ORIGINAL ARTICLE

IL-10-specific autoantibodies predict major adverse cardiovascular events in kidney transplanted patients - a retrospective cohort study

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ABSTRACT

End-stage renal failure is associated with persistent systemic inflammation. The aim of this study was to investigate if systemic inflammation at the time of kidney transplantation is linked to poor graft survival, major adverse cardiovascular events (MACE), and increased mortality, and if these processes are modulated by naturally occurring cytokine-specific autoantibodies (c-aAbs), which have been shown to regulate cytokine activity *in vitro*. Serum levels of cytokines, high-sensitivity C-reactive protein (hsCRP) and c-aAbs specific for interleukin (IL)-1α, tumor necrosis factor (TNF)-α, IL-6, and IL-10 were measured at the time of transplantation in a retrospective cohort study of 619 kidney transplanted patients with a median follow-up of 4.9 years (range 1.2–8.2 years). Systemic inflammation was associated with all-cause mortality in simple and multiple Cox regression analyses. IL-10-specific c-aAbs were associated with MACE after transplantation, suggesting that IL-10 may be a protective factor. Similarly, patients with a history of MACE before transplantation had lower levels of TNF-α-specific c-aAbs, hence we hypothesized that TNF may be a risk factor of MACE. These findings support that pro-inflammatory activity before transplantation is a pathological driver of MACE and all-cause mortality after transplantation. This information adds to pretransplantation risk estimation in renal transplant candidates.

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Key words
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Introduction

Cardiovascular disease (CVD) is the most frequent cause of morbidity and mortality in end-stage renal failure (ESRD) patients [1]. Renal transplantation reduces the risk of CVD although not to the level in the background population [1].

Systemic inflammation predisposes individuals to insulin resistance, dyslipidemia, endothelial dysfunction, and accelerated atherosclerosis [2]. Persistent systemic inflammation is a predictor of mortality and major adverse cardiovascular events (MACE) in the background population [3,4]. The inflammatory hypothesis of atherothrombosis suggests that reducing vascular inflammation in the absence of concomitant lipid-lowering medication reduces the rate of cardiovascular events [5]. Moreover, comorbidities are likely to contribute to a positive feedback loop that further

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exacerbates systemic inflammation [2]. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β initiate inflammatory processes and constitute upstream triggers of IL-6 production, which stimulates the liver production of acute phase reactants, such as C-reactive protein (CRP), and initiates a regulatory anti-inflammatory response that inhibits the production or signaling of pro-inflammatory cytokines. Thus, IL-10 inhibits TNF-α synthesis, and IL-1 receptor antagonist (RA) inhibits signaling through the IL-1 receptor [6]. In patients with inflammatory rheumatic diseases, persistent systemic inflammation is considered to contribute to an increased risk of CVD, whereas anti-TNF-α therapy modulates vascular risk factors in a beneficial direction [7]. Additionally, the recently published CANTOS study has shown that specific anti-IL-1β therapy leads to a lower rate of recurrent cardiovascular events in patients experiencing MACE and reduces the risk and mortality from lung cancer in patients with atherosclerosis [8,9]. This new paradigm may also be applicable to ESRD and be operational in kidney transplantation. Thus, ESRD is strongly associated with persistent systemic inflammation [10], which is associated with increased CVD-related mortality [4]. Furthermore, systemic inflammation after kidney transplantation is associated with graft loss as well as mortality with a functioning graft [11,12].

Natural cytokine-specific autoantibodies (c-aAbs) are common and have been measured in both health and disease states [13]. It is not known whether c-aAbs represent a regulatory mechanism or a dysfunction of immunologic tolerance. In support of a regulatory potential is the finding that neutralizing the levels of c-aAbs toward pro-inflammatory IL-1α is associated with less joint erosion and disease activity in chronic polyarthritis [14,15]. Moreover, IL-6-specific c-aAbs have been associated with obesity, type 2 diabetes (DM) and severe infections [16,17], indicating that c-aAb-induced imbalances in the cytokine network may lead to immune dysfunction. Moreover, GM-CSF-specific c-aAbs have been associated with pulmonary alveolar proteinosis [18]. IL-6-specific c-aAbs were negatively correlated with the IL-6-induced CRP levels in Danish blood donors, indicating cytokine neutralizing levels of c-aAbs even in healthy populations [19]. Whereas correlational studies suggest a link between elevated levels of IL-6 and MACE, chronically elevated levels of IL-6 likely reflect ongoing TNF production [20].

It is likely that in ESRD patients, a high level of systemic inflammation predisposes and accelerates microvascular injury and atherosclerosis in the transplanted graft as well as in the recipient's own vascular system after renal transplantation. Moreover, it is possible, but unexplored, that these processes are modulated by natural c-aAbs, e.g., TNF-α-specific c-aAbs and IL-1α-specific c-aAbs, are possible protective factors in relation to graft loss, MACE, and mortality after transplantation as a result of a blockade/decline of pro-inflammatory activity, whereas IL-6-specific c-aAbs may fuel metabolic syndrome. Additionally, IL-10-specific c-aAbs may enhance pro-inflammatory activity through the blockade of the anti-inflammatory activities mediated by IL-10. The aim of this study was to test these hypotheses in a retrospective cohort study of Danish kidney transplanted patients. We investigated if systemic inflammation, as well as c-aAbs at the time of renal transplantation, is associated with the rate of graft loss, major adverse cardiovascular events and mortality. We focused on the TNF↑→IL-6↑→CRP↑ axis as these inflammatory proteins are powerful inflammatory biomarkers in epidemiological studies of the background population and in cohorts of kidney patients. Additionally, we included the anti-inflammatory and immunoregulatory IL-10, which represents a central inhibitor of TNF-α production in the TNF↑→IL-6↑→IL-10↑→TNF↓ circuit [6,21].

Material and methods

Study population

This study was a retrospective cohort study of all patients who underwent kidney transplantation between January 1st, 2009 and December 31st, 2015 in one of two transplantation centers (Herlev Hospital and Rigshospitalet, University Hospital of Copenhagen, Denmark). Patients were followed until March 17th, 2017 or until death, with a median follow-up of 4.9 years (range of 1.2–8.2 years) after transplantation. A total of 632 patients were transplanted in this period. Thirteen patients were excluded because of a low volume of serum samples before transplantation (<1 ml); thus, 619 patients were included in the study. A negative result of cross-matching for IgG B cell complement-dependent cytotoxicity was required for all recipients. Immunosuppression protocols and treatment of allograft rejection episodes after transplantation were similar between the two transplantation centers.

This study was approved by the regional ethical authority (code number H-16028690). The research biobank and research database were approved by the Danish Data Protection Agency (RH-2016-240, I-Suite nr: 04840).

