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ORIGINAL ARTICLE

Plasma neutrophil gelatinase-associated lipocalin and kidney graft outcome

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ABSTRACT

Background. Plasma neutrophil gelatinase-associated lipocalin (pNGAL) has been investigated extensively in acute kidney injury. This study investigated its pathophysiological significance and utility as marker for graft failure and mortality in stable kidney transplant recipients (KTR).
Methods. Baseline pNGAL was measured in 698 KTR (58% male, age 53 ± 13 years, estimated glomerular filtration rate 52.4 ± 20.4 mL/min/1.73 m^2) at median 5.4 (interquartile range 1.8–12.0) years after transplantation, enrolled in the prospective TransplantLines Food and Nutrition Biobank and Cohort Study.
Results. pNGAL concentrations were higher in males, younger patients, patients with a deceased-donor kidney and higher serum creatinine. Independent of these, pNGAL was positively associated with urinary protein excretion, systemic inflammation parameters and calcineurin inhibitor use. During median follow-up of 5.3 (4.5–6.0) years, death-censored graft failure rates were 3.9%, 7.3% and 25.0% across increasing tertiles of pNGAL ($P_{log\text{-}rank} < 0.001$). Cox-regression analyses showed no independent associations of pNGAL with mortality, but strong associations with graft failure (hazard ratio, per doubling 4.16; 95% confidence interval 3.03–5.71; $P < 0.001$), which remained independent of adjustment for confounders. These associations were present only in patients with pre-existent proteinuria and poor kidney function.
Conclusions. pNGAL is associated with parameters of kidney graft damage and with graft failure. The latter association is particularly present in KTR with pre-existent poor kidney function and proteinuria.
Trial Registration: ClinicalTrials.gov NCT02811835.

Keywords: graft failure, inflammation, kidney transplant, mortality

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INTRODUCTION

Graft failure remains a major clinical problem for kidney transplant recipients (KTR). The condition implies the need for re-initiation of dialysis or re-transplantation, and is associated with increased morbidity and mortality, reduced quality of life and higher economic cost [1]. To provide optimal long-term care to KTR, it is pivotal to recognize patients at risk for graft failure not only shortly after transplantation, but also in the following years. A main parameter used to assess risk of graft failure in current clinical practice is proteinuria. Unfortunately, proteinuria is a late phenomenon, occurring when a certain degree of damage to kidney cells has already taken place [2]. In addition, while routine proteinuria measurements are recommended and have good specificity, proteinuria has limited sensitivity (<50%) as a marker for graft failure [2]. Other clinical parameters, such as the estimated glomerular filtration rate (eGFR), also provide limited utility in predicting long-term outcome of the transplanted kidney [3]. Therefore, there is a great clinical need for markers that identify patients at risk of (subclinical) kidney injury and graft failure in a timely manner, before the injury has taken place [2].

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein that is synthesized in the bone marrow during myelopoiesis. It is then stored in the granulae of neutrophils, but NGAL is also expressed in other tissues, including the kidney epithelium [4]. NGAL (in urine and plasma) was first recognized as a biomarker for acute kidney injury and was extensively studied in patients with this condition [5]. NGAL appeared to be an important mediator in post-ischaemic injury, tubular damage, apoptosis and increased cell proliferation [6, 7]. These properties raise the hypothesis that NGAL may be upregulated in KTR experiencing chronic kidney damage, putting them at increased risk of graft failure [1]. The value of NGAL measurements on short-term outcomes, such as delayed graft function, has been assessed in previous studies [8]. However, the utility of plasma NGAL (pNGAL) measurements longer after transplantation has remained largely unexplored.

Therefore, we investigated the association of pNGAL with graft failure in KTR at least 1 year after transplantation. Additionally, we assessed cross-sectional associations of pNGAL with clinical and biochemical parameters, and additional prospective associations with mortality.

MATERIALS AND METHODS

Study population

We included KTR who were included in the TransplantLines Food and Nutrition Biobank and Cohort Study [9]. In brief, this cohort study included 707 adult KTR (≥18 years old) with a functional graft ∼1 year or longer after transplantation, who visited the University Medical Center Groningen (UMCG, Groningen, The Netherlands) outpatient clinic, between November 2008 and June 2011. The study was approved by the UMCG institutional review board (METc 2008/186) and adheres to the declarations of Helsinki and Istanbul (ClinicalTrials.gov: NCT02811835). All patients provided written informed consent.

All 698 participants with available plasma samples were included in the current analyses. The flow of the study population is presented in Supplementary data, Figure S1. The primary endpoint was death-censored graft failure, defined as requirement of dialysis or re-transplantation, and all-cause mortality was a secondary endpoint. The continuous surveillance system of the outpatient program ensured up-to-date information on patient status. There were no losses to follow-up.

Data collection and definitions

All measurements were performed during a morning visit to the outpatient clinic. Blood pressure was measured using a semi-automatic device following a strict protocol [9]. Demographic and clinical characteristics including medical history were extracted from patient records. Diabetes was defined using the American Diabetes Association criteria [10]. Kidney function was assessed by means of eGFR, according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [11]. Proteinuria was defined as a urinary protein excretion higher than 0.5/24 h, as measured in 24 h collection urine.

