Original Paper

Neutrophil Gelatinase-Associated Lipocalin, but Not Kidney Injury Marker 1, Correlates with Duration of Delayed Graft Function

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Abstract
Background: No specific early biomarker is available to measure kidney injury after kidney transplantation (KT). Both neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury marker 1 (KIM-1) increase after oxidative injury. Their potential as early biomarkers was evaluated in this one-arm pilot study. ***Materials and Methods:*** Twenty consecutive KT patients receiving a kidney from a donation after circulatory death donor were included. Graft perfusate was collected, as well as serum samples before transplantation, at the end of surgery, and 1, 4, and 7 days after transplantation. NGAL and KIM-1 were measured using ELISA. Kidney function and delayed graft function (DGF) were monitored. ***Results:*** In this cohort, 85% of the KT patients developed DGF. Perfusate NGAL correlated with donor age (r^2 = 0.094, p = 0.01) and serum creatinine (r^2 = 0.243, p = 0.05). A cardiac cause of death was associated with higher NGAL in the perfusate (p = 0.03). Serum NGAL at day 1 was significantly higher in patients with DGF (730 ng/ml, range 490–1,655, vs. 417 ng/ml, range 232–481; p = 0.01). Serum NGAL levels at day 1, 4, and 7 correlated with the duration of DGF. KIM-1 was not detectable in the perfusate or in the serum until postoperative day 4 in 80% of patients. ***Conclusions:*** NGAL in the perfusate correlates with known donor risk factors for DGF. For the first time, we describe that serum NGAL at day 1 can discriminate between DGF and immediate graft function. Also, serum NGAL levels at day 1, 4, and 7 correlate with the duration of DGF. No association with KIM-1 was found. These data suggest that NGAL may be used as an early biomarker to detect DGF and warrants further study.

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Introduction

In recent years, there has been a rise in the use of extended criteria kidney donors [including donation after circulatory death (DCD) donors] for kidney transplantation (KT). Grafts from these donors have an increased risk of delayed graft function (DGF). DGF is associated with prolonged hospitalization, acute rejection, and graft loss [1]. DGF is mostly caused by oxidative damage to the graft due to extensive ischemia-reperfusion injury (IRI), which is inevitable in transplantation. In clinical practice, serum creatinine is the most widely used marker to assess graft function. Unfortunately, serum creatinine is a nonspecific biomarker for IRI after KT. Another disadvantage of serum creatinine is that it is a late biomarker [2]. An increase in serum creatinine is observed when kidney damage has already led to deterioration in function. An early and more specific biomarker could be helpful to assess the quality of the graft even before transplantation or to predict the risk of developing DGF. Such a biomarker may create the possibility to discard grafts with a high risk of poor outcome or to accept grafts that would otherwise be discarded.

Both neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury marker 1 (KIM-1) are promising biomarkers to detect oxidative damage early after KT. NGAL is a member of the lipocalin family and was first identified in neutrophils [3], although it is found in most tissues. In epithelial cells, it is synthesized during inflammation [4–6]. In 2003, NGAL was found to be expressed in renal tubular epithelial cells early after ischemia. After kidney damage, NGAL is released in serum and urine [7]; in urine, it can be measured as early as 6 h after transplantation, and in plasma, after 12 h [8, 9]. KIM-1 is a member of the immunoglobulin superfamily molecules that are involved in the regulation of Th-1 and Th-2 cell-mediated immunity. After oxidative kidney damage, KIM-1 is expressed on the surface of damaged kidney epithelial cells, especially in the proximal tubule [10]. KIM-1 is a sensitive biomarker of acute kidney injury (AKI) in both native kidneys and allografts as it is absent in normal kidneys and is expressed at high levels on proximal tubular cells after (oxidative) damage [11]. KIM-1 is mostly measured in urinary samples, although measuring KIM-1 in the serum is feasible [12–15]. In urine, KIM-1 increases between 9 and 18 h after renal IRI [16].