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Circulating levels of cytokines, high-sensitivity CRP (hsCRP), and c-aAbs

Inflammatory biomarkers were measured in pretransplantation serum samples isolated at the day of transplantation or up to 2 months earlier. Serum was stored for 1–8 years at −20°C before analyses were performed. Serum concentrations of TNF-α, IL-6, and IL-10 were analyzed using the MSD® V-PLEX Custom Human Biomarkers assay (Meso Scale Diagnostics LLC, Rockville, Maryland, US) according to the instructions from the manufacturer. The limits of detection (LOD) were 0.28 pg/ml for TNF-α, 0.21 pg/ml for IL-6, and 0.21 pg/ml for IL-10 in our laboratory. All samples were run as duplicates, and the mean values were calculated. Deviations in duplicates had to be < 20% to accept the analysis (intra-assay CV). Inter-assay CV was 10–12% for IL-10, 9.4–9.7% for IL-6 and 15% for TNFα in our laboratory. hsCRP was analyzed at the Department of Clinical Biochemistry, University Hospital of Copenhagen, Denmark, using the routine assay Cobas, Cardiac C-Reactive Protein (Latex) High Sensitive reagent (CRPHS) (Roche Diagnostics Limited, Switzerland). The detection range was 0.15–20 mg/l. Values below and above the detection limit were replaced by the detection limit for all further analyses.

IL-1α-specific, IL-6-specific, IL-10-specific, and TNFα-specific c-aAbs were analyzed using a custom made Luminex® 100 (Luminex corporation, US/Netherlands) based assay, as described previously by Guldager *et al.* [13]. The LOD for the assay were defined as the blank sample + 4 standard deviations (SD), as described previously [19].

Registry data

Data regarding donor age, donor sex, recipient age, recipient sex, HLA types, HLA mismatches, ABO types and ABO mismatches were obtained from the Scandiatransplant Database with data available for all 619 included patients. Primary kidney disease, cold ischemia time, graft onset, acute rejections, graft loss, and death were obtained from the Danish Nephrology Registry (DNR). Data regarding MACE and DM were obtained from the Danish National Registry 10th revision (ICD-10) thereafter. Delayed graft function was defined as the requirement for dialysis in the first postoperative week. Available data on Cytomegalovirus (CMV) serostatus was obtained from the PERSIMUNE Data Warehouse, Rigshospitalet, Denmark.

Outcomes

Outcomes were defined as patient death, graft loss, and MACE defined as acute myocardial infarction, apoplexy or thromboembolism in a major artery.

Statistics

Statistical analyses were performed using IBM SPSS Statistics 22 and R [22]. Duration of follow-up was calculated as the time from inclusion (transplantation) until data retrieval on March 17th, 2017. Cytokines, hsCRP, c-aAb values and cold ischemia time were $\log_2$-transformed prior to statistical analysis. Geometric means and normal range for biomarkers and ischemic time in Table 1 were calculated as back-transformed (inverse $\log_2$) means and means ± 2SD of $\log_2$ transformed data. We compared continuous variables across groups by two-sided *t*-tests. Chi-squared tests were used to compare categorical data. Spearman's correlation analyses were used to assess monotone associations between continuous variables. Survival and cumulative incidence curves were estimated by the Kaplan–Meier and Aalen–Johansen estimators, respectively. Gray's test was used for testing equivalence of cumulative incidence functions [23]. Log-rank tests and Cox-regression analyses were used to investigate for associations between pretransplantation levels of circulating cytokines, hsCRP, and c-aAb (explanatory variables) and the mortality, graft loss, and MACE hazards (outcome variables). When analysing graft loss and MACE, death was treated as a competing risk. Multiple Cox-regression analyses were adjusted for factors considered to have potential influences on associations between inflammatory biomarkers and the outcome variables. This included the recipient factors age, sex, CMV sero status, primary kidney disease, pretransplantation DM and pretransplantation MACE when post-transplantation MACE or all-cause mortality was the outcome variable. The validity of the proportional hazards assumption and the functional form of covariates were assessed by model diagnostics based on cumulative sum of martingale residuals [24]. Missing data in the multiple Cox regressions models were handled by multiple imputations (using 25 imputed data sets and treating death as a competing risk) as implemented in the R package SMCFCS [24]. Results of complete-case analyses were like the multiple imputation analyses. Binary logistic regression analyses were used to test for associations between inflammatory mediators, c-aAb, and delayed graft function. $P < 0.05$

Table 1. Baseline characteristics in the study population

Variable*	Female (*n* = 232)	Male (*n* = 387)	All (*n* = 619)	*P*
Recipients				
Age - years	45.6 ± 15.5	47.2 ± 15.3	46.6 ± 15.4	0.19
Retransplantation - no. (%)	32 (14)	65 (17)	97 (16)	0.38
Pretransplantation dialysis‡ - no. (%)	198 (88)	324 (87)	522 (87)	0.96
Pretransplantation MACE - no. (%)	19 (8)	58 (15)	77 (12)	0.02
Pretransplantation DM§ - no. (%)	23 (10)	64 (17)	87 (15)	0.02
Pretransplantation CMV IgG positive¶ - no. (%)	172 (80)	234 (65)	406 (70)	<0.001
Primary kidney disease				
Glomerulonephritis - no. (%)	42 (18)	107 (28)	149 (24)	0.009
Diabetes mellitus - no. (%)	20 (9)	50 (13)	70 (11)	
Hypertension - no. (%)	23 (10)	43 (11)	66 (11)	
Interstitial or pyelonephritis - no. (%)	26 (11)	25 (7)	51 (8)	
Polycystic disease - no. (%)	34 (15)	47 (12)	81 (13)	
Other - no. (%)	87 (38)	115 (30)	202 (33)	
Donors				
Donor age - years	50.0 ± 15.9	50.1 ± 15.1	50.1 ± 15.4	0.90
Donor male sex - no. (%)	125 (54)	186 (48)	311 (50)	0.19
Deceased donor - no. (%)	149 (64)	236 (61)	385 (62)	0.47
Transplantation details				
AB0 incompatible - no. (%)	26 (11)	28 (7)	54 (9)	0.12
HLA-A/B/DR mismatch	2.8 ± 1.4	2.7 ± 1.5	2.7 ± 1.5	0.50
Anti-HLA immunized – no. (%)	69 (30)	87 (23)	156 (25)	0.06
Cold-ischemia time**				
Living donor - hours [geometric mean (normal range)]†	2.7 (1.4–5.2)	2.7 (1.3–5.6)	2.7 (1.3–5.4)	0.89
Deceased donor - hours [geometric mean (normal range)]†	16.5 (5.6–48.3)	16.5 (5.7–47.7)	16.5 (5.7–47.9)	0.98
Inflammatory markers				
TNF-α - pg/ml [geometric mean (normal range)]†	5.17 (1.87–14.30)	6.01 (1.71–21.11)	5.68 (1.74–18.50)	0.002
IL-6 - pg/ml [geometric mean (normal range)]†	1.29 (0.08–19.84)	1.68 (0.07–42.82)	1.52 (0.07–32.68)	0.04
IL-10 - pg/ml [geometric mean (normal range)]†	0.30 (0.06–1.55)	0.32 (0.05–2.04)	0.31 (0.05–1.85)	0.42
hsCRP - pg/ml [geometric mean (normal range)]†	1.80 (0.14–22.47)	2.42 (0.20–28.93)	2.16 (0.18–26.69)	0.005

c-aAb, cytokine-specific autoantibodies; CI, confidence interval; CMV, Cytomegalovirus; DM, diabetes mellitus; geo. mean, geometric mean; HLA, human leukocyte antigen; hsCRP, high-sensitivity C-reactive protein; IgG, immunoglobulin G; IL, interleukin; MACE, major cardiovascular events; SD, standard deviations; TNF, tumor necrosis factor.

