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ORIGINAL ARTICLE

High plasma hemopexin activity is an independent risk factor for late graft failure in renal transplant recipients

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Summary

Chronic low-grade inflammation is involved in late renal transplant dysfunction. Recent studies suggest a role for hemopexin, an acute phase protein, in kidney damage. We investigated whether hemopexin activity (Hx) predicts graft failure in renal transplant recipients (RTRs). In 557 RTRs with functioning grafts for ≥1 year, Hx was measured in citrate-plasma. RTRs were divided according to Hx into two groups; A: sextile 1–5 (464 RTRs, 83%) and B: sextile 6 (92 RTRs, 17%). Hx [median (IQR) 11.1 (3.3–19.1) arbitrary units] was measured at 6.0 (2.6–11.5) years post-transplant. RTRs with high Hx (group B) had significantly higher urinary protein excretion (UP) and diastolic blood pressure than group A, despite significantly more prevalent use of renin-angiotensin-aldosterone system inhibitors. After follow-up [4.6 (3.8–5.2) years], incidence of graft failure in group A was 25 (5%) and in group B 14 (15%, $P = 0.0009$). After adjustment for high-sensitivity C-reactive protein (hsCRP), UP and other potential confounders, Hx remained an independent predictor of graft failure [HR = 2.5 (95% CI 1.2–5.3), $P = 0.01$]. In conclusion, elevated Hx predicts late graft failure in RTRs, independent of hsCRP and UP. This suggests that Hx measurement, next to measurement of creatinine clearance and UP, could be of value for the identification of RTRs at risk for graft failure.

Introduction

One-year survival of renal allografts has steadily improved over the last few decades. However, improvement in late allograft survival strongly lags behind, with more or less consistent loss of almost half of all kidneys transplanted from post mortal donors within 10 years after transplantation [1]. Chronic transplant dysfunction (CTD) is one of the leading causes of this late allograft loss [2]. CTD is characterized clinically by a gradual decline in renal function with proteinuria and hypertension [3,4]. Its pathophysiology is, however, still poorly understood [2–5].

The plasma protein hemopexin is known for binding heme with the highest affinity of any known protein. Its function of scavenging free heme released following turnover of heme proteins such as hemoglobin protects the body from the oxidative damage associated with free heme. However, hemopexin has also been found to affect the glomerular filtration barrier. Intrarenal infusion of human plasma hemopexin in rats leads to transient proteinuria [6]. *In vitro*, active hemopexin has been shown

to cause nephrin-dependent remodeling of podocytes and to affect permeability of the glomerular filtration barrier by degrading the glycocalyx [7]. Furthermore, plasma levels of activated hemopexin are high in proteinuric children with minimal change disease [8]. Importantly, plasma hemopexin is one of the acute phase proteins of the hepatic response to inflammation [9].

Thus, ongoing chronic low-grade inflammation and release of cytokines from the transplanted kidney into the circulation may lead to relatively high production and activation of plasma hemopexin, which in turn may enhance glomerular permeability and facilitate existence of proteinuria. We therefore hypothesize that high plasma Hx in renal transplant recipients (RTRs) is a risk factor for late graft failure.

We aimed to investigate whether high circulating levels of activated HX are associated with increased plasma concentrations of C-reactive protein and with increased urinary protein excretion in RTRs. We furthermore aimed to investigate prospectively whether high HX activity predicts decline of renal function and development of graft failure in RTRs.

Materials and methods

Study design and patients

In this longitudinal prospective study, all RTRs who visited our out-patient clinic between August 2001 and July 2003 and had a functioning graft for at least 1 year were eligible to participate at their next visit to the out-patient clinic. Recipients were asked to participate during a subsequent visit to the out-patient clinic if they were ill or had signs of an infection. A total of 606 RTRs signed written informed consent, from an eligible 847 (72% consent rate). The group that did not sign informed consent was comparable with the group that signed informed consent with respect to age, gender, body mass index, plasma creatinine, creatinine clearance, and proteinuria. Hx was determined in 557 RTRs. In the remaining 49 subjects, no plasma was available (for the purpose of determining Hx). Further details of this study have been published previously [10,11]. The Institutional Review Board approved the study protocol (METc 01/039) which was in adherence to the Declaration of Helsinki. Funding resources had neither a role on the collection and analysis of data, nor in the submission and publication of the manuscript.