In this study, we measured NGAL and KIM-1 in the perfusate and serum of DCD kidney allografts immediately before and after transplantation and studied their predictive values for early graft outcome. This is the first report in which these biomarkers were measured in the perfusate and related to the risk of developing DGF.

Materials and Methods

Between November 2011 and July 2012, all adult patients who were admitted to the Renal Transplant Unit of the Erasmus MC, University Medical Center Rotterdam, the Netherlands, for a DCD KT were asked to participate in the 'PINK' study, a one-arm, intervention pilot study to assess the feasibility and safety of ischemic postconditioning (Dutch trial registry, No. TC-3117, October 20, 2011). Ethical approval for the study was obtained from the Medical Ethics Committee of the Erasmus MC (No. MEC-2011–067; NL 34987.078.11, version 4, 04/19/2011). The study was monitored by an independent Data Safety Monitoring Board. The clinical results of the 'PINK' study have been published recently [17]. Within this clinical study, we collected perfusate and serum samples to search for biomarkers predictive of DGF and its recovery. The results of these investigations are presented here. Patients gave their written informed consent for ischemic postconditioning and biobanking. All kidneys underwent postconditioning according to a previously described protocol [17]. None of the kidneys had been maintained on machine perfusion before implantation.

The following donor characteristics were collected: age, gender, BMI, first warm ischemia time (WIT), hypotensive episodes, need for cardiopulmonary resuscitation, last measured serum creatinine before procurement, and cause of death. For recipients, age, gender, BMI, time on dialysis, kidney disease, cold

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Table 1. Donor characteristics

Characteristics	Donors (n = 20)
Age, years	57.7 (20–71)
Gender	
Female	8 (40)
Male	12 (60)
Height, cm	176 (144–190)
Weight, kg	74.1 (48–95)
BMI	24.0 (21–27)
Cause of death	
Neurological	10 (50)
Cardiac	4 (20)
Respiratory	4 (20)
Trauma	0 (0)
Not specified	2 (10)
Hypotension	5 (25)
Creatinine[a], µmol/l	79 (40–156)
eGFR, ml/min per 1.73 m^2	100 (39–197)
Storage solution	
HTK	16 (80)
UW	4 (20)
WIT1, min	17 (9–36)
CIT, min	879 (495–1,380)
WIT2, min	27 (16–40)
TIT, min	923 (541–1,415)

Categorical data are expressed as number (%). Continuous data are expressed as mean (range). eGFR = Estimated glomerular filtration rate; HTK = histidine-tryptophane-ketoglutarate; UW = University of Wisconsin; WIT1 = first WIT; WIT2 = second WIT; TIT = total ischemic time.

[a] Last measured prior to circulatory arrest.

ischemia time (CIT), and second WIT were recorded. Incidence of DGF (defined as the need for dialysis within the first week after transplantation), serum creatinine, and estimated glomerular filtration rate in the first 7 days after transplantation were listed.

Perfusate

At arrival, kidneys were inspected during bench surgery just before transplantation. Kidneys were flushed with cold HTK preservation solution (Tramedico International BV, Weesp, The Netherlands) through the renal artery until the effluent was clear. The first 25 ml of perfusate was collected from the renal vein and stored at –80°C.

Serum Samples

Whole blood was collected using Vacutainer Serum Separator Tubes (Becton Dickinson). Samples were collected before transplantation, at the end of transplantation (about 30–45 min after reperfusion), and 1, 4, and 7 days after transplantation. Samples were left to clot at room temperature for at least 30 min and centrifuged for 10 min at 2,000 rpm. Serum was removed and aliquoted and stored at –80°C. Serum creatinine and C-reactive protein (CRP) were measured as part of our routine post-KT protocol.

ELISA Assay

NGAL and KIM-1 were measured in perfusate and serum samples with an ELISA assay kit (R&D Systems, Minneapolis, Minn., USA). For KIM-1, samples were processed according to the manufacturer's instructions. For NGAL, the perfusate and serum were diluted 1:40 and 1:160, respectively. Color intensity was measured with a Wallac 1420 Victor^2 microplate reader (Perkin Elmer, Groningen, The Netherlands) at 450 and 540 nm.