Cold-ischemia time and inflammatory markers were $\log_2$-transformed prior to statistical analysis. Chi-square test was used for the comparison of categorical variables.

*Mean ± SD are shown for continuous variables.

†Backtransformed means ± 2 SD from $\log_2$-transformed data.

‡Data regarding pretransplant dialysis status was available for 598 patients, corresponding to 97% of the included patients.

§Data regarding pretransplantation DM were available for 598 patients, corresponding to 97% of the included patients.

¶Data regarding pretransplantation CMV IgG status were available for 577 patients, corresponding to 93% of the included patients.

**Data regarding cold-ischemia time were available for 586 patients, corresponding to 95% of the included patients. *t*-test was used for the comparison of independent groups. P values are for the comparison between male and female sex.

was considered to indicate statistical significance in all analyses.

Results

Characteristics of the cohort

The baseline characteristics of the 619 kidney transplanted patients are shown in Table 1. Most of the cohort was men and first-time transplantations with deceased donors. The most common primary kidney disease was glomerulonephritis. Men more often had a history of MACE and DM as well as higher levels of TNF-α, IL-6, hsCRP, and IL-1α-specific c-aAbs compared to that in women. Additionally, men were less immunized with HLA antibodies and CMV IgG antibodies. There was no evidence for a difference between men and women regarding outcome variables in the study including MACE, graft loss, and mortality during follow-up. The time of dialysis duration was evaluated in patients with first time transplantations. Data were available for 418 patients (missing data in 22 patients) and the median was 3 years of dialyses (range 12–5866 days).

Thirty-one patients had hsCRP concentrations in serum below the limit of detection and 36 participants had levels above the standard curve. As sensitivity analysis we replaced the values under the lower detection limit for hs-CRP with half or one-fourth of the limit, and correspondingly the values above the detection limit with twice or four times the limit. This had only minor effect on all subsequent results and did not alter any conclusion.

C-aAbs in relation to systemic inflammation, epidemiological data, and comorbidity at baseline (Table 2)

Patients with a pretransplantation history of MACE had lower levels of TNF-α-specific c-aAbs and a similar, although not significant, pattern of IL-1α-specific c-aAb levels. Men had higher levels of IL-1α-specific c-aAbs and IL-10-specific c-aAbs compared to that of women, and the IL-10-specific c-aAbs were inversely correlated with age. All measured c-aAbs were inter-correlated. Additionally, the TNF-α-specific c-aAbs were negatively correlated with circulating levels of IL-10 (table 2). Similarly, the IL-1α-specific c-aAb and IL-10-specific c-aAbs were negatively correlated with circulating levels of IL-10 although associations did not reach significance ($P = 0.08$). Scatter plots of correlations are found in the Appendix S1.

The duration of dialysis was weakly correlated with levels of CRP ($r_s = 0.16$; $N = 418$, $P = 0.001$) and IL-6 ($r_s = 0.17$; $N = 418$, $P < 0.001$) in Spearman correlation analyses but not with TNF-α, IL-10, or any of the c-aAbs (data not shown). Re-transplantations had higher levels of IL-10 compared with first-time transplantations: Geometric mean (95% CI) was 0.30 (0.28–0.33) pg/ml versus 0.37 (0.30–0.44) pg/ml, $P = 0.05$. There were no statistical differences in other inflammatory markers (data not shown).

Associations between pretransplantation factors (explanatory variables) and mortality in the follow-up period (outcome)

During the follow-up period 46 patients died. The Kaplan–Meier curves for inflammatory biomarkers in relation to all-cause mortality are shown in Fig. 1a–d. Patients are divided into low, medium, and high levels defined according to quartiles. The log-rank tests showed that all markers of systemic inflammation (hsCRP TNF-α, IL-6 and IL-10) were significantly associated with all-cause mortality. When inflammatory markers were included as continuous variables in a simple Cox regression analysis, high pretransplantation levels of hsCRP, TNF-α, IL-6, and IL-10 were associated with death (Table 3, model 1). Log_2 was chosen to simplify the interpretation of hazard ratios (HR), e.g., an HR = 1.48 for TNF-α means that a doubling of the circulating TNF-α concentration is associated with a 48% increase in mortality rate. Other factors associated with death in the simple Cox regression analyses included age, pretransplantation DM, donor age and donation type. The specific c-aAbs, pretransplantation MACE and CMV serostatus were not associated with death. In the multiple Cox regression analysis, TNF-α, IL-6, hsCRP, and IL-10 were all associated with death after adjusting for age, sex, pretransplantation DM, pretransplantation MACE, CMV serostatus and primary kidney disease (Table 3, model 2).

Effects of cytokines were not affected by specific c-aAbs in survival analyses. Compared with the model presented in Table 2, HR increased for TNF-α (HR = 1.69 (95% CI: 1.27–2.24), $P < 0.001$) if TNF-α-specific c-aAbs were further added to model 2 whereas TNF-α-specific c-aAbs had no significant effect ($P = 0.3$). Similar effects were found for IL-6 (HR = 1.22 (95% CI: 1.08–1.39), $P = 0.002$) when IL-6-specific c-aAbs were added and for IL-10 (HR = 1.47 (95% CI: 1.21–1.78), $P < 0.001$) when IL-10-specific c-aAbs were added.

Table 2. Pretransplantation associations between cytokine-specific autoantibodies, epidemiological data, co-morbidity, and systemic biomarkers

	IL-1α c-aAb (MFI)	TNFα c-aAb (MFI)	IL-6 c-aAb (MFI)	IL-10 c-aAb(MFI)
Inflammatory biomarkers±				
TNFa - pg/ml	$r_s = -0.06$	$r_s = -0.06$	$r_s = -0.03$	$r_s = 0.02$
IL-6 - pg/ml	$r_s = -0.06$	$r_s = -0.02$	$r_s = -0.07$	$r_s = -0.05$
IL-10 - pg/ml	$r_s = -0.07$	$r_s = -0.09$	$r_s = -0.05$	$r_s = -0.07$
hsCRP - mg/l	$r_s = -0.05$	$r_s = -0.03$	$r_s = -0.03$	$r_s = 0.02$
IL-1α c-aAb - MFI		$r_s = 0.26$	$r_s = 0.23$	$r_s = 0.21$
TNFα c-aAb - MFI	$r_s = 0.26$		$r_s = 0.23$	$r_s = 0.36$
IL-6 c-aAb - MFI	$r_s = 0.23$	$r_s = 0.23$		$r_s = 0.30$
IL-10 c-aAb - MFI	$r_s = 0.21$	$r_s = 0.36$	$r_s = 0.30$	
Epidemiological data and pretransplantation comorbidity				
Recipient age - years†	$r_s = 0.01$	$r_s = -0.04$	$r_s = -0.03$	$r_s = -0.14$
Recipient sex*	$P = 0.002$	$P = 0.84$	$P = 0.12$	$P = 0.01$
Male - MFI [geometric mean (95% CI)]	281 (247–319)	214 (200–229)	306 (269–348)	108 (100–117)
Female - MFI [geometric mean (95% CI)]	204 (174–239)	217 (195–240)	260 (223–304)	92 (83–102)
Pretransplantation DM*	$P = 0.12$	$P = 0.85$	$P = 0.38$	$P = 0.69$
Neg - MFI [geometric mean (95% CI)]	239 (214-266)	216 (202-231)	284 (255-316)	102 (95-109)
Pos - MFI [geometric mean (95% CI)]	305 (228–408)	219 (195–247)	323 (242–431)	106 (89–125)
Pretransplantation MACE*	$P = 0.07$	$P = 0.028$	$P = 0.12$	$P = 0.90$
Neg - MFI [geometric mean (95% CI)]	257 (230–286)	220 (207–235)	297 (267–330)	102 (95–109)
Pos - MFI [geometric mean (95% CI)]	202 (159–257)	181 (158–208)	233 (177–308)	101 (80–126)
Pretransplantation CMV IgG*	$P = 0.95$	$P = 0.82$	$P = 0.26$	$P = 0.35$
Neg - MFI [geometric mean (95% CI)]	245 (204–294)	217 (193–244)	260 (213–318)	97 (87–107)
Pos - MFI Mean (95% CI)	243 (215–276)	214 (199–229)	297 (263–335)	104 (95–113)