Outcome events

All participating subjects visited the out-patient clinic at least once a year. Information on graft failure and mortality was recorded by our renal transplant center and through close collaboration with general practitioners as well as referring nephrologists. Graft failure was defined as the return to dialysis or re-transplantation and was censored for death. Graft failure and mortality of all RTRs were recorded until August 2007. There was no loss to follow-up.

Renal transplant characteristics

Relevant transplant characteristics were taken from the Groningen Renal Transplant Database. This database holds information on all renal transplantations performed at our center since 1968, including dialysis history. Standard immunosuppression consisted of the following: from 1968 until January 1989, prednisolone and azathioprine (100 mg/day); from January 1989 to February 1993, cyclosporine standard formulation (Sandimmune, Novartis; 10 mg/kg; trough levels of 175–200 mg/l in first 3 months, 150 mg/l between 3 and 12 months post-transplant, and 100 mg/l thereafter) combined with prednisolone (starting with 20 mg/day, rapidly tapered to 10 mg/day). From March 1993 to May 1997, cyclosporine microemulsion (Neoral; Novartis Pharma b.v., Arnhem, The Netherlands; 10 mg/kg; trough levels idem) and prednisolone. From May 1997 to date, mycophenolate mofetil (Cellcept; Roche b.v., Woerden, The Netherlands; 2 g/day) was added. Current medication was extracted from the medical record.

Body mass index, waist circumference, body surface area, and blood pressure were measured as described previously [11]. Smoking status and cardiovascular history were recorded with a self-report questionnaire. Cardiovascular disease history was considered positive if there was a previous myocardial infarction (MI), transient ischemic attack (TIA) or cerebrovascular accident (CVA).

Baseline laboratory and clinical assessments

Hemopexin activity was measured in citrate-plasma with a standard amidolytic assay with the artificial substrate S2302 (H-D-Pro-Phe-Arg-pNA.2HCl) (Chromogenix, Milano, Italy) as described previously [12]. This assay has been shown to match with the extracellular matrix ECM stripping assay whereby loss of glomerular apyrase expression after incubation of kidney sections with Hx, with or without anti Hx IgG, was quantified as a standard for Hx activity [12]. As previous pilot studies indicated that the protease activity of the samples could not be inhibited by α-2- macroglobulin, it is unlikely that plasma kallikrein activity occurs in the samples tested.

High sensitivity C-reactive protein (hsCRP) concentrations were determined using in-house enzyme-linked immunosorbent assays (ELISA); as described before, the

lowest limit of detection was 0.002 mg/l [13]. Total cholesterol was determined using the CHOD PAP method (MEGA AU 510; Merck Diagnostica, Darmstadt, Germany). Low density lipoprotein (LDL) was calculated using the Friedewald formula. High density lipoprotein cholesterol (HDLc) was determined using the CHOD PAP method on a Technikon RA-1000 (Bayer Diagnostics b.v., Mijdrecht, The Netherlands). Plasma glucose was determined by the glucose-oxidase method (YSI 2300 Stat plus; Yellow Springs, OH, USA). Serum creatinine levels were determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Assessment of class I and class II HLAab was performed by ELISA (LATM20 × 5, One Lambda, Canoga Park, CA, USA). Samples were classified as negative, borderline and positive according to instructions of the manufacturer. Total protein concentration was analysed using the Biuret reaction (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany) and proteinuria was defined as urinary protein excretion ≥0.5 g/24 h.

Statistical analyses

Analyses were performed with SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) and SIGMA PLOT version 10 (Systat software Inc., Erkrath, Germany). Parametric parameters are given as means ± standard deviation (SD), whereas nonparametric parameters are given as median [interquartile range]. Hazard ratios (HRs) are reported with [95% confidence interval (CI)]. A two-sided *P*-value $<P < 0.05$ indicated statistical significance.

Table 1. Recipient-related characteristics at inclusion according to subgroups of hemopexin.