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Table 2. Recipient characteristics

Characteristics	Recipients (n = 20)
Age, years	57.0 (31–75)
Gender	
Female	3 (15)
Male	17 (85)
Height, cm	173 (155–200)
Weight, kg	83.9 (49–118)
BMI	28.0 (19.5–39.5)
Primary kidney disease	
Hypertensive nephropathy	2 (10.0)
Diabetic nephropathy	3 (15.0)
Glomerulonephritis	3 (15.0)
Polycystic kidney disease	5 (25.0)
Reflux/obstructive nephropathy/congenital	1 (5.0)
Unknown	2 (10.0)
Other	4 (20.0)
First transplantation[1]	14 (70)
Retransplantation	5 (25)

Categorical data are expressed as number (%). Continuous data are expressed as mean (range).

[1] One first transplantation was preemptive.

Statistics

Results are expressed as means ± SD unless stated otherwise. Categorical data were compared using the χ^2 test. Correlation analyses were done according to Pearson's correlation coefficient or Spearman's test, whichever was appropriate. Normality was determined with the Shapiro-Wilk test. p values <0.05 were considered significant. All analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corporation, Armonk, N.Y., USA).

Results

Patients

Donor and recipient characteristics are depicted in tables 1 and 2. Eighteen donations resulted in 20 transplantations. Seventeen recipients (85%) experienced DGF. Three recipients (15%) suffered one or more episodes of acute rejection within the first 3 months after transplantation, for which treatment was initiated.

NGAL Levels in the Graft Perfusate

NGAL was detectable in 19 out of 20 perfusate samples (95%) and ranged between 7.39 and 112.10 ng/ml (49.0 ± 31.2). NGAL levels in the perfusate correlated with the last measured serum creatinine in the donor before procurement (r^2 = 0.243, p = 0.05) and with donor age (r^2 = 0.094, p = 0.01). No correlations were found between NGAL levels and WIT (p = 0.88), CIT (p = 0.66), or duration of DGF (p = 0.61). There was no difference in NGAL levels between both sexes (p = 0.11), donors with or without a period of hypotension (p = 0.27), or grafts with DGF and immediate graft function (IGF; p = 0.24). Furthermore, there was no difference between grafts with or without rejection after transplantation (p = 0.89). Cardiac cause of death was associated with a higher value of NGAL (77.4 ± 22.5 vs. 41.9 ± 29.4 ng/ml; p = 0.04).

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Fig. 1. Serum NGAL after KT. Black circles represent serum NGAL levels of recipients suffering from DGF. Grey squares represent recipients with IGF. Preop = Preoperatively.
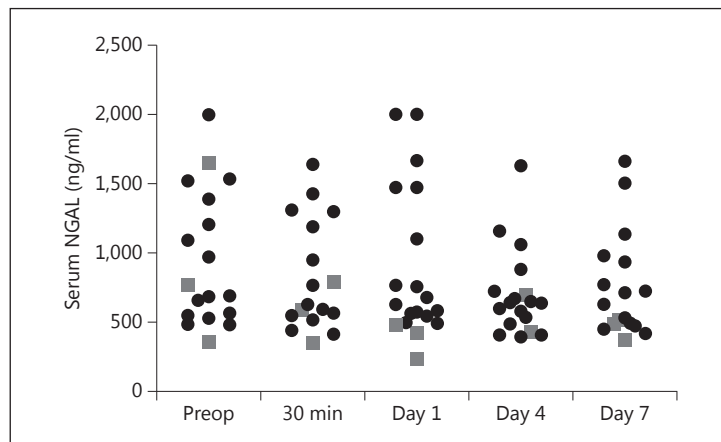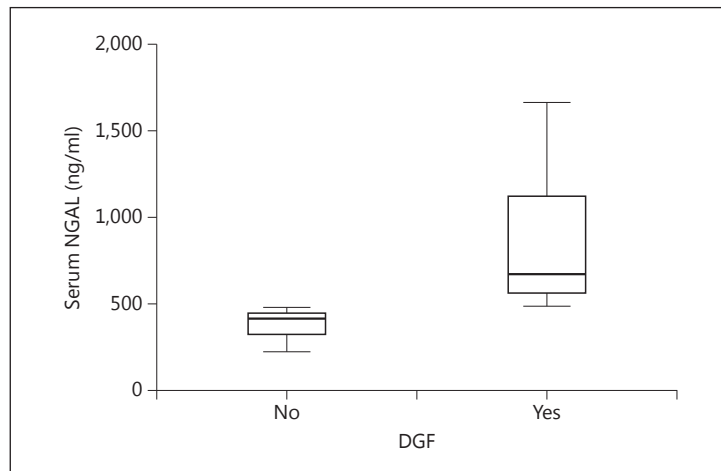