c-aAb, cytokine-specific autoantibodies; CI, confidence interval; CMV, Cytomegalovirus; DM, diabetes mellitus; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; MACE, major cardiovascular events; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.

Data regarding pretransplantation DM were available for 598 patients, corresponding to 97% of the included patients.

Data regarding pretransplantation CMV IgG status were available for 577 patients, corresponding to 93% of the included patients.

*Student's *t*-test was used for the comparison of $\log_2$-transformed c-aAb between groups. Geometric mean and 95% CI is indicated for MFI values.

†Spearman's correlation was used for assessing montone relationships between continuous variables.

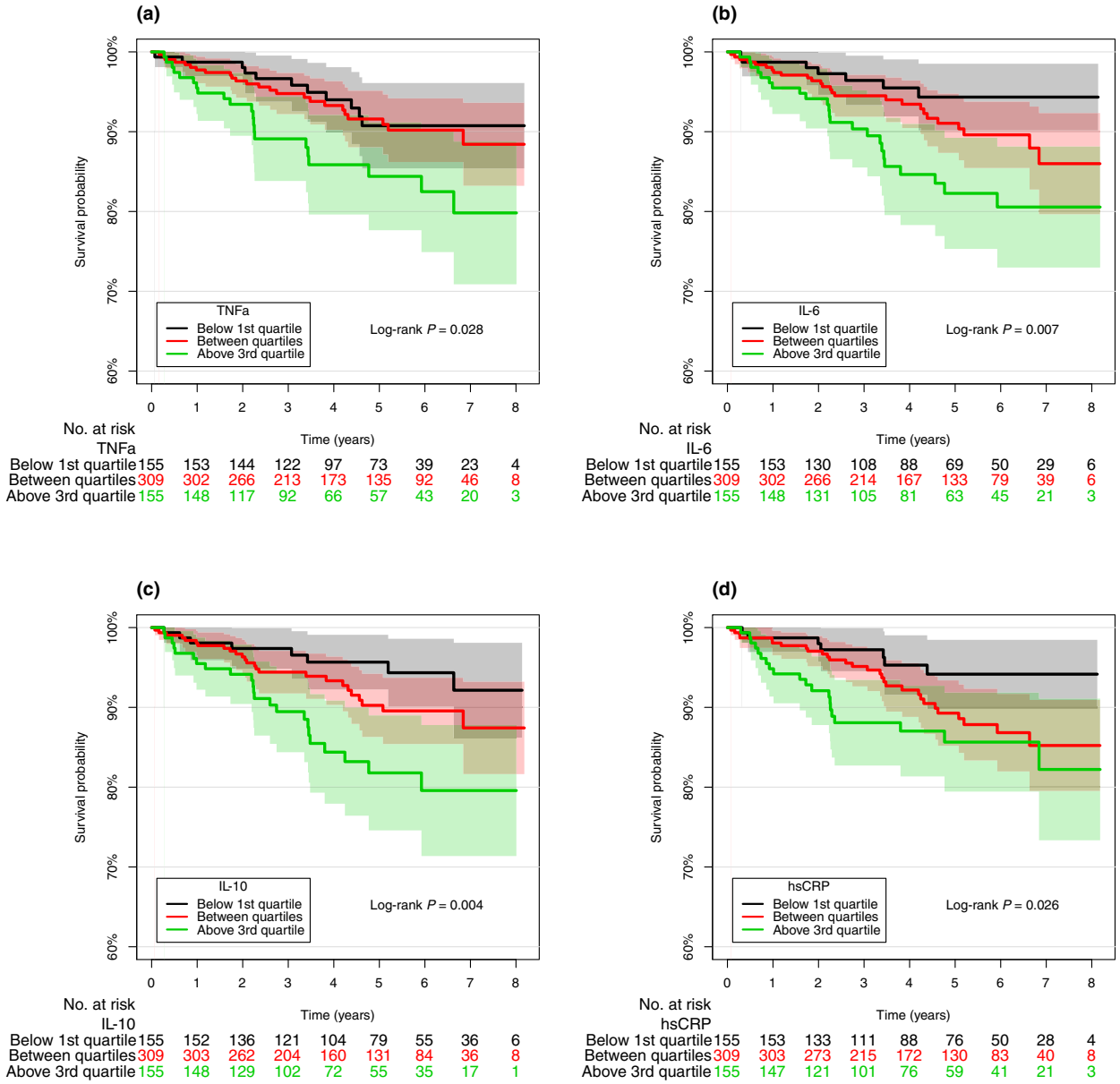

Figure 1 Kaplan–Meier curves for all-cause mortality, according to systemic inflammation before transplantation. The cohort is divided into three groups based on concentrations of cytokines categorized by quartiles: <25% (black line), 25–75% (red line) and > 75% (green line). Below the graphs are numbers at risk. Confidence intervals are shown. hsCRP, high-sensitivity C-reactive protein; IL, interleukin; TNF, tumor necrosis factor.

The inflammation marker estimates in the multiple Cox regression models in Table 3 were not mutually adjusted for each other. If cytokines and hsCRP were included in the same model effects of TNF-α, IL-6, and IL-10 were difficult to separate because of collinearity, while hsCRP caught aspects of the data not described by the other three markers. Reducing the model containing $\log_2$TNF-α, $\log_2$IL-6, $\log_2$IL-10, and $\log_2$hsCRP by dropping $\log_2$TNFa and $\log_2$IL-6 (both models adjusted for the same variables as Model 2 in Table 3) did not result in a significantly poorer model fit (likelihood ratio test $P = 0.79$).

