Artificial Organs

Review Article

Neutrophil Gelatinase-Associated Lipocalin as a Biomarker of Allograft Function After Renal Transplantation: Evaluation of the Current Status and Future Insights

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Abstract: Neutrophil gelatinase-associated lipocalin (NGAL), a protein belonging to the lipocalin superfamily initially found in activated neutrophils, is expressed by several cell types, including kidney tubule. The increase in NGAL production and release from tubular cells in response to various insults has been proven to predict acute kidney injury (AKI). For this reason, it has emerged as a valuable noninvasive biomarker of AKI in clinical nephrology. Also in the renal transplant setting, different studies have indicated NGAL as a valuable tool, especially in the early postoperative period, since the currently available clinical and laboratory parameters remain poorly sensitive to monitor immediate posttransplant graft function. This is an analysis of the recent literature to assess the utility of plasma and urinary NGAL, exosomal mRNA for NGAL, and NGAL levels in the perfusate of machine-perfused kidneys for the prediction of graft function recovery in the early postsurgery phase after renal transplantation. We found that NGAL appears as a promising troponin-like biomarker to detect short-term impairment of graft function after renal transplant, but there are still some limitations in its clinical application, essentially related to its low specificity. Moreover, comparing NGAL assayed in serum, urine, machine-perfusate, or as exosomal mRNA, each one has shown limitations and benefits in terms of predictive performance for DGF, according to various existing studies, feasibly due to different cut-off levels, designs and patient sample sizes. **Key Words:** Biomarker—Neutrophil gelatinase-associated lipocalin—Kidney transplantation—Plasma NGAL—Urinary NGAL.

The necessity of early biomarkers in acute kidney injury and renal allograft outcomes

Acute kidney injury (AKI), is a common problem in critically ill patients, and is defined as the abrupt (e.g., within 48 h) and sustained decrease in renal function. In current clinical practice, the diagnosis and classification of AKI stages relies on serum creatinine, glomerular filtration rate (GFR), and urine output (1).

However, there are some major limitations to the use of creatinine, since it is an unreliable and delayed indicator of the deterioration of kidney function. To overcome these difficulties, an extensive search for more suitable and timely laboratory markers monitoring impaired renal function is required.

In renal transplant recipients, the need of noninvasive and early biomarkers to detect delayed graft function (DGF), defined as the need for dialysis during the first posttransplant week, is of paramount importance in current research.

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The large interindividual differences in clinical outcomes immediately after renal transplantation can range from early recovery observed after living donation to slow or delayed recovery of graft function, and primary allograft failure in the worst cases (2). DGF can be viewed as a form of AKI following kidney transplantation, and has been associated with 40% higher risk of graft loss at one year posttransplant (3), increased susceptibility to acute and chronic rejection and poorer long-term outcomes (4,5). However DGF diagnosis can be complicated because there are several definitions based on a variety of clinical parameters (6). Although the use of dialysis in the first postoperative week is the most widely adopted to define DGF in both clinical practice and scientific literature, it is important to underline that this criterion might be misleading in those cases when a single postoperative dialysis is performed for the management of hyperkalemia, volume overload or for the safe administration of blood products, or when dialysis is avoided due to a good urine output from the native kidney (7).

Moreover, clinicians have still to deal with the poor performance of serum creatinine and creatinine-based equations to estimate GFR and to predict graft and patient survival in kidney transplant recipients (8,9). The main limitations with use of serum creatinine in the very early posttransplant phases are related to the effect of dialysis sessions immediately prior to or after surgery, or native urine output.

The Cockcroft-Gault formula (10) and the Modification of Diet in Renal Disease (MDRD) equation (11) are the most widely used to assess kidney function also in the transplant setting, but with some well-known limitations, particularly in the elderly patients (<65 years) and those with extreme body mass indexes (BMIs).

In addition, an important point to consider is that the applicability of these formulas in renal transplant recipients can be reduced by some factors affecting serum creatinine levels: possible changes in muscle mass due to steroid treatment, enhanced creatinine catabolism triggered by opportunistic infections, and the effect of certain drugs such as cimetidine, trimethoprim, pyrimethamine, phenacemide, salicylates, corticosteroids, and active vitamin D metabolites, able to determine a rise in serum creatinine without influencing glomerular filtration (12).

Role of NGAL in renal and nonrenal clinical settings

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin 2, uterocalin, siderocalin, or oncogene 24p3, was initially isolated from the supernatant of human activated neutrophils (13).

In year 2000, a young graduate student David Goetz at the University of California in San Francisco, under the supervision of Professor Roland Strong, first described the three-dimensional structure of NGAL, revealing a high sequence similarity to a protein superfamily called lipocalins. Professor Strong defined lipocalins as "small proteins that cells send out to bind things and carry them back" (14). Successive studies proved the ability of NGAL to bind with high affinity bacterial siderophores or endogenous compounds in mammals (15,16), its key role in iron transport into cells, and iron-mediated downstream cellular responses (17,18). Afterwards, NGAL has been implicated in several pathways, including bacteriostasis, control of apoptosis, and induction of renal tubule proliferation, a possible mechanism of NGAL-mediated renal protection during AKI (19,20). NGAL expression has been reported in different human cell types, including kidney tubular cells, in response to various insults, highlighting the multifaceted role of NGAL in both renal and nonrenal clinical settings (21–29).

Within this framework, NGAL has also emerged, among the many candidate molecules, as a promising early predictor of AKI. Thus, NGAL has been regarded as the "troponin of the kidney" (30,31).

In renal transplant settings, different studies have indicated NGAL as a valuable tool to monitor allograft function, especially in the early postoperative period.

NGAL is involved in cellular immunity, for its ability to induce immune tolerance by upregulating HLA-G expression and expansion of T-regulatory cells in healthy donors, providing the basis for further studies to evaluate its possible role in immunomodulation and tolerance induction in transplant recipients (32).

A cornerstone in the field of kidney transplant research was laid by Mishra et al. (25) who used immunochemical staining of protocol biopsy specimens from renal allografts obtained at approximately one hour of reperfusion after surgery to demonstrate a correlation of increased NGAL expression with prolonged cold ischemia time, elevated serum creatinine levels, and DGF. These findings suggested that enhanced local production of NGAL by the tubular epithelium of DGF allografts results in increased plasma and urine NGAL levels, as a consequence of the ischemia/reperfusion stress applied to the transplanted kidney before organ withdrawal, during the ischemic storage and successive reperfusion. However, there are

different mechanisms underlying the rise of NGAL in urine and plasma. The main fraction of urinary NGAL (uNGAL) during AKI is likely to be related to an impaired reabsorption of the filtered NGAL by the proximal tubule together with an increased local synthesis by the distal nephron. Conversely, it is known that the injured kidney is not the main source of plasma NGAL (pNGAL), but increased NGAL mRNA expression by other distant organs, mainly liver and lungs, gives the most substantial contribution to NGAL plasma pool (31).

Urinary NGAL and DGF

A large proportion of current research is focused on the urine medium, since it represents an ideal model to reflect the molecular constitution of the transplanted organ. However, the changes in urine levels of a given molecule might result from different underlying mechanisms, namely passive or active release, filtration across the glomerular basement membrane, and resorption or catabolism.

The main utility of uNGAL as a biomarker for predicting kidney injury in the early posttransplant period is its potential applicability for a