Biochemical analyses

Blood was drawn at baseline after an overnight fasting period of at least 8 h, including no medication use. Samples were stored at room temperature for a maximum of 1 h, centrifuged at 2800 *g* at 4°C for 10 min and stored at −80°C until analysis. NGAL concentrations were measured in ethylenediamine tetraacetic acid (EDTA)-plasma using a validated particle-enhanced turbidimetric immunoassay (Gentian, Moss, Norway) [12]. Other clinical chemistry assays including leukocyte count and high-sensitivity C-reactive protein (hs-CRP) and glucose were performed using automated routine spectrophotometric-based methods (Roche Diagnostics, Basel, Switzerland).

Statistical analyses

Baseline characteristics were summarized in tertiles of pNGAL concentrations. Significance of differences between tertiles was assessed using analysis of variance, Kruskal–Wallis or Chi-squared tests, depending on distribution. Univariable linear regression analyses were performed with binary logarithmically transformed ($\log_2$) pNGAL as the dependent variable. Normality of the residuals was evaluated by visual inspection of QQ-plots, where variables were transformed using a $\log_2$ transformation if necessary to reach assumptions for linear regression. After univariable analyses, we adjusted for age, sex, creatinine and donor status.

Kaplan–Meier curves and log-rank tests were used to assess differences of graft and patient survival between tertiles of pNGAL. Cox proportional hazards regression analyses were used to assess associations of pNGAL with death-censored graft failure and all-cause mortality. In multivariable Cox regression analyses, we included variables known to be associated with graft failure risk, including age, sex, eGFR, 24-h urinary protein excretion and presence of circulating anti-human leukocyte antigen (HLA) Class II antibodies [13]. We additionally adjusted for other potential confounders and parameters that were suggested to be associated with pNGAL according to the literature, including pre-emptive transplantation, rejection treatment, history of cardiovascular (CV) disease, leukocyte count and hs-CRP. Associations of pNGAL with age, sex, eGFR and urinary protein excretion were assessed by adding interaction terms to the final models. Schoenfeld residuals were visually checked and the final models did not violate the assumption for proportionality of hazards ($P = 0.18$ and $P = 0.74$ graft failure and mortality models, respectively). Hazard ratios are presented per doubling of pNGAL, with 95% confidence intervals (95% CI). We tested

for differences in the −2 log likelihood of the final prediction models with and without pNGAL.

As we identified an interaction of eGFR and 24-h urinary protein excretion with pNGAL, we repeated the Cox regression analyses in subgroups based upon median values of patients with graft failure (eGFR ~35 mL/min/1.73 m^2) or clinically accepted cut-off values (urinary protein excretion ≥0.5 g/24 h). These additional Cox regression models, including adjustment for the final model, are presented in a forest plot. For all prospective associations, multiple imputation using fully conditional specification was performed using the R package 'mice', to account for missing data among variables other than data on pNGAL, where the number of imputed values is reported in table footnotes. The imputed values were visually checked for biological plausibility. All data were analysed using IBM SPSS software, version 23.0 (SPSS Inc., Chicago, IL, USA) and R version 3.2.3 (Vienna, Austria). For all analyses, $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Baseline characteristics

Baseline characteristics according to tertiles of pNGAL are presented in Table 1. We included 698 KTR with a mean ± standard deviation (SD) age of 53 ± 13 years, of whom 58% were male, at a median (interquartile range) time after transplantation of 5.4 (1.8–12.0) years. Mean eGFR at baseline was 52.4 ± 20.4 mL/min/1.73 m^2 and pNGAL concentration was 170 (133–232) mg/mL. Male prevalence increased, whereas the prevalence of having a living donor kidney decreased across increasing tertiles of pNGAL ($P = 0.02$ and $P = 0.002$, respectively). Leukocyte count and hs-CRP concentrations increased, whereas eGFR decreased across increasing tertiles of pNGAL.

Associations of pNGAL with clinical and biochemical parameters

pNGAL concentrations were lower in females, older patients and patients that received a living donor kidney, as shown in Table 2. Among all variables, parameters of kidney function were most strongly associated with pNGAL, where serum creatinine showed the strongest association [standardized (st.) β 0.62, $P < 0.001$]. After adjustment for age, sex, creatinine and donor status, variables including time after transplantation (st. β 0.09, $P = 0.005$) and use of calcineurin inhibitors (st. β 0.11, $P < 0.001$) remained positively associated with pNGAL concentrations, while pre-emptive transplantation was associated with lower pNGAL concentrations (st. β −0.08, $P = 0.012$). While cold ischaemia time was positively associated with pNGAL in univariable analyses (st. β 0.19, $P = 0.001$), this association disappeared after adjustment for donor status, age, sex and creatinine (st. β 0.09, $P = 0.14$). Additional analyses showed that the association between cold ischaemia time and pNGAL remained materially unchanged after adjustment for age, sex and $\log_2$ creatinine, while it disappeared after additional adjustment for donor type. Among laboratory measurements, the positive association of pNGAL was strongest with urea (st. β 0.19, $P < 0.001$), leukocyte count (st. β 0.28, $P < 0.001$), hs-CRP (st. β 0.18, $P < 0.001$) and 24-h urinary protein excretion (st. β 0.09, $P = 0.005$).