Associations between pretransplantation factors (explanatory variables) and post-transplantation MACE as the outcome

Fifty-seven patients experienced a MACE during the follow-up period. Cumulative incidence for IL-1α-specific, IL-6-specific, IL-10-specific, and TNFα-specific c-aAbs with regard to post-transplantation MACE is shown in Fig. 2a–d, with high and low levels of c-aAbs arbitrarily defined as MFI ≥ 500 and MFI < 500. Figure 2d illustrates that high levels of IL-10-specific c-aAbs at the time of transplantation were associated with an increased risk of

Table 3. Pretransplantation clinical and immunologic factors and associations with all-cause mortality

Variable	No. of patients	Model 1		Model 2	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Inflammation					
TNFa per doubling (log_2 transformed)	619	1.48 (1.24–1.78)	<0.001	1.38 (1.15–1.66)	0.001
IL-6 per doubling (log_2 transformed)	619	1.15 (1.07–1.24)	0.001	1.12 (1.03–1.22)	0.007
IL-10 per doubling (log_2 transformed)	619	1.34 (1.17–1.52)	<0.001	1.29 (1.13–1.46)	<0.001
hsCRP per doubling (log_2 transformed)	619	1.21 (1.04–1.42)	0.001	1.22 (1.04–1.43)	0.016
Cytokine-specific autoantibodies					
IL-1α c-aAb per doubling (log_2 transformed)	619	1.10 (0.96–1.25)	0.19	1.10 (0.96–1.26)	0.17
TNFα c-aAb per doubling (log_2 transformed)	619	1.07 (0.84–1.36)	0.58	1.16 (0.91–1.48)	0.23
IL-6 c-aAb per doubling (log_2 transformed)	619	1.02 (0.88–1.17)	0.84	1.03 (0.90–1.20)	0.65
IL-10 c-aAb per doubling (log_2 transformed)	619	1.02 (0.81–1.28)	0.87	1.07 (0.88–1.30)	0.53
Recipients factors					
Age per 1- years increment	619	1.09 (1.07–1.12)	<0.001		
Recipient sex					
Female	232	1	0.36		
Male	387	1.29 (0.74–2.27)			
Pretransplantation MACE					
No	542	1	0.12		
Yes	77	1.74 (0.90–3.36)			
Pretransplantation DM*					
No	511	1	0.01		
Yes	87	2.39 (1.29–4.43)			
Graft rank					
First transplant	522	1	0.43		
Subsequent transplant	97	1.31 (0.68–2.54)			
Pretransplantation CMV IgG†					
Neg	171	1	0.52		
Pos	406	1.24 (0.64–2.39)			
Pretransplantation dialysis‡					
No dialysis	76	1	0.12		
Dialysis (hemo/peritoneal)	522	2.25 (0.70–7.24)			
Primary kidney disease					
Glomerulonephritis	149	1	0.002		
Diabetes mellitus	70	5.72 (2.22–14.74)			
Hypertension	66	3.57 (1.27–10.03)			
Interstitial or pyelonephritis	51	2.01 (0.57–7.11)			
Polycystic disease	81	1.27 (0.36–4.51)			
Other	202	2.59 (1.03–6.52)			
Transplantation					
Donor age per 1-year increment	619	1.04 (1.02–1.06)	<0.001		
Donor sex					
Female	308	1	0.26		
Male	311	1.35 (0.80–2.29)			
Donation type					
Deceased	385	1	<0.001		
Living	234	0.32 (0.16–0.66)			
AB0 match					
AB0 compatible	565	1	0.81		
AB0 incompatible	54	0.89 (0.32–2.45)			
No. of HLA-A/B/DR mismatches	619	0.93 (0.77–1.12)	0.42		
Anti-HLA immunized					
No	463	1	0.13		
Yes	156	1.57 (0.89–2.75)			

Transplant International 2019; 32: 933–948

Table 3. Continued.

Variable	No. of patients	Model 1 HR (95% CI)	Model 1 *P*	Model 2 HR (95% CI)	Model 2 *P*
Cold ischemia§ time per doubling					
Deceased donor (log_2 transformed)	363	1.02 (0.64–1.62)	0.95		
Living donor (log_2 transformed)	223	0.71 (0.17–2.90)	0.63		

c-aAb, cytokine-specific autoantibodies; CMV, Cytomegalovirus; DM, diabetes mellitus; HLA, human leukocyte antigen; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; MACE, major cardiovascular events; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.

Model 1: Simple, unadjusted Cox-regression.

Model 2: Multiple Cox regression. Estimates for inflammation markers and c-aAb are not mutually adjusted for each other but from separate individual models. Missing data is handled by multiple imputation. Model 2 is adjusted for age, sex, pretransplant MACE, pretransplant DM, CMV serostatus and primary kidney disease.

*Data regarding pretransplantation DM were available for 598 patients, corresponding to 97% of the included patients.

†Data regarding pretransplantation CMV IgG status were available for 577 patients, corresponding to 93% of the included patients.

‡Data regarding pretransplant dialysis status were available for 598 patients, corresponding to 97% of the included patients.

§Data regarding cold-ischemia time were available for 586 patients, corresponding to 95% of the included patients.

MACE compared to patients with low levels of IL-10-specific c-aAbs. In a simple unadjusted Cox regression analysis with c-aAbs as continuous variables, high levels of IL-10-specific c-aAbs were associated with an increased rate of MACE in the follow-up period after transplantation (Table 4, model 1). Effects of IL-1α-specific c-aAbs, TNF-α-specific c-aAbs, or IL-6-specific c-aAbs did not reach statistical significance in similar models (Table 4, model 1 and Fig. 2). Other factors associated with post-transplantation MACE included age, pretransplantation MACE, and pretransplantation DM (Table 4). We considered age, sex, pretransplant MACE, pretransplant DM, CMV serostatus, and primary kidney disease to have potential effects on the association between IL-10-specific c-aAbs and MACE. However, effects of IL-10-specific c-aAbs on MACE were almost unaffected when these factors were included in a multiple Cox regression analysis (Table 4, model 2). Additionally, the effect of IL-10-specific c-aAbs were unaffected when model 2 was adjusted for IL-10 (data not shown).

Associations between pretransplantation factors (explanatory variable) and graft loss (outcome)

Sixty-one patients experienced a graft loss during the follow-up period. We found no association between inflammatory markers or c-aAbs and graft loss (data not shown) in a simple Cox regression analysis, whereas receiving a transplant from a deceased compared to a living donor was associated with increased graft loss hazard (HR = 2.49; 95% CI = 1.32–4.68; $P = 0.005$).

We performed extra analyses to explore in more details if acute rejection or delayed graft function were potential intermediate factors in the association between IL-10-specific c-aAbs and post-transplant MACE. We had access to information about acute rejection for 615 patients. Acute rejection was reported in 151 of these patients (25%) during the first year after transplantation. We found no significant association between IL-10-specific c-aAbs (log_2 transformed) and acute rejection (outcome variable) in a Cox regression analysis: HR = 1.02 (95% CI: 0.90–1.15). We had access to information for 590 patients regarding delayed graft function. Among these participants 137 patients (23%) experienced delayed graft function and 13 patients (2%) obtained no graft function at all. The latter group was excluded from further analyses of delayed graft function as an outcome variable. A significant association was found between log_2 transformed IL-10-specific c-aAbs (explanatory variable) and delayed graft function (outcome) in a binary logistic regression analysis: IL-10-specific c-aAbs: Odds ratio (OR) = 1.17, 95% CI = 1.00–1.37, $P = 0.046$. The latter finding made us investigate if systemic inflammation including TNF-α, IL-6, IL-10, and hsCRP (explanatory variables) individually associated with mortality were also associated with delayed graft function (outcome). We found significant