	Hemopexin		
	Group A	Group B	*P*-value
n (%)	465 (83)	92 (17)	
Hemopexin activity (arbitrary units)	7.9 [2.1–14.4]	30.5 [26.1–46.1]	<0.0001
Recipient demographics			
Age (years)	51.7 ± 12.3	49.9 ± 11.5	0.2
Male gender, *n* (%)	259 (56)	51 (55)	1.0
Body composition			
BMI (kg/m^2)	26.0 ± 4.4	25.8 ± 4.1	0.6
Waist circumference (cm)	97.2 ± 11.0	95.7 ± 13.1	0.3
Smoking, *n* (%)	102 (22)	20 (22)	1.0
Blood pressure			
Systolic pressure (mmHg)	152.8 ± 23.0	155.9 ± 21.1	0.2
Diastolic pressure (mmHg)	89.7 ± 9.7	92.0 ± 9.8	0.04
Use of ACE-inhibitor or AII-antagonist, *n* (%)	148 (32)	39 (42)	0.04
Use of β-blocker, *n* (%)	287 (62)	58 (63)	0.8
No. anti-hypertensive drugs, (*n*)	2.0 [1.0–3.0]	2.0 [1.0–3.0]	0.2
Prior history of cardiovascular disease			
MI, *n* (%)	37 (8)	9 (10)	0.6
TIA/CVA, *n* (%)	28 (6)	5 (5)	0.8
Lipids			
Total cholesterol (mmol/l)	5.6 [4.9–6.2]	5.9 [5.1–6.4]	0.04
LDL (mmol/l)	3.5 [2.9–4.1]	3.7 [3.1–4.4]	0.02
HDL (mmol/l)	1.0 [0.9–4.1]	1.1 [0.9–1.4]	0.07
Triglycerides (mmol/l)	1.9 [1.4–2.6]	1.7 [1.2–2.7]	0.2
Use of statin at inclusion, *n* (%)	229 (49)	45 (49)	1.0
Glucose homeostasis			
Glucose (mmol/l)	4.5 [4.1–5.0]	4.5 [4.0–5.1]	0.8
Insulin (μmol/l)	11.0 [8.0–16.0]	10.7 [7.6–15.2]	0.3
Diabetes after transplantation, *n* (%)	80 (17)	15 (16)	0.8
Use of antidiabetic drugs (%)	64 (14)	9 (10)	0.3
CRP (mg/l)	1.9 [0.7–4.8]	2.4 [1.1–4.8]	0.4

Groups of hemopexin activity: A; sextile; 1–5, and B sextile 6. Values are presented as mean ± standard deviation, median [interquartile range] or percentages. Differences between groups were tested for statistical significance with Student's *t*-test for normally distributed variables, Mann–Whitney test for skewed distributed variables, and chi-squared test for categorical variables.
MI, myocardial infarction; TIA, transient ischemic attack; CVA, cerebrovascular accident.

Table 2. Transplanted kidney-related at inclusion characteristics according to subgroups of hemopexin.

	Hemopexin, *n* (%)		
	Group A	Group B	*P*-value
Donor demographics			
Age (years)	36.6 ± 15.5	37.7 ± 14.4	0.5
Male gender, *n* (%)	260 (56)	45 (49)	0.2
Renal allograft function			
Serum creatinine concentration (μmol/l)	134.0 [114.0–163.0]	140.5 [109.5–186.3]	0.3
Creatinine clearance (ml/min)	62.1 ± 21.2	59.4 ± 26.1	0.3
Urinary protein excretion (g/24 h)	0.2 [0.0–0.5]	0.3 [0.1–0.7]	0.03
Proteinuria, *n* (%)	122 (26)	31 (34)	0.1
Primary renal disease, *n* (%)			
Primary glomerular disease	132 (28)	24 (26)	0.5
Glomerulonephritis	26 (6)	10 (11)	
Tubular interstitial disease	74 (16)	11 (12)	
Polycystic renal disease	81 (17)	14 (15)	
Dysplasia and hypoplasia	16 (3)	5 (5)	
Renovascular disease	24 (5)	7 (8)	
Diabetes mellitus	20 (4)	3 (3)	
Other or unknown cause	92 (20)	18 (20)	
Prior dialysis duration (months)	26.0 [12.0–49.0]	27.5 [16.0–45.0]	0.9
Transplantation type, *n* (%)			
Deceased donor	385 (83)	73 (79)	0.3
Living donor	63 (14)	17 (19)	
Ischemia times			
Cold ischemia times (h)	21.0 [14.5–27.0]	21.5 [14.0–27.8]	0.8
Warm ischemia times (min)	35.0 [30.0–45.0]	35.0 [30.0–43.0]	0.5
HLA mismatches, *n*			
HLA-AB	1.3 ± 1.1	1.2 ± 1.1	0.3
HLA-DR	0.4 ± 0.6	0.3 ± 0.5	0.07
Class I HLA antibodies			
Negative, *n* (%)	378 (81.3)	73 (79.3)	0.9
Borderline, *n* (%)	17 (3.7)	4 (4.3)	
Positive, *n* (%)	70 (15.1)	15 (16.3)	
Class II HLA antibodies			
Negative, *n* (%)	410 (88.2)	81 (88.0)	0.8
Borderline, *n* (%)	15 (3.2)	4 (4.3)	
Positive, *n* (%)	40 (8.6)	7 (7.6)	
Acute rejection, *n* (%)	214 (46)	40 (43)	0.7
Acute rejection treatment, *n* (%)			
High doses corticosteroids	150 (32)	26 (28)	0.7
Antilymphocyte antibodies	64 (14)	14 (15)	
Immunosuppression			
Prednisolone dose, (mg/day)	10.0 [7.5–10.0]	10.0 [7.5–10.0]	0.9
Calcineurin inhibitor, *n* (%)	364 (78)	77 (84)	0.2