Associations of pNGAL with death-censored graft failure

In total, 86 patients (12.3%) developed graft failure during a median follow-up time of 5.3 (4.5–6.0) years after baseline. Rates of graft failure were 3.9%, 7.3% and 25.0% across increasing tertiles of baseline pNGAL (Figure 1A, $P_{log\text{-}rank} < 0.001$). Cox regression analyses showed that pNGAL was associated with an increased risk of graft failure [hazard ratio (HR) per doubling 4.16; 95% CI 3.03–5.71; $P < 0.001$]. As presented in Table 3, this association weakened, but remained strong, independent of adjustment for predefined potential confounders (HR per doubling 1.97; 95% CI 1.20–3.24; $P = 0.008$). The prospective association between pNGAL and graft failure is visualized in Figure 2. Addition of pNGAL to this Cox regression model, which included age, sex, eGFR, 24-h urinary protein excretion, presence of anti-HLA class II antibodies, pre-emptive kidney transplantation, history of rejection, history of cardiovascular disease, hs-CRP and leukocyte count, significantly improved the model ($P_{likelihood\ ratio} = 0.009$).

Sensitivity analyses

There were no interactions between pNGAL and age, sex or time after transplantation for the associations of pNGAL with outcomes ($P > 0.05$ for all). However, importantly, there was a significant interaction of kidney function and 24-h urinary protein excretion for the association of pNGAL with graft failure ($P = 0.002$ and $P = 0.008$, respectively). Sensitivity analyses in subgroups were therefore performed for the full model of age, sex, time after transplantation, eGFR, $\log_2$ 24-h urinary protein excretion, anti-HLA class II antibodies, pre-emptive transplantation, history of rejection, history of cardiovascular disease, $\log_2$ leukocyte count and $\log_2$ hs-CRP. These analyses indicated that pNGAL was only significantly associated with graft failure in patients with eGFR <35 mL/min/1.73 m^2 (HR 3.24; 95% CI 1.62–6.48; $P = 0.002$), but not in patients with better kidney function (HR 0.98; 95% CI 0.42–2.27; $P = 0.95$), as presented in Figure 3. Similarly, higher pNGAL was associated with an increased risk of graft failure in patients with proteinuria (HR 3.14; 95% CI 1.47–6.71; $P = 0.004$), but not in patients without proteinuria (HR 1.25; 95% CI 0.55–2.87; $P = 0.58$).

Associations of pNGAL with all-cause mortality

In secondary analyses, we assessed associations of pNGAL with all-cause mortality. During a median follow-up time of 5.4 (4.8–6.1) years after baseline, 150 patients (21.5%) died. Mortality rates were 15%, 21% and 28% across increasing tertiles of baseline pNGAL (Figure 1B, $P_{log\text{-}rank} = 0.001$). Cox regression analyses with pNGAL as continuous variable were consistent with a positive association of pNGAL with risk of all-cause mortality (HR per doubling 1.55; 95% CI 1.21–2.00; $P < 0.001$). This association was no longer significant after adjustment for predefined potential confounders. There were no interactions of age, sex and eGFR with pNGAL in the final model.

DISCUSSION

This study shows that clinical parameters including increased urinary protein excretion, worse kidney function, use of calcineurin inhibitors and higher parameters of systemic inflammation were independently associated with higher

Table 1. Baseline characteristics of 698 renal transplant recipients in tertiles of pNGAL

