of bad graft outcome.

Recent reports have further investigated the role played by CXCR3 and its ligand in organ transplant. In detail, several experimental models of HLA-mismatched mouse cardiac transplantation have shown a relatively little prolongation of survival of full MHC-mismatched allografts in CXCR3<sup>-/-</sup> recipients and no effects on cardiac vasculopathy [40,41]. Moreover, the disruption or blockade of recipient CXCR3 had relatively little effects on rejection of MHC-mismatched mouse cardiac allografts [42,43]. These data leave many questions unanswered about the role of CXCR3 and its ligands in allograft rejection, and also the question as to why CXCR3 ligands CXCL9, CXCL10 and CXCL11 are so abundant. In this regard, it is to be noted that CXCR3 ligands are not only CXCR3 agonists but also are antagonists for other chemokine receptors [44]; moreover, CXCR3 may act as a decoy receptor [45]. Undoubtedly we need to develop a critical view of the roles of ligand-receptor systems operating in the inflammatory allograft, and our study attempts to examine these processes during renal acute allograft rejection, even though in a small cohort of patients with different HLA-mismatch (mean HLA-mismatch = 2.6).

In this study, we have also confirmed the predictive role of CXCL10 in relation to graft outcome and have assessed whether combined measurement of serum CXCL9 and CXCL10 allows pretransplant estimation of the risk for graft failure. Despite our results suggesting that the measurement of both CXCL9 and CXCL10 might indeed represent a more robust predictor of graft outcome (Kaplan–Meier's survival analysis and log-rank test  $P < 0.001$ ), no statistically significant differences have been observed among the four different groups of patients according to their pretransplant serum chemokines status. This finding is probably resulting from the too small number of patient analysed in our study and this does not allow drawing firm conclusions. Thus, the predictive role of the combined measurement of CXCL9 and CXCL10 in predicting graft outcome, as well as the possible combined role of different targets (CXCL16, CXCR6, FT3) in the perspective of a multi-parametric evaluation of risk factor for graft loss, should be evaluated in a larger number of patients.

In our cohort of patients, we observed a high incidence of AR episodes, but this data may be explained by the close selection criteria; in fact none of the patients that



were included in this study received any induction therapy. The main reason is that all the selected patients were not hyper-immune time of transplantation (PRA < 20%). Nevertheless few graft recipients, who had received an induction therapy with basiliximab as participants of Clinical Trials between 1991 and 2002, were excluded in order to evaluate a homogeneous population with regard to immunosuppressive therapy. In fact it is well known that basiliximab reduces AR incidence in renal transplant recipients when combined with the standard dual- or triple immunotherapy [46]. This choice, even if questionable, has allowed us to rule out a confounding factor in order to establish a possible correlation between high pre-transplant levels of serum CXCL9 and onset of early AR episodes.

Moreover, in this study, we have underlined the early onset of AR because it is reasonable to infer that a pre-transplant factor may affect the immune system during an early period post-transplant, while after the first year, the role of the post-transplant immunosuppressive regimen becomes prevalent.

The results of the kidney biopsy obtained from patients affected by acute allograft rejection show high expression of CXCL9 but do not permit drawing conclusion with regards to the pathophysiologic mechanisms sustaining the high levels of expression of the CXCR3-binding chemokines during rejections. However, the elevated expression of the CXCR3-binding chemokines in clinical acute rejection samples and the results of studies in murine models of transplantation in which this chemokine axis was genetically manipulated, would suggest that CXCR3 and its ligands are involved in recruiting effector T cells to the graft parenchyma [28,37,47–49]. Furthermore, a recent study demonstrated that CXCL9 from both donor and recipient sources would act in synergy during rejection of class II major histocompatibility complex disparate skin allografts [50].

In conclusion, we show that the measurement of CXCL9 serum levels before transplantation allows the identification of highly reactive recipients with higher risk of graft failure. Moreover, consistent with the well-established involvement of CXCL9 in the immune response towards the transplant, a higher pretransplant serum level of CXCL9 characterized those patients experiencing AR in the first post-transplant year.

A cut-off level (272.1 pg/ml) for CXCL9 was established, which may be useful in clinical setting to identify candidates for kidney transplantation with higher immunologic risk. Although our findings should be confirmed by larger randomized prospective studies, it seems reasonable to propose that measuring the pretransplant serum levels of CXCL9 in patients undergoing renal transplantation may be useful to estimate the acute allograft rejection risk, tailor

the peritransplant immunosuppressive regimens, with the final aim of prolonging the long-term graft outcome.

### Authorship

MR, GSN and EL: designed and performed the study. GS, EB and MS: collected data. LC and GG: analysed data. LG, FPS, PR and MS: wrote the paper.

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