Groups of hemopexin activity: A; sextile; 1–5, and B sextile 6. Values are presented as mean ± standard deviation, median [interquartile range] or percentages. Differences between groups were tested for statistical significance with Student's *t*-test for normally distributed variables, Mann–Whitney test for skewed distributed variables, and chi-square test for categorical variables.

In exploring data for linearity of association with outcome, RTRs were divided into tertiles and sextiles according to Hx. In case of a nonlinear relationship, we proceed with analyses according to tertiles or sextiles, depending on the nature of the relationship. It is considered statistically inappropriate to search for an 'optimal' cut-off point for dividing the population and perform analyses according to that cut-off point [14]. For analyses, we combined sextile 1–5 to one group (group A) and compared this group with sextile 6 (group B). Differences between groups were tested for statistical significance with Student's *t*-test for normally distributed variables, Mann–Whitney test for skewed distributed variables, and chi-squared test for categorical variables.

In our prospective analyses, we first investigated whether Hx was associated with a decline in renal

function. Renal function decline was calculated as the change in creatinine clearance, expressed as percentage of baseline creatinine clearance, per year of follow up. Subjects with <1 year of follow-up for renal function were excluded from analyses of change in renal function. Difference in change of renal function between groups was investigated by Student's *t*-test. We then proceeded with investigating Hx as potential predictor of graft failure in a Kaplan–Meier analysis, first in the full population and then only in subjects without proteinuria. Finally, we performed univariate and multivariate Cox regression analyses in the full population. In the multivariate analyses, we adjusted for recipient age and gender (model 2), for creatinine clearance and time between transplantation and inclusion date (model 3), all factors that were univariately associated with Hx ($P < 0.1$, Tables 1 and 2, model 4), and for high-sensitivity C-reactive protein (hsCRP) concentration and urinary protein excretion (model 5). In an additional model (model 6), we also further adjusted for variables associated with graft failure [number of human leukocyte antigen (HLA)-mismatches, presence of HLA antibodies, delayed graft function, donor age, plasma triglycerides, use of calcineurin inhibitors and acute rejection].

Results

A total of 557 RTRs (56% male subjects, aged 51.4 ± 12.2 years, 86% transplants from post mortal donors) were analysed. Median [interquartile range (IQR)] time between transplantation and baseline measurements was 6.0 [2.6–11.5] years. Median [IQR] Hx was 11.1 [3.3–19.1] arbitrary units. Recipient-related and transplanted kidney-related baseline characteristics according to plasma Hx status are given in Tables 1 and 2 respectively. Median [IQR] Hx was 7.9 [2.1–14.4] in group A and 30.5 [26.1–46.1] in group B ($P < 0.0001$). In cross-sectional analyses, RTRs with high plasma Hx (group B) had significantly higher urinary protein excretion and diastolic blood pressure than group A, despite significantly more prevalent use of angiotensin-converting enzyme (ACE) inhibitors or AII-antagonists. Other differences were in plasma lipids, with higher values in subjects with high plasma Hx (group B).