	Tertile 1 ($n = 233$)	Tertile 2 ($n = 233$)	Tertile 3 ($n = 232$)	P-value
pNGAL (µg/L)	119 (101–133)	171 (158–189)	264 (232–309)	<0.001
Recipient				
Male sex [*n* (%)]	117 (50.2)	134 (57.5)	147 (63.4)	0.02
Age at visit (years)	54.3 ± 12.5	53.2 ± 12.7	51.6 ± 12.9	0.06
Pre-emptive transplantation [*n* (%)]	52 (22.3)	29 (12.4)	27 (11.6)	0.002
Time after transplantation (years)	5.0 (1.3–10.0)	6.1 (2.3–12.4)	5.9 (1.9–13.4)	0.04
Anti-HLA Class II antibodies [*n* (%)]	26 (11)	43 (18)	51 (22)	<0.001
History of rejection [*n* (%)]	47 (20)	54 (23)	84 (36)	<0.001
Primary renal disease [*n* (%)]				0.05
Diabetic nephropathy	6 (2.6)	16 (6.0)	15 (6.5)	
Glomerulonephritis	54 (23.2)	60 (25.8)	68 (29.3)	
Interstitial nephritis	27 (11.6)	33 (14.2)	28 (12.1)	
Cystic kidney disease	52 (22.3)	45 (19.3)	46 (19.8)	
Renal vascular disease	16 (6.9)	6 (2.6)	16 (6.9)	
Other congenital/hereditary disease	17 (7.3)	10 (4.3)	12 (5.2)	
Other multisystem diseases	15 (6.4)	20 (8.6)	11 (4.7)	
Other or unknown cause	46 (19.7)	45 (19.3)	36 (15.5)	
Diabetes [*n* (%)]	59 (25.3)	58 (24.9)	52 (22.4)	0.7
History of CV disease [*n* (%)]	78 (36.4)	83 (40.5)	89 (43.2)	0.4
Body mass index (kg/m^2)	26.6 ± 4.8	26.8 ± 4.4	26.6 ± 5.1	0.9
Systolic blood pressure (mmHg)	135.4 ± 16.7	135.3 ± 17.8	137.1 ± 17.6	0.5
Current smoking [*n* (%)]	24 (10.3)	23 (9.9)	37 (15.9)	0.1
Kidney transplant characteristics				
Donor age (years)	41.4 ± 15.4	43.2 ± 15.7	44.0 ± 15.2	0.2
Living donor [*n* (%)]	99 (42.5)	77 (33.0)	63 (27.2)	0.002
Cold ischaemia time (h)	13.1 (2.6–20.1)	15.1 (2.8–22.0)	16.6 (3.1–21.0)	0.12
Warm ischaemia time (min)	2.0 (0.0–4.0)	0.0 (0.0–4.0)	0.0 (0.0–4.0)	0.9
Delayed graft function	10 (5.4)	16 (8.2)	17 (8.9)	0.4
Laboratory measurements				
Leukocytes ($\times 10^9$/L)	7.3 ± 2.1	8.3 ± 2.6	8.7 ± 2.9	<0.001
Haemoglobin (mmol/L)	8.43 ± 0.96	8.31 ± 1.12	7.92 ± 1.09	<0.001
Sodium (mmol/L)	141.1 ± 2.7	141.0 ± 2.5	140.5 ± 3.1	0.03
Potassium (mmol/L)	3.9 ± 0.4	4.0 ± 0.5	4.1 ± 0.5	<0.001
eGFR (mL/min/1.73 m^2)	65.1 ± 17.5	53.6 ± 18.6	38.5 ± 15.6	<0.001
Creatinine (µmol/L)	99 (85–120)	123 (104–149)	167 (136–211)	<0.001
Urea (mmol/L)	8.1 ± 2.7	10.2 ± 4.1	14.3 ± 5.8	<0.001
hs-CRP (mg/L)	1.4 (0.6–3.0)	1.5 (0.7–4.5)	2.0 (0.9–6.5)	0.001
Proteinuria	28 (12.1)	47 (20.2)	82 (35.3)	<0.001
Medication use [*n* (%)]				
Diuretics	72 (30.9)	98 (42.1)	113 (48.7)	<0.001
RAAS blocker	105 (45.1)	106 (45.5)	128 (55.2)	0.05
Calcium antagonist	54 (23.2)	53 (22.7)	64 (27.6)	0.4
Beta blocker	141 (60.5)	141 (60.5)	160 (69.0)	0.09
Prednisolone	233 (100.0)	231 (99.1)	227 (97.8)	0.06
Calcineurin inhibitor	104 (44.6)	127 (54.5)	168 (72.4)	<0.001
Proliferation inhibitor	207 (88.8)	199 (85.4)	176 (75.9)	0.001
mTOR inhibitor	10 (4.5)	7 (3.0)	6 (2.6)	0.5

Normally distributed data are presented as means ± SD, skewed data as median (interquartile range), and categorical data as number (valid percentage). P-values represent significance of differences between tertiles as assessed with analysis of variance, Kruskal–Wallis or Chi-squared tests, depending on distribution. CV, cardiovascular; eGFR, estimated glomerular filtration rate as calculated using CKD-EPI formula; LDH, lactate dehydrogenase; RAAS, renin-angiotensin-aldosterone system. Diabetes mellitus was defined as blood glucose >7 mmol/L or use of antidiabetics.

pNGAL concentrations in stable KTR. Importantly, elevated levels of pNGAL were strongly associated with an increased risk of death-censored graft failure. In further analyses, we unravelled that the association between pNGAL and graft failure was driven only by the association in patients with an eGFR <35 mL/min/1.73 m^2, and in patients with proteinuria. The association of pNGAL with all-cause mortality was present in univariable Cox regression models, but lost significance after further adjustment for confounders.

NGAL is freely filtered by the glomerulus and almost completely reabsorbed in the proximal tubules [14]. In addition, NGAL is expressed by renal tubular cells, especially during stress, and is related to morphological changes in patients with renal disease [15, 16]. NGAL is therefore regarded as a marker of kidney injury and plays a role in both acute kidney injury and chronic kidney disease [17, 18]. NGAL in blood and urine has been studied extensively in acute kidney injury and to a lesser extent in KTR [8, 19–21]. NGAL in both