Fig. 2. Serum NGAL 1 day after KT. Serum NGAL levels are significantly higher in patients with DGF.

KIM-1 levels were not detectable in 16 out of 20 samples, with low levels in the remaining 20% of the samples (0.50 ± 0.15 ng/ml; data not shown). No correlations or differences could be found in this small group of KIM-1-positive perfusates.

Serum Measurements of NGAL and KIM-1

Baseline levels of NGAL and KIM-1 were measured in blood samples of recipients before transplantation. NGAL levels ranged from 355 to 1,654 ng/ml (951 ± 489). KIM-1 was detectable in 20% of the samples, and levels ranged from 0.53 to 0.79 ng/ml. NGAL levels did not correlate with CRP. Preoperative NGAL levels were not associated with worse transplantation outcome (DGF, rejection).

Patient Outcome

NGAL levels could be detected at every time point (immediately after KT and at day 1, 4, and 7 after transplantation; fig. 1). At day 1 after transplantation, NGAL levels were significantly higher in recipients developing DGF (range 491–1,666 µmol/l) compared to recipients with IGF (range 232–481 µmol/l, p = 0.008; fig. 2). This discriminative effect of serum NGAL was not seen immediately after KT and at day 4 and 7 after transplantation (p = 0.25, 0.53, and 0.15 respectively). There was no correlation with serum creatinine and serum NGAL, and serum creatinine could not differentiate between DGF (range 365–1,781 µmol/l) and IGF

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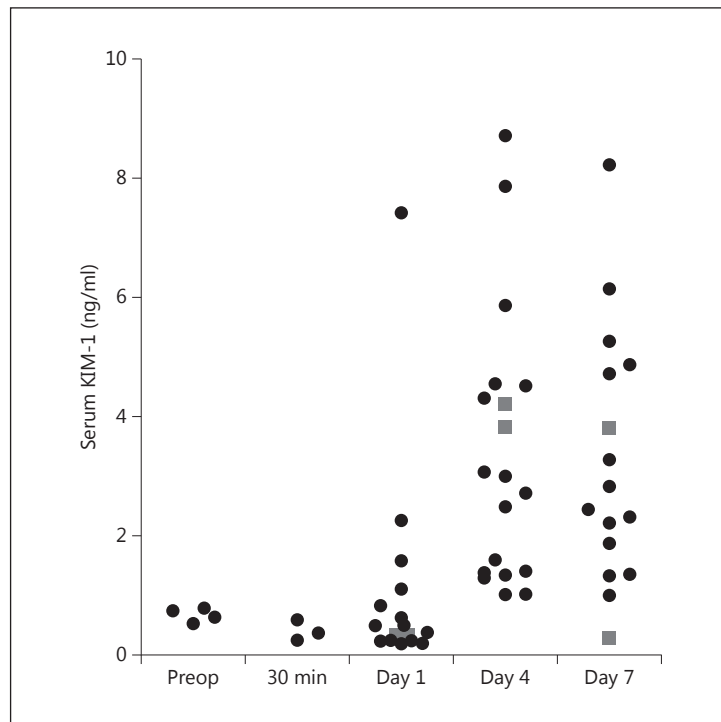
Fig. 3. Serum KIM-1 after KT. Black circles represent serum KIM-1 levels of recipients suffering from DGF. Grey squares represent recipients with IGF. Preop = Preoperatively.