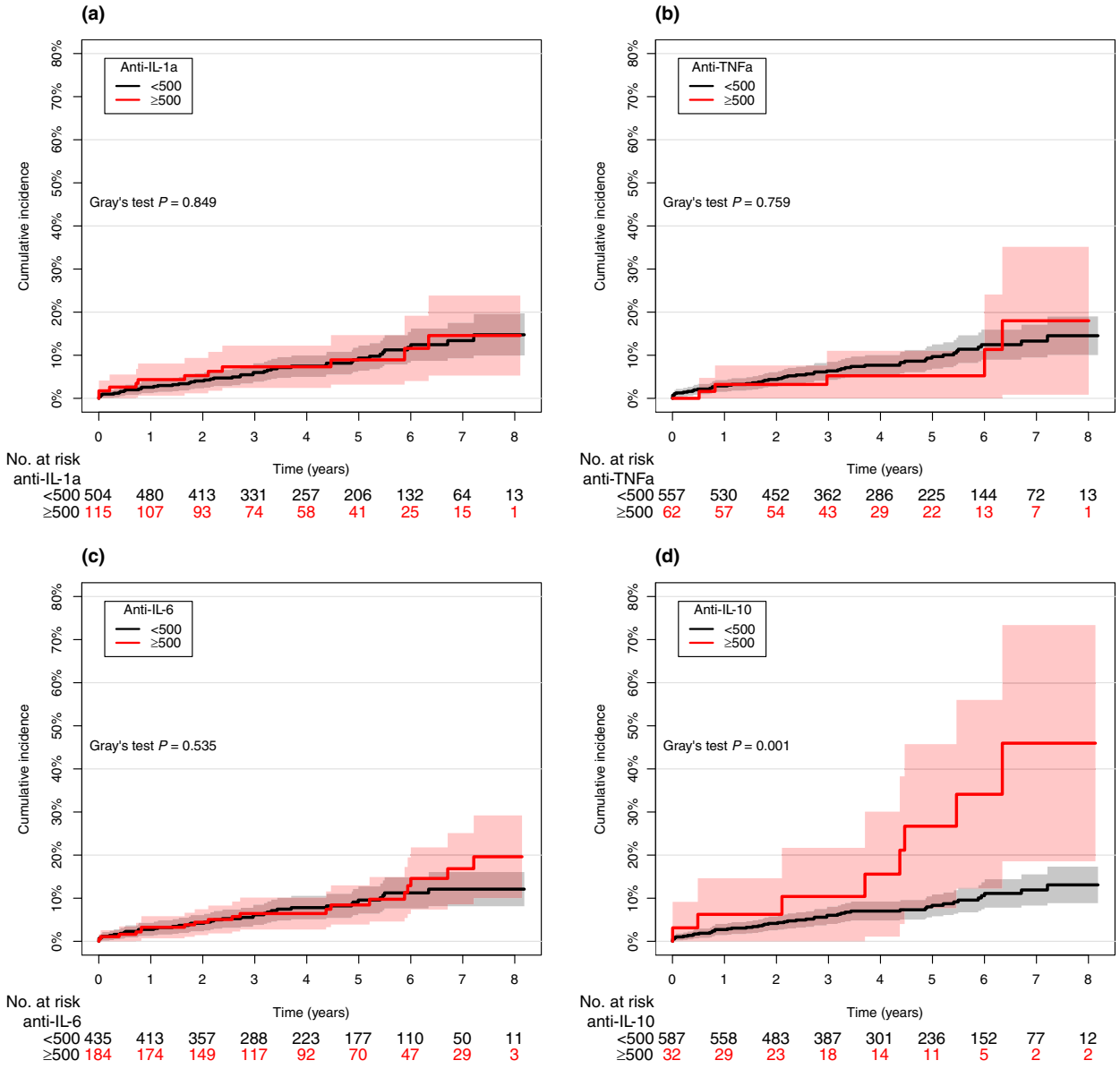

Figure 2 Cumulative incidence for MACE, according to cytokine-specific autoantibodies before transplantation. The cohort is divided into two groups based on c-aAb with MFI<500 (black line) and MFI≥500 (red line). Fig. 1d illustrates a specific association of IL-10 c-aAb with a MFI value ≥ 500 (n = 32), that is not seen for the other c-aAb. Below the graphs are numbers at risk. Confidence intervals are shown. c-aAb: cytokine-specific autoantibodies. IL, interleukin; MACE, major cardiovascular events; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.

associations for all these $\log_2$ transformed variables in binary logistic regression analyses: TNF-α (OR = 1.41, 95% CI = 1.14–1.73); IL-6 (OR = 1.13, 95% = 1.05–1.22); IL-10 (OR = 1.24, 95% CI = 1.07–1.43); hsCRP (OR = 1.20, 95% CI = 1.07–1.33).

Discussion

In this retrospective study of 619 kidney transplanted patients, we observed that enhanced pretransplantation levels of TNF-α, IL-6, hsCRP, and IL-10 were consistently associated with all-cause mortality after transplantation, adjusted for other clinically relevant factors. We found similar effects of TNF-α, IL-6, IL-10, and hsCRP in survival analyses, demonstrating robustness of the models but hsCRP caught additional aspect of the data not described by the three cytokines in mutual models. This finding supports that persistent low-grade activation of the TNF↑→IL-6↑→IL-10↑→TNF↓ circuit and the TNF↑→IL-6↑→CRP↑ axis at the time of

Transplant International 2019; 32: 933–948

Table 4. Associations between pretransplantation clinical and immunologic factors and post-transplantation MACE

Variable	No. of patients	Model 1		Model 2	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Inflammation					
TNFa per doubling (log_2 transformed)	619	1.17 (0.90–1.53)	0.27	1.22 (0.92–1.63)	0.17
IL-6 per doubling (log_2 transformed)	619	1.07 (0.98–1.18)	0.17	1.07 (0.95–1.19)	0.27
IL-10 per doubling (log_2 transformed)	619	0.96 (0.77–1.19)	0.70	0.99 (0.79–1.25)	0.96
hsCRP per doubling (log_2 transformed)	619	1.15 (0.99–1.32)	0.06	1.07 (0.92–1.24)	0.37
Cytokine-specific autoantibodies					
IL-1α c-aAb per doubling (log_2 transformed)	619	1.00 (0.86–1.15)	0.98	1.02 (0.88–1.19)	0.75
TNFα c-aAb per doubling (log_2 transformed)	619	0.92 (0.71–1.19)	0.52	0.95 (0.72–1.24)	0.68
IL-6 c-aAb per doubling (log_2 transformed)	619	1.07 (0.94–1.23)	0.31	1.09 (0.95–1.25)	0.24
IL-10 c-aAb per doubling (log_2 transformed)	619	1.31 (1.11–1.56)	0.005	1.26 (1.07–1.48)	0.005
Recipient factors					
Age per 1-year increment	619	1.04 (1.02–1.06)	<0.001		
Recipient sex					
Female	232	1	0.89		
Male	387	1.04 (0.61–1.78)			
Pretransplantation MACE					
No	542	1	0.002		
Yes	77	2.83 (1.57–5.10)			
Pretransplantation DM*					
No	511	1	0.014		
Yes	87	2.28 (1.24–4.20)			
Graft rank					
First transplant	522	1	1.00		
Subsequent transplant	97	1.00 (0.49–2.04)			
Pretransplantation CMV IgG†					
Neg	171	1	0.029		
Pos	403	2.11 (1.03–4.33)			
Pretransplantation dialysis‡					
No dialysis	76	1	0.91		
Dialysis (hemo/peritoneal)	522	0.96 (0.43–2.11)			
Primary kidney disease					
Glomerulonephritis	149	1	0.04		
Diabetes mellitus	70	2.48 (1.15–5.35)			
Hypertension	66	1.56 (0.65–3.76)			
Interstitial or pyelonephritis	51	1.39 (0.53–3.67)			
Polycystic disease	81	0.45 (0.13–1.56)			
Other	202	0.92 (0.43–1.95)			
Transplantation					
Donor age per 1-year increment	619	1.01 (0.99–1.02)	0.43		
Donor sex					
Female	308	1	0.47		
Male	311	1.21 (0.72–2.04)			
Donation type					
Deceased	385	1	0.07		
Living	234	0.59 (0.33–1.06)			
AB0 match					
AB0 compatible	565	1	0.78		
AB0 incompatible	54	1.15 (0.46–2.87)			
No. of HLA-A/B/DR mismatches	619	0.96 (0.79-1.15)	0.63		
Anti-HLA immunized					
No	463	1	0.035		
Yes	156	0.46 (0.21–1.02)			