Subsequently, we proceeded with prospective analyses, in which we first investigated whether Hx was associated with change in renal function during follow up. Change in renal function in group A of Hx was significantly lower than in groups B of Hx (−2.1% (SD 12.3) per year vs. −6.6% (SD 11.6) per year respectively ($P = 0.002$, Fig. 1). Results of multivariate linear regression analyses for change in renal function in the population with follow-up for 1 year or more ($n = 433$ for group A and $n = 86$ for

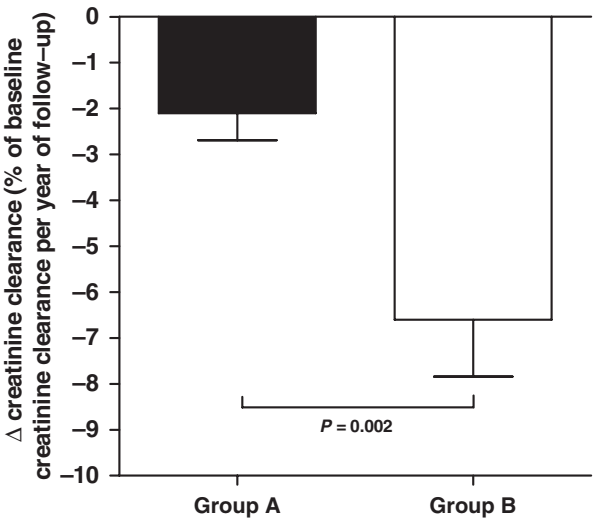

Figure 1 Change in creatinine clearance (mean ± SEM) between baseline and follow-up according to groups of hemopexin activity.

Table 3. Univariate and multivariate Cox and linear regression analyses for late graft failure and change in renal function respectively in RTR according to groups of hemopexin activity.

	Hemopexin activity		
	Group A Reference	Group B	*P*
Change in renal function*		B [95% CI]	
Model 1	0.0	−4.6 [−7.4; −1.7]	0.002
Model 2	0.0	−4.6 [−7.4; −1.8]	0.001
Model 3	0.0	−4.8 [−7.6; −2.0]	0.001
Model 4	0.0	−5.1 [−7.9; −2.2]	0.0004
Model 5	0.0	−4.9 [−7.7; −2.1]	0.001
Model 6	0.0	−4.8 [−7.6; −2.0]	0.001
Graft failure		HR [95% CI]	
Model 1	1.0	3.0 [1.6; 5.8]	0.0009
Model 2	1.0	3.0 [1.6; 5.8]	0.001
Model 3	1.0	2.4 [1.2; 4.6]	0.01
Model 4	1.0	2.2 [1.1; 4.3]	0.03
Model 5	1.0	2.2 [1.1; 4.6]	0.02
Model 6	1.0	2.5 [1.2; 5.3]	0.01

Groups of hemopexin activity: A; sextile 1–5 2, B; sextile 6.
*Change in renal function is expressed in % change in creatinine clearance per year
Model 1: Crude model.
Model 2: Model 1 + adjustments for recipient age and gender.
Model 3: Model 2 + adjustments for creatinine clearance and time between transplantation and baseline.
Model 4: Model 3 + adjustments for LDL, diastolic blood pressure, and use of ACE-inhibitor or AII-antagonist.
Model 5: Model 4 + CRP and urinary protein excretion.
Model 6: Model 5 + adjustments for number of HLA mismatches, presence of HLA ab, delayed graft function, donor age, concentration triglycerides, use of calcineurin inhibitor, and acute rejection.

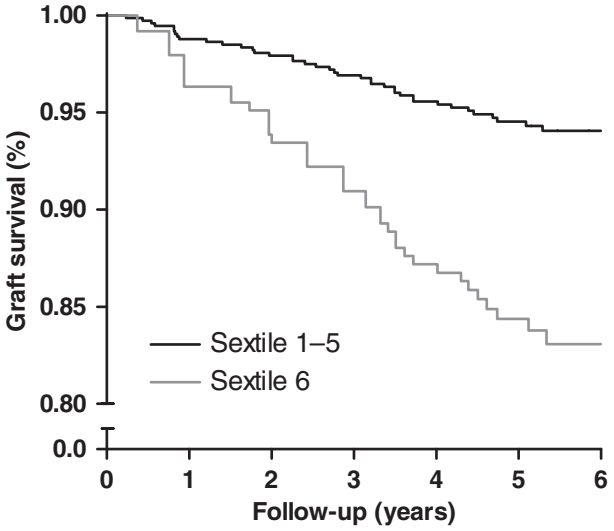

Figure 2 Kaplan–Meier curves for graft survival groups of Hemopexin activity. Group A: sextile 1–5 and group B sextile 6 of hemopexin activity ($P = 0.0009$).