(range 453–694 μmol/l; p = 0.22). The duration of DGF varied from 0 to >84 days (median 8 days). A significant correlation was found with NGAL levels at day 1 after transplantation and duration of DGF (in days; p = 0.004, r^2 = 0.19). This correlation remained significant at day 4 and 7 (p = 0.02, r^2 = 0.28, and p = 0.03, r^2 = 0.27, respectively). NGAL levels could not predict rejection during follow-up (3 months) and were not correlated with postoperative CRP. There was no correlation between NGAL serum levels in the first week after KT and kidney function after 3 months.

KIM-1 levels remained mostly negative at day 1 after transplantation. At day 4 after transplantation, KIM-1 was detectable in 100% of the samples (fig. 3). No differences in KIM-1 levels were seen between patients with DGF and those with IGF. No correlations were found with duration of dialysis or between KIM-1 and NGAL expression at any time point.

Discussion

The aim of the present study was to investigate the value of NGAL and KIM-1 as predictive biomarkers for DGF and transplantation outcome.

Perfusate NGAL
We found that NGAL is detectable in graft perfusate at the end of CIT, with a positive correlation between NGAL and both donor age and last measured serum creatinine, which are both known risk factors for DGF. A cardiac cause of death was associated with higher levels of NGAL in the perfusate as well. Despite the feasibility of measuring NGAL in the perfusate, its relevance remains undetermined. It is interesting that perfusate NGAL levels correlate with risk factors for DGF, but we did not find any predictive value for DGF. However, this is potentially due to the low number of patients. Therefore, studies investigating a larger number of patients are needed.

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The possibility to measure NGAL in the perfusate after first flush during bench surgery immediately after procurement could enable an early quality assessment. Machine preservation certainly paves the way towards more standardized perfusate biomarker research to determine graft quality, since NGAL is detectable in the perfusate during machine preservation. Even more, higher levels of NGAL in the perfusate seem to be correlated with increasing WIT [18]. NGAL could serve as a biomarker to measure the (re-)conditioning of machine perfusion or provide an even earlier quality assessment method; it may direct decision-making as to whether a kidney needs longer reconditioning using machine perfusion or whether it can be transplanted after the standard machine perfusion. Whether NGAL levels are associated with outcome remains to be elucidated.

Serum NGAL

To our knowledge, our study reports the highest levels of serum NGAL found in recipients to date [19]. Elevated serum NGAL levels are associated with a variety of kidney diseases [20–22], progressive kidney disease [23–25], and various comorbidities, although evidence is limited [26–28]. Furthermore, it is speculated that dialysis causes an inflammatory state, resulting in high NGAL levels [19]. Nevertheless, we did not find correlations between morbidity, CRP, and NGAL levels. Thus, the relevance of this finding is not clear and the cause remains to be elucidated. Still, these high preoperative NGAL levels are not associated with DGF or rejection.

After KT, serum NGAL levels were significantly lower in recipients with IGF. Although the number of patients was low, and the incidence of DGF was high (85%), NGAL levels could differentiate between DGF and IGF on day 1, when no difference was seen in serum creatinine. These data strongly suggest that NGAL is an early predictor of DGF. Furthermore, NGAL levels of recipients with IGF were all below 490 ng/ml. Thus, there may be a cutoff value that distinguishes between DGF and IGF. This predictive value of NGAL can be seen as early as 12 h after transplantation [9, 29, 30]. Our first post-KT serum samples were taken at the end of the transplantation procedure, approximately 30–45 min after reperfusion. At this point, NGAL was not able to predict the outcome. To validate the findings of this study, it must be repeated with more patients and earlier measurements of NGAL.