Table 4. Continued.

Variable	No. of patients	Model 1 HR (95% CI)	Model 1 *P*	Model 2 HR (95% CI)	Model 2 *P*
Cold ischemia§ time per doubling					
Deceased donor (log_2 transformed)	363	0.63 (0.45–0.88)	0.007		
Living donor (log_2 transformed)	223	0.40 (0.14–1.15)	0.09		

c-aAb, cytokine-specific autoantibodies; CMV, Cytomegalovirus; DM, diabetes mellitus; HLA, human leukocyte antigen; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; MACE, major cardiovascular events; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.

Model 1: Simple, unadjusted Cox-regression.

Model 2: Multiple Cox regression. Estimates for inflammation markers and c-aAb are not mutually adjusted for each other but from separate individual models.

Missing data is handled by multiple imputation treating death as a competing risk. Model 2 is adjusted for age, sex, pre-transplant MACE, pre-transplant DM, CMV serostatus and primary kidney disease.

*Data regarding pretransplantation DM were available for 598 patients, corresponding to 97% of the included patients.

†Data regarding pretransplantation CMV IgG status were available for 574 patients, corresponding to 93% of the included patients.

‡Data regarding pretransplant dialysis status were available for 598 patients, corresponding to 97% of the included patients.

§Data regarding cold-ischemia time were available for 586 patients, corresponding to 95% of the included patients.

transplantation predicts subsequent high mortality risk. Additionally, this study is the first to investigate if natural c-aAbs modulate the outcome of kidney transplantation. We observed that pretransplantation IL-10-specific c-aAb levels were positively associated with the post-transplantation MACE rate adjusted for other clinically relevant factors, indicating that IL-10 may be a protective factor. In accordance with this finding, we observed that patients with a history of MACE before transplantation were characterized by lower levels of TNF-α-specific c-aAbs, and hence we hypothesized that TNF-α may be a risk factor in MACE; although not significant, a similar trend was found with regard to IL-1α-specific c-aAbs. Thus, it is possible that c-aAbs mediate a natural brake in the cytokine network, affecting the balance between anti-inflammatory and pro-inflammatory activity. These findings are in accordance with the inflammatory hypothesis of atherothrombosis [8] and support our hypotheses that natural c-aAbs modulate inflammatory activity and systemic inflammation in the kidney transplantation candidate. Moreover, our data demonstrate that biomarkers of systemic inflammation identify clinical risk phenotypes that should be taken into consideration in the risk estimation of the individual transplantation candidate in the clinical setting at the time of transplantation.

Inflammation is involved in all stages of atherosclerosis [25], and inflammation is associated with obesity and type 2 DM [25,26]. Cytokines such as TNF-α and IL-1 induce endothelial dysfunction, leukocyte adherence and hypercoagulability, and TNF-α causes insulin resistance [2]. Clinical TNF blockade modulates vascular risk factors in a beneficial direction in the treatment of inflammatory arthritis [7]. Most epidemiological studies, however, focus on CRP and IL-6 as biomarkers of chronic inflammation, although cytokines such as IL-10, TNF-α, and IL-1 could be more important from a pathophysiological point of view [27]. Our finding that natural IL-10 c-aAb cytokine inhibitors are associated with the rate of MACE after transplantation supports this consideration. Additionally, this observation is consistent with the findings that the pharmacological targeting of IL-1β leads to a significantly lower rate of recurrent cardiovascular events than placebo [9] and reduced CRP levels [28].

IL-10 is an anti-inflammatory cytokine linked to anti-atherosclerotic effects, but we found high pretransplant levels of IL-10 to be a risk factor in all-cause mortality. In accordance with our data, it has been reported that IL-10 increases along with reduced kidney function, and high IL-10 levels are associated with MACE in patients with chronic kidney disease [29]. The production of IL-10 is induced by IL-6 [30], and IL-10 subsequently inhibits further TNF-α production [31]. Moreover, IL-6 inhibits the production of TNF-α [32]. Accordingly, the production and levels of these cytokines are tightly linked, and in this regard, systemic levels of IL-10 are likely to act as a biomarker of persistent inflammation and balances in the complex cytokine

Transplant International 2019; 32: 933–948

networks. Consistent with this, it is well-established that high levels of the natural occurring IL-1RA is a prognostic risk biomarker in a wide range of diseases with an inflammatory component, including rheumatoid arthritis, systemic lupus erythematosus, sepsis, hepatitis, tuberculosis, etc., although it is considered to be preventive and not harmful in these diseases [33]. Considering the complexity of the interplay in the cytokine network and the lack of post-transplant IL-10 measurements in the present study pharmaceutical IL-10 blockade in prospective studies are needed for conclusions about IL-10-mediated anti-atherosclerotic activities in kidney transplanted patients.