group B) are shown in Table 3, models 2–6. Subsequent adjustments did not materially affect the association. Second, we investigated whether plasma Hx was associated with renal survival. During median [IQR] follow-up beyond baseline for 4.6 [3.8–5.2] years, 39 (7%) RTRs experienced graft failure in the full cohort. Of these cases, 25 (5%) occurred in group A and 14 (15%) in group B [HR = 3.0 (95% CI 1.6–5.8), $P = 0.0009$, Table 3, model 1, Fig. 2]. Biopsies were performed in 22 (56%) of the 39 cases of graft failure. One biopsy showed signs of recurrence of primary disease (oxalosis), the rest was classified as chronic allograft nephropathy (CAN). A biopsy was performed in 15 (60%) cases in group A versus 7 (50%) in group B ($P = 0.55$). The case of graft failure resulting from recurrence of oxalosis occurred in group A. If this case is excluded from analyses, crude HR [95% CI] for development of graft failure in group B versus group A is 3.2 [1.6–6.1], $P = 0.0006$. Transplant glomerulopathy was present in nine (43%) of the cases classified as CAN. In these cases, transplant glomerulopathy was present six (42.9%) in group A and three (42.9%) in group B ($P = 1.0$). If plasma Hx was entered into Cox-regression analyses as a continuous variable, the association of with graft failure was not significant, indicating that the association of plasma Hx with graft failure is strongly nonlinear, with an increased risk only in the high range of values. These results were essentially similar when we restricted our analysis to subjects without proteinuria ($n = 403$), in which there were 12 cases of graft failure during follow-up. Of these cases, seven (2%) occurred in group A and five (8%) in group B [HR = 4.2 (95% CI 1.3–13.3), $P = 0.01$]. Results of multivariate Cox regression analyses for late graft failure in the full population are shown in Table 3, models 2–6. Adjustment for recipient age and gender (Table 3, model 2) did not materially change results. Further adjustment for creatinine clearance and time between transplantation and inclusion date (Table 3, model 3) slightly weakened the association of hemopexin with graft failure [HR = 2.4 (95% CI 1.2–4.6), $P = 0.01$]. Additional adjustments for LDL cholesterol, total cholesterol, diastolic blood pressure, and use of ACE-inhibitor or AII-antagonist did not materially change these results. The association of Hx with graft failure remained significant after final adjustment for hsCRP and urinary protein excretion [HR = 2.3 (95% CI 1.1–4.6), $P = 0.02$, Table 3, model 5]. This was also true after adjustment for other potential confounders, including presence of class I and/or class II HLAab (Table 3, model 6).

Discussion

We found no association of high plasma Hx with plasma concentrations of CRP. However, significant associations of high plasma Hx with increased urinary protein excretion and diastolic blood pressure, despite higher frequency of use of anti-proteinuric ACE-inhibitors and AII-antagonists in RTRs with high plasma Hx could be demonstrated. We furthermore identified high plasma Hx as a risk factor for development of graft failure in RTRs, independent of plasma concentrations of CRP and urinary protein excretion. We moreover found that high plasma Hx is a risk factor for graft failure in RTRs even without proteinuria.

Hemopexin belongs to the class I acute phase proteins and is mainly produced in the liver after stimulation by pro-inflammatory cytokines IL-1 and IL-6 [15]. Therefore, it is likely that increased Hx might reflect activation of the inflammatory cascade. The observation in this study that hsCRP, mainly produced by the liver, was not associated with enhanced Hx activity, may indicate that increased Hx activity in these subjects reflects the possibility of, in addition to Hx synthesis by hepatocytes, also production by other cells i.e. inflammatory, or mesangial cells [16,17]. Indeed, Kapojos *et al.* [16] showed that human mesangial cells obtained from normal human kidney tissue, stimulated by tumor necrosis factor-α (TNF-α) are able to release hemopexin *in vitro*. This suggests that hemopexin can be locally produced in the kidney after stimulation with a pro-inflammatory agent. As interstitial inflammation of the kidney is linked to proteinuria in humans [18], it is conceivable that local production of active Hx by mononuclear inflammatory or mesangial cells [16,17] may promote enhanced glomerular permeability. In humans, the idea for a role of hemopexin

in proteinuria is supported by a study in children with minimal change nephritic syndrome, in which, during relapse of the disease, enhanced Hx has been demonstrated as compared with controls [8]. Also in the experimental model, active Hx is able to induce proteinuria as well as loss of glomerular anionic sites in the rat kidney [6,12]. Finally, a recent study underscores the idea that active hemopexin may be involved in kidney damage. In this study, it was shown that hemopexin is able to alter the cytoskeleton organization of human podocytes, and is capable of increasing glomerular permeability by degrading the glycocalyx [7].