Table 2. Linear regression analysis of log_2 pNGAL

	Univariable		Adjusted for age, sex, creatinine and donor status	
Baseline variables	St. β (95% CI)	P-value	St. β (95% CI)	P-value
Recipient				
Female sex	−0.16 (−0.24 to −0.09)	<0.001	–	–
Age at visit	−0.11 (−0.18 to −0.03)	0.005	–	–
Pre-emptive transplantation	−0.13 (−0.20 to −0.06)	0.001	−0.08 (−0.15 to −0.02)	0.012
Time after transplantation[a]	0.07 (−0.01 to 0.14)	0.078	0.09 (0.03 to 0.15)	0.005
Anti-HLA Class II antibodies	0.12 (0.05 to 0.19)	0.001	0.07 (0.01 to 0.13)	0.015
History of rejection	0.16 (0.09 to 0.24)	<0.001	0.06 (0.00 to 0.12)	0.035
Primary renal disease				
Unknown	Ref.		Ref.	Ref.
Glomerulonephritis	0.09 (−0.01 to 0.20)	0.080	0.04 (−0.04 to 0.12)	0.4
Interstitial nephritis	−0.00 (−0.09 to 0.09)	1.0	0.00 (−0.07 to 0.07)	1.0
Cystic kidney disease	0.03 (−0.07 to 0.13)	0.5	−0.02 (−0.10 to 0.06)	0.7
Other congenital/hereditary disease	−0.03 (−0.11 to 0.06)	0.5	−0.06 (−0.13 to 0.00)	0.055
Renal vascular disease	0.03 (−0.05 to 0.11)	0.5	−0.01 (−0.07 to 0.06)	0.8
Diabetic nephropathy	0.04 (−0.04 to 0.12)	0.3	−0.01 (−0.07 to 0.06)	0.9
Other multisystem disease	−0.02 (−0.11 to 0.06)	0.6	−0.02 (−0.09 to 0.04)	0.5
Other	−0.08 (−0.16 to 0.00)	0.06	−0.06 (−0.12 to −0.00)	0.047
History of cardiovascular disease	0.00 (−0.07 to 0.08)	0.9	0.01 (−0.05 to 0.07)	0.7
Diabetes	−0.04 (−0.11 to 0.04)	0.3	−0.01 (−0.07 to 0.05)	0.8
Body mass index	−0.01 (−0.09 to 0.06)	0.8	−0.03 (−0.09 to 0.02)	0.3
Systolic blood pressure	0.06 (−0.02 to 0.13)	0.12	−0.01 (−0.06 to 0.05)	0.9
Current smoking	0.08 (0.01 to 0.16)	0.032	0.05 (−0.01 to 0.10)	0.13
Kidney transplant characteristics				
Donor age	0.07 (−0.00 to 0.15)	0.054	−0.05 (−0.12 to 0.02)	0.13
Living donor	−0.13 (−0.20 to −0.06)	0.001	–	–
Cold ischaemia time (h)	0.14 (0.05 to 0.22)	0.001	0.09 (−0.03 to 0.21)	0.14
Warm ischaemia time (min)	−0.03 (−0.11 to 0.05)	0.5	−0.06 (−0.12 to 0.01)	0.10
Delayed graft function	−0.00 (−0.08 to 0.08)	0.9	−0.08 (−0.14 to −0.01)	0.023
Laboratory measurements				
Haemoglobin	−0.19 (−0.26 to −0.12)	<0.001	−0.01 (−0.08 to 0.05)	0.7
Sodium	−0.09 (−0.17 to −0.02)	0.015	−0.04 (−0.10 to 0.02)	0.19
Potassium	0.24 (0.17 to 0.31)	<0.001	0.05 (−0.01 to 0.11)	0.11
Creatinine[a]	0.62 (0.56 to 0.68)	<0.001	–	–
eGFR	−0.54 (−0.60 to −0.48)	<0.001	0.01 (−0.20 to 0.23)	0.9
Urea	0.55 (0.48 to 0.61)	<0.001	0.19 (0.09 to 0.29)	<0.001
Leukocytes	0.28 (0.21 to 0.35)	<0.001	0.28 (0.22 to 0.33)	<0.001
hs-CRP[a]	0.21 (0.14 to 0.29)	<0.001	0.18 (0.12 to 0.24)	<0.001
Urinary protein excretion[a]	0.30 (0.23 to 0.37)	<0.001	0.09 (0.03 to 0.15)	0.005
Medication				
Diuretics	0.17 (0.19 to 0.49)	<0.001	0.06 (−0.00 to 0.25)	0.051
RAAS blocker	0.07 (−0.01 to 0.28)	0.067	−0.01 (−0.14 to 0.09)	0.7
Calcium antagonist	0.09 (0.04 to 0.38)	0.018	0.05 (−0.03 to 0.24)	0.11
Beta blockers	0.07 (−0.00 to 0.31)	0.052	0.02 (−0.09 to 0.16)	0.6
Prednisolone	−0.07 (−0.15 to 0.00)	0.051	−0.06 (−0.11 to 0.00)	0.052
Calcineurin inhibitor	0.23 (0.15 to 0.30)	<0.001	0.11 (0.05 to 0.17)	<0.001
Proliferation inhibitor	−0.14 (−0.21 to −0.06)	<0.001	−0.08 (−0.14 to −0.03)	0.005
mTOR inhibitor	−0.06 (−0.13 to 0.02)	0.14	−0.04 (−0.10 to 0.02)	0.2

[a]Variables were log_2 transformed. LDH, lactate dehydrogenase; RAAS, renin-angiotensin-aldosterone system. $^{*}P < 0.050$; $^{**}P < 0.010$; $^{***}P < 0.001$.

blood and urine has prognostic qualities for delayed graft function [8]. However, NGAL in blood or urine early after transplantation has limited added value on top of creatinine and urine output in predicting graft function up to 1 year after transplantation [22–24].