Natural c-aAbs are found both in healthy and diseased individuals but their precise etiology is still undefined. We found that all measured c-aAbs were correlated with each other in ESRD patients. This finding may reflect chronic disease and uremia-associated systemic inflammation in the investigated cohort, but it has also been reported in a study of 8,972 healthy Danish blood donors [19]. Associations between post-transplantation MACE and IL-10-specific c-aAbs only are consistent with observations that individual c-aAbs are linked to lacunar defects [16,34–37]. We found no significant correlations between levels of IL-10-specific c-Abs and markers of systemic inflammation. Additionally, effects of IL-10-specific c-aAbs on MACE were unaffected by adjustment for systemic IL-10 levels in Cox regression analysis. The latter observation suggested that IL-10-specific c-aAbs were not another surrogate marker of the TNF↑→IL-6↑→IL-10↑→TNF↓ circuit or the TNF↑→IL-6↑→CRP↑ axis. Consistent with our findings, high levels of IL-10-specific c-aAbs are not associated with CRP levels in the Danish blood donor cohort study [19]. Experimental studies have shown that IL-10-specific c-aAbs block IL-10-induced STAT3 phosphorylation in normal blood leukocytes [38]. Moreover, it has previously been demonstrated that 0.4% of apparently healthy Danish blood donors produce high concentrations of polyclonally derived IL-10-specific c-aAbs of IgG class. These autoantibodies are stable from months to years and they bind IL-10 with extremely high avidity and act as competitive IL-10 inhibitors *in vitro*, substantially inhibiting cellular IL-10 receptor binding and neutralizing IL-10 activity *in vitro* [39]. Thus, there is evidence from experimental studies that IL-10-specific c-aAbs modulate the signaling and function/activities of IL-10 but not the production of IL-10. Accordingly, a strong linear relation is not expectable between system levels of IL-10 and IL-10-specific c-aAbs. We were not able to investigate if the presence of IL-10-specific c-aAbs dampens the IL-10 function or modulates the acute response in response to acute/chronic triggers *in vivo* or *in vitro* before and after transplantation in our study population. However, we speculate that the finding of IL-10 c-aAb as a risk factor in MACE supports that the biological role of IL-10 is to mediate anti-inflammatory/immunoregulatory and anti-atherosclerotic activities in kidney transplanted patients. Additionally, IL-10 may theoretically serve as a marker of regulatory B cells [40]. Therefore, natural occurring IL-10-specific c-aAbs at the time of transplantation might influence the development of rejection. Regarding acute rejection, we evaluated clinical data for the first year after transplantation when we consider this diagnosis to best defined, but we were not able to demonstrate associations with IL-10-specific c-aAbs. Moreover, we found no association between IL-10-specific c-aAbs and graft loss but we observed associations between IL-10-specific c-aAbs, systemic low-grade inflammation and delayed graft function. This makes us speculate that inflammatory activity at the time of transplantation is a contributing risk factor to delayed graft function, which is considered to have a detrimental long-term impact after kidney transplantation [41].

In accordance with our data, persistent systemic inflammation is associated with all-cause mortality in populations without kidney disease [42–46], and CRP is associated with death from coronary heart disease and ischemic stroke as well as nonvascular mortality in a large meta-analysis [4,47]. In regard to patients with advanced kidney disease, persistent inflammation is common and is associated with CVD and high mortality risk [10,48–50]. We found no association between systemic inflammation or c-aAbs before transplantation and the rate of graft loss although systemic inflammation was associated with delayed graft function. It is possible that the follow-up period with a median of 4.9 years in our study is only sufficient to detect clinical atherosclerosis in native vessels, whereas a longer follow-up period is needed to detect accelerated atherosclerosis in the allograft. Similar results were, however, reported in a small study of 115 kidney transplanted patients in whom pretransplantation CRP was associated with all-cause and cardiovascular mortality but not with graft loss [51]. This study is the only other study that evaluates associations between pre-transplantation levels of inflammation in relation to the outcomes after transplantation. Compared to this study, our study offers a larger study cohort and a more extensive inflammatory profile measured before transplantation. Accordingly, we are able to suggest

biological/pathological key players (TNF-α and IL-10) in the inflammatory cascade before transplantation with clinical importance of MACE and mortality after transplantation and to identify special phenotypes in the individual transplantation candidate. More studies evaluate systemic inflammation several years after transplantation in relation to transplantation outcome variables, but the clinical importance of such data is different, as it evaluates a clinical risk much later and does not provide individual risk estimation before transplantation. In accordance with our data, post-transplantation CRP was associated with all-cause mortality but not with graft loss in kidney transplanted patients with a median 7.8 years of follow-up [52]. A J-shaped association between post-transplantation hsCRP and mortality has been reported in kidney transplant recipients when hsCRP values were divided into quartiles for values below 5 mg/l. Associations between the lowest levels of CRP and high mortality risk has not been confirmed in other studies. Post-transplantation levels of TNF-α and IL-6 measured more than 3 months after kidney transplantation were associated with death with a functioning graft over a 6-year follow-up period in patients [53]. CRP measured more than 12 months after kidney transplantation predicts MACE [54], and circulating hsCRP and IL-6 levels measured more than 6 months after kidney transplantation are associated with MACE, all-cause mortality [12] and graft loss [11].

Limitations of the study should be considered. Persistent inflammation is strongly associated with obesity, smoking, and physical fitness in epidemiological studies [55]. These data were only available for a limited number of patients in our study. We cannot rule out that inflammatory markers in the study simply reflect these factors. Furthermore, the clinical dataset is incomplete with regard to cold ischemia time, CMV serostatus, pretransplantation DM and pretransplantation dialysis status, and we do not have information about the profile of lipids. We did not include multiple cytokines and hsCRP simultaneously in the multiple regression models in Table 3 because the cytokines and autoantibodies are highly collinear, making their roles difficult to separate. The aim of the models was to show robustness of inflammatory markers for outcome after transplantation, and indeed we found similar associations between the measured cytokines and hsCRP and outcome. If all inflammation markers were included in the same model, the roles of TNF-α, IL-6 and IL-10 were difficult to separate because of collinearity, while hsCRP caught aspects of the data not described by the other three markers. A model with hsCRP and one out of the three measured cytokines did not result in a significantly decrease in model fit than the model with hsCRP together with all three cytokines. Retransplantion is likely to affect the transplantation outcome. However, re-transplantation had no effect on survival, MACE or graft loss in univariate models of our cohort and accordingly we found no rationale to exclude these patients. The serum samples were stored at −20°C prior to the cytokine analyses, which may have caused some protein degradation. At least hsCRP has been proven to be a stable marker [56,57], and despite a potential limitation because of protein degradation, the cytokine data still had a high clinical impact in our study cohort. It is possible the induction therapies and maintenance immunosuppression change levels of cytokines and cytokine specific autoantibodies after transplantation and this may influence associations with MACE. Checking post-transplant changes in these biomarkers would be helpful in clarifying associations. Unfortunately, we did not have plasma available to perform such analyses. Moreover, the present study does only test associations and not causality. However, the main purpose of this study was to evaluate if inflammatory activity in the transplantation candidate at the time of transplantation is a risk factor that can be used as a new base of risk scoring and decision making in the clinical setting at the time of transplantation.

In conclusion, systemic inflammation in kidney transplantation candidates predicts all-cause mortality in the years after transplantation. TNF-α, IL-6, and IL-10 had similar roles whereas hsCRP added extra effects in multiple regression survival models. Markers of systemic inflammation can therefore add to the individual risk estimation before transplantation. IL-10-specific c-aAbs are associated with increased rate of MACE after transplantation, suggesting these autoantibodies modulate the inflammatory network in relation to universal atherosclerosis in kidney transplanted patients.

Authorship

KPL and HB: developed the hypotheses of the study and they designed, coordinated, and conducted the study and were main responsible for the writing of this paper. KL: established the clinical biobank and databases and she performed statistical analyses under the supervision of Frank Eriksson. JHS and MBH: developed and performed laboratory analyses of the cytokine-specific autoantibodies. FE: supervisor of statistical

Transplant International 2019; 32: 933–948

analyses and perform statistical model controls. BKP: housed analyses of for the inflammatory markers. SSS: responsible for the clinical patient data. All authors contributed to the writing process and the scientific discussion of data.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supplemental scatter plots of cytokines, cytokine specific autoantibodies, and hsCRP.

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