We found that the change in creatinine clearance per follow-up year, expressed as percentage of baseline creatinine clearance, was significantly associated with plasma Hx. We also found plasma Hx to be significantly associated with graft failure late after transplantation, independent of CRP and urinary protein excretion. High plasma Hx was also a predictor of graft failure in the sub-cohort without proteinuria. CTD is one of the leading causes of late graft failure [2]. The mechanisms of CTD are complex and not fully understood, but chronic low-grade inflammation is likely to be involved [2,5]. There are several possible explanations for the association of Hx and graft failure. First, high Hx might reflect ongoing intrarenal alloreactivity with ongoing chronic low-grade intrarenal inflammation and progressive damage, ultimately leading to graft failure [5]. Second, the mechanism linking increased Hx to graft failure may lie in proteinuria. Proteinuria is one of the hallmarks of CTD [3,4] and is linked to interstitial inflammation in the kidney [18]. As said before, human mesengial cells obtained from normal kidney cortexes stimulated by tumor necrosis factor-α (TNF-α) are able to release hemopexin *in vitro* [16]. Thus, our finding of high Hx to predict graft failure may be a reflection of the release of hemopexin into the circulation by renal mesangial cells in response to interstitial inflammation in the context of proteinuria. Importantly, however, we also found hemopexin to be at least equally predictive for development of graft failure in RTRs without proteinuria. An explanation may be that the cascade with interstitial inflammation is already fully activated when the amount of protein in urine is still below the upper limit of the reabsorption capacity of the tubular epithelial cells. There is recent evidence that much more protein is filtered than has previously been thought and that active processing by tubular epithelial cells prevents it from appearing in urine [19–21]. Hx will then already be elevated in RTRs when proteinuria is not yet detectable. In multivariate analysis the association of Hx with graft failure was independent of urinary protein excretion, which is in concordance with this line of reasoning. Hemopexin is widely known for its function in heme scavenging. It is unlikely that the association of high Hx with graft failure is explained by this. Free iron is known to be toxic for kidneys. So, if this would play a role one would have expected low plasma Hx to be associated with increased risk for graft failure rather than the other way around.

This study has some limitations. First, this study is a single center study and the predictive value of hemopexin needs to be confirmed in other centers and/or through multicenter studies. Also in our study, Hx was assessed from samples taken at one time point in each patient. It would be interesting to investigate in a future study whether sequential measurements of Hx could be used as an even earlier marker, and can be used to predict development of proteinuria and increases in plasma creatinine. Third, this study includes RTRs that were transplanted in multiple immunosuppressive eras, and it may be difficult to extrapolate our findings to current immunosuppressive regimens. However, immunosuppressive therapy was not significantly associated with Hx and adjustment for immunosuppressive era in the multivariate analyses did not materially change the outcomes (data not shown). Furthermore, our study only includes stable RTRs, some of whom have already established very stable, long-term graft function. Future studies have to be performed to investigate whether hemopexin, measured in the early post-transplant period, also predicts late graft failure and mortality. An important strength of this study is that follow-up was complete for all patients.

In conclusion, elevated plasma Hx predicts graft failure late after renal transplantation, independent of hsCRP and urinary protein excretion. These results suggest that measurement of Hx could be of additional value, next to measurement of creatinine clearance and proteinuria, for the identification of RTR at risk for graft failure. Although the risk associated with high plasma Hx was independent of proteinuria, current evidence suggests that high plasma Hx could be a damaging factor for podocytes, and thus causally involved in occurrence of CTD and finally graft failure. Future studies are needed to confirm the association of Hx with higher risk for graft failure and to elucidate not only the mechanism underlying the link of Hx with graft failure late after renal transplantation, but also whether interventions aiming at reduction of plasma Hx might be renoprotective.

Authorship

JAK and RMVR: participated in writing of the paper and the data analysis. AK: retrieved patients files and performed assays for presence of class I and class II HLA antibodies. MAS: participated in the preparation of the

paper. TB: performed the analysis of hemopexin. SPML: supervised the HLA- typing. JPS: participated in and gave expert advice in the data-analysis. WWB, ROBG and GN: participated in writing of the paper. SJLB: participated in the research design, the writing of the paper and supervised the project.

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