Unfortunately, the value of NGAL measurements in KTR has mainly been assessed with urine or blood taken only hours or days after transplantation, yet the utility of NGAL measurements >1 year after transplantation is incompletely investigated [8]. To the best of our knowledge, the current study is the first to show that in a population of stable KTR 1 year or longer after transplantation, pNGAL concentrations are strongly associated with graft failure. This is interesting, since current clinically used prognostic factors, including eGFR and proteinuria, are mainly related to consequences of graft dysfunction [2, 3]. In contrast, the association between NGAL and graft outcome may reflect underlying pathophysiology of graft dysfunction, rather than its consequences.

The associations of pNGAL with hs-CRP and leukocyte count in our study further confirm the established relation between

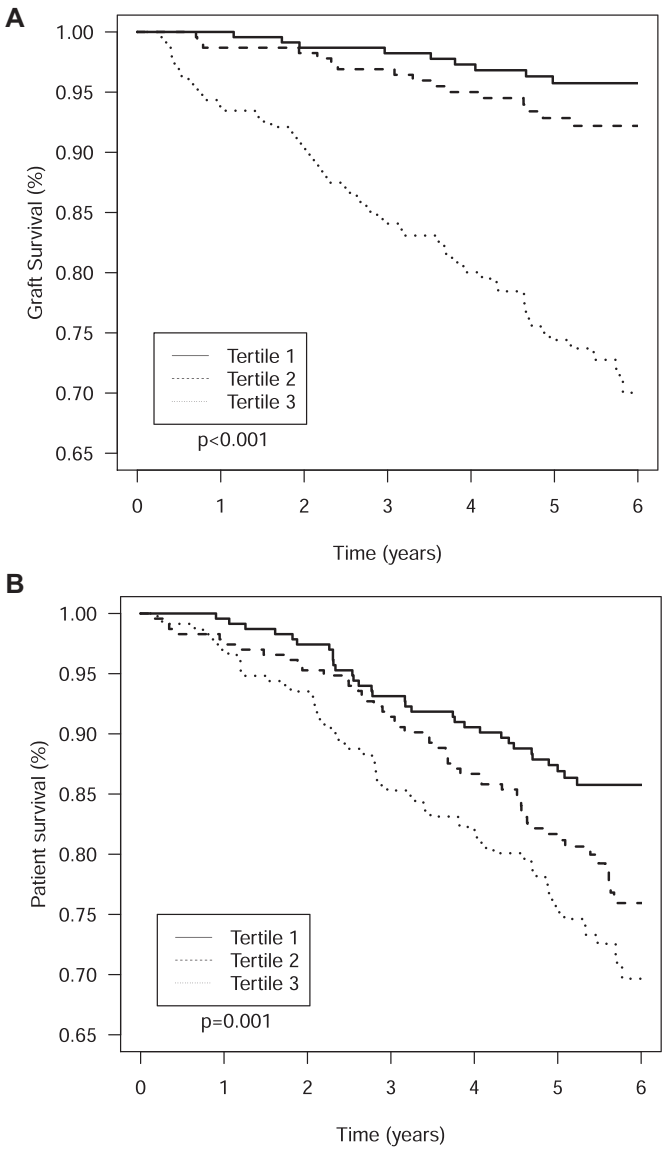

FIGURE 1: Kaplan–Meier analyses for **(A)** death-censored graft survival and **(B)** patient survival across tertiles of pNGAL. P-values represent significance of differences between the groups, as assessed using log-rank tests.

inflammation and NGAL [25]. In addition, our study indicates that NGAL expression may indicate ongoing stress or damage in renal tubular cells, because factors that come with more kidney damage, such as having a deceased donor, longer cold ischaemia time, a longer time after transplantation and proteinuria were all associated with higher pNGAL concentrations. Although we found no consistent association between pNGAL and delayed graft function, these associations add to a previous study showing associations between NGAL and delayed graft function [8]. The relation of kidney damage with NGAL is further reflected by the finding that worse kidney function (e.g. lower eGFR, higher creatinine and urea concentrations in blood) was also associated with higher pNGAL concentrations, which is in line with studies in different study populations [26]. The associations of pNGAL with both inflammation and graft failure that we found in this study therefore raise the hypothesis that ongoing inflammation may contribute to progressive renal damage, which may eventually lead to graft failure [1, 18]. Thus, despite the use of immunosuppressive medication to prevent graft rejection, underlying inflammation may still contribute to graft damage. Interestingly, adjustment for hs-CRP and leukocyte count strengthened the association between pNGAL and graft failure, but strongly reduced the association between pNGAL and mortality. This suggests that hs-CRP and leukocyte count may potentially reflect different aspects of inflammation, compared with NGAL. Where hs-CRP and leukocyte count are regarded as general markers of systemic inflammation, with direct effects on the risk of mortality [27], NGAL may be a more specific marker of intrarenal inflammation, as reflected by the strong association with graft failure. This notion that NGAL reflects local intrarenal inflammation, rather than systemic inflammation, appears biologically plausible, especially since NGAL is abundantly expressed