Serum NGAL levels after KT, and not serum creatinine levels, correlated with the duration of DGF, expressed as the number of days patients received dialysis. At 1, 4, and 7 days after transplantation, this correlation was seen. We could not find predictive values for discontinuing dialysis.

NGAL correlated with the duration of DGF and therefore may aid in the decision process of whether or not to continue dialysis after transplantation, as it reflects the level of kidney injury. However, the requirement for dialysis is also based on clinical assessment. NGAL levels in the perfusate and preoperative serum NGAL levels in recipients did not predict DGF, which confirms earlier findings in previous research showing that serum NGAL levels in both living and deceased donor KT fail to predict DGF in recipients [30, 31].

Serum KIM-1

Although KIM-1 is considered as an early and specific biomarker for kidney damage, our results show that elevation of KIM-1 serum levels is delayed by days compared to NGAL. No predictive value for DGF and no correlations with renal function were found. One explanation could be that we measured KIM-1 in serum and not in urine. Serum KIM-1 is still being studied as a potential biomarker of AKI; results so far have been ambivalent. Sabbisetti et al. [14] found increased serum KIM-1 levels in patients with AKI after cardiac surgery. Tekce et al. [15] showed a significant increase in urinary KIM-1 one day after cisplatin treatment in patients developing AKI, with an AUC of 0.94. However, serum KIM-1 was not significantly

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altered. Urinary KIM-1 is well studied and shown to be increased 2 h after an ischemic insult [32]. Although we collected urinary samples after transplantation, both of the transplanted kidney (via a splint) and the native kidneys (via a bladder catheter), we ended up with very few samples due to little or no (rest) diuresis caused by end-stage renal disease. Furthermore, 85% of recipients suffered from DGF, with little or no urine production from the transplanted kidney. Thus, although KIM-1 was not detectable in our early serum samples, it remains undetermined if these kidneys produced or excreted KIM-1. Another possibility is that KIM-1 is not a marker for damage, but for regeneration after IRI. It makes it difficult though to implement KIM-1 as an early marker in these extended criteria kidney donor KTs, where high DGF rates are to be expected.

Study Limitations

The sample size of this study was small, especially in the group with IGF. For that matter, additional experiments are needed including larger patient numbers. Another shortcoming of this study is the diversity of the perfusate product. The quantity of biomaterials in the perfusate (from bloody to very clear) depends on the procurement procedure and the amount of preservation fluid used for the initial washout during cold perfusion, as well as on the final flush of the graft after procurement, just before cold storage. We processed the perfusate samples in a standardized manner, but first flush after procurement was done in another center. Thus, we cannot state that every kidney graft was flushed similarly. Additional research with standardized samples of the perfusate is needed to determine whether NGAL can be used to measure graft viability and quality in the perfusate. Although we planned in the initial design of this study to measure NGAL and KIM-1 in urine samples, very low numbers of urine samples were collected, due to low diuresis. Furthermore, it must be noted that all kidneys had undergone ischemic postconditioning during KT as part of a single-arm pilot study [13]. Whether or not this has affected our data remains uncertain.

In conclusion, the present study demonstrated the feasibility to measure NGAL in the perfusate at the end of CIT. Furthermore, NGAL levels correlate with known donor risk factors for DGF. More research under standardized conditions needs to be done to verify the potential of perfusate NGAL levels as a graft quality marker. NGAL serum levels on posttransplant day 1 strongly predict DGF and correlate with the duration of DGF, which could serve as a biomarker for dialysis withdrawal. This predictive value and correlation was not seen in serum creatinine, which is still used as the gold standard for measuring kidney damage after KT. Therefore, we hypothesize that NGAL has potential as a biomarker for early transplant outcome. This needs to be confirmed in subsequent larger studies.

Disclosure Statement

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