Table 3. Cox regression analysis of the association of baseline log_2 pNGAL with death-censored graft failure and mortality

	Graft failure		All-cause mortality	
Model	HR per doubling (95% CI)	P-value	HR per doubling (95% CI)	P-value
Univariable	4.16 (3.03–5.71)	<0.001	1.55 (1.21–2.00)	<0.001
Model 1	4.36 (3.13–6.08)	<0.001	1.79 (1.39–2.32)	<0.001
Model 2	1.86 (1.21–2.86)	0.005	1.50 (1.10–2.05)	0.011
Model 3	1.69 (1.10–2.59)	0.017	1.41 (1.02–1.94)	0.037
Model 4	1.76 (1.14–2.73)	0.012	1.40 (1.02–1.93)	0.038
Model 5	1.81 (1.16–2.84)	0.010	1.39 (1.09–1.92)	0.045
Model 6	1.81 (1.15–2.84)	0.011	1.37 (0.99–1.90)	0.054
Model 7	1.80 (1.15–2.83)	0.011	1.37 (0.99–1.89)	0.059
Model 8	1.97 (1.20–3.24)	0.008	1.25 (0.79–1.65)	0.2

Model 1, adjustment for age, sex and time after transplantation. Model 2, adjusted for variables in Model 1 + eGFR. Model 3, adjusted for variables in Model 2 + log_2 24-h urinary protein excretion. Model 4, adjusted for variables in Model 3 + anti-HLA class II antibodies. Model 5, adjusted for variables in Model 4 + pre-emptive transplantation. Model 6, adjusted for variables in Model 5 + rejection treatment (yes versus no). Model 7, adjusted for variables in Model 6 + history of cardiovascular disease. Model 8, adjusted for variables in Model 7 + log_2 leukocyte count and log_2 hs-CRP.

A total of 84 patients (12.0%) encountered death-censored graft failure during a median follow-up time of 5.3 (4.5–6.0) years after baseline, and 150 patients (21.5%) died during a median follow-up time of 5.4 years [4.8–6.1 years] after baseline. Addition of log_2 pNGAL significantly augmented the model of sex, age and time after transplantation, eGFR, log_2 24-h urinary protein excretion, anti-HLA class II antibodies, log_2 leukocyte count and log_2 hs-CRP for graft failure ($P_{likelihood\ ratio} = 0.009$), but not for mortality ($P_{likelihood\ ratio} = 0.14$). Multiple imputation was used to account for two missing values (0.3%) of eGFR, one missing value (0.1%) of urinary protein excretion and 40 (5.7%) missing values of hs-CRP.

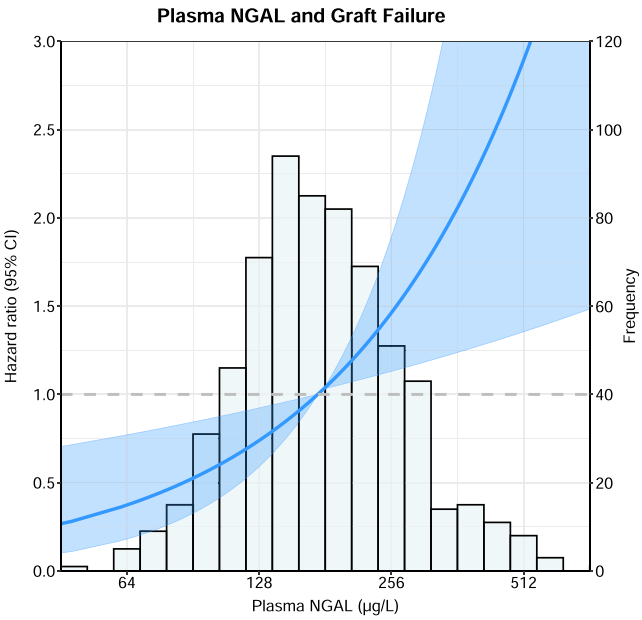

FIGURE 2: Graphical representation of the association between pNGAL and risk of death-censored graft failure, based on a multivariable Cox proportional hazards regression analyses. The model was adjusted for the full model of age, sex, time after transplantation, eGFR, log_2 24-h urinary protein excretion, anti-HLA class II antibodies, pre-emptive transplantation, history of rejection, history of cardiovascular disease, log_2 leukocyte count and log_2 hs-CRP, and presented in relation to the histogram of pNGAL. The blue line represents the hazard ratio and the shaded blue area about the line the associated pointwise 95% CI.

specifically by kidney tubular cells during stress [15, 16], although this notion needs to be further evaluated using more precise methods to evaluate systemic and intrarenal inflammation.

The interactions of eGFR and urinary protein excretion with pNGAL indicate that this ongoing inflammation may be especially detrimental for graft outcome in high-risk patients. Subgroup analyses indeed showed that pNGAL was only strongly associated with graft failure in patients with eGFR <35 mL/min/1.73 m^2, and in patients with proteinuria. pNGAL therefore appears to be of most clinical significance in patients that already have a higher risk of graft failure, as defined by low eGFR and high urinary protein excretion.

Strengths and limitations

This is the first study to show associations of pNGAL with many clinical and biochemical parameters in KTR. Importantly, this study is also the first to show the potential pathophysiological role and clinical value of pNGAL in stable KTR with a functioning graft. Another strength of this study is the large number of study participants and the fact that we were able to adjust for many potential confounders in this well-defined prospective cohort study. A limitation of this study is that the used NGAL assay (and other commercially available assays) cannot distinguish between the monomeric and dimeric forms of NGAL, even though these may reflect distinct information regarding pathophysiological processes or prognosis [30]. Additionally, this was a single-centre study with a mostly Caucasian population, which calls for prudence when extrapolating results to other populations with different ethnicities. In addition, data on kidney graft biopsies may confirm the suggested association of pNGAL with intrarenal inflammation, but such data are lacking in this study. Similarly, the lack of data regarding cytokines and inflammatory cell subpopulations allowed us only to speculate, but not to draw conclusions regarding the role of pNGAL in intrarenal and systemic inflammatory processes. In addition, even though the reported associations are consistent and robust after extensive adjustment for potential confounders, residual confounding (e.g. by kidney function) cannot be excluded, due to the observational design of this study. Also due to its observational design, this study can only describe associations and cannot prove causality. Ideally, the strong association of pNGAL with graft failure should be validated in a future study in an external cohort to confirm clinical value. In such future studies, it may also be attractive to perform similar analyses with urinary NGAL, in addition to pNGAL.

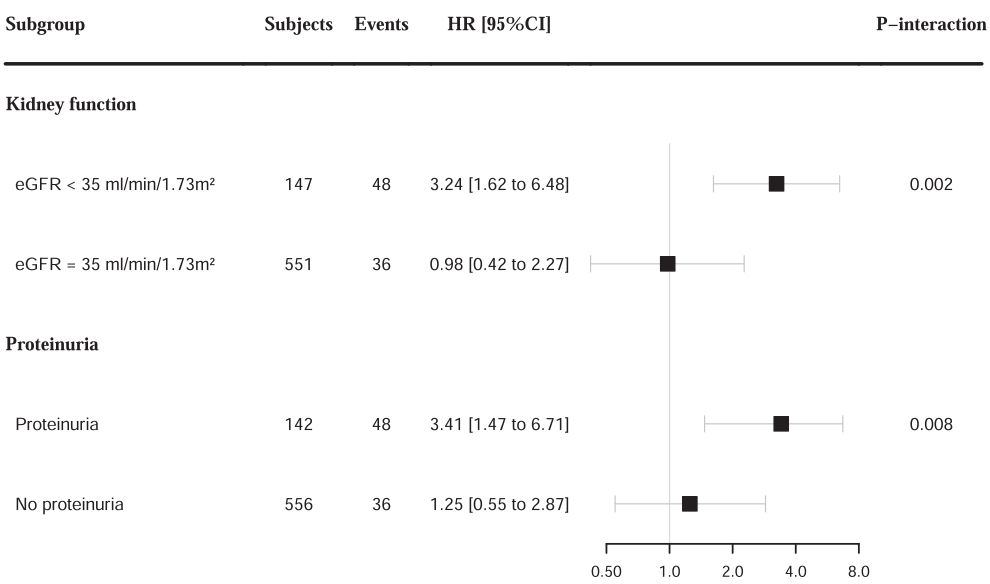

FIGURE 3: Forest plot for the association of pNGAL with death-censored graft failure in subgroups. The model was adjusted for the full model of age, sex, time after transplantation, eGFR, $\log_2$ 24-h urinary protein excretion, anti-HLA class II antibodies, pre-emptive transplantation, history of rejection, history of cardiovascular disease, $\log_2$ leukocyte count and $\log_2$ hs-CRP.

CONCLUSION

Our results highlight the relationship between pNGAL and kidney graft damage in a population of stable kidney transplant recipients. In addition, pNGAL was strongly and independently associated with graft failure, especially in patients with pre-existent poor kidney function and proteinuria.

SUPPLEMENTARY DATA

Supplementary data are available at *ckj* online.

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AUTHORS' CONTRIBUTIONS

D.K. wrote the draft manuscript. D.K., A.P., D.G. and S.K.K. performed data analyses. A.W.G.-N. and M.F.E. performed the research and contributed to data collection, interpretation and manuscript revisions. T.N., C.H. and E.S. provided essential materials and performed laboratory analyses. G.N. and S.J.L.B. were responsible for the research design, and contributed to data interpretation and manuscript revisions. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

T.N., C.H. and E.S. are employed by Gentian, but since the company has discontinued the manufacturing of this kit, they declare no conflicts of interest. The other authors of this manuscript also declare no conflicts of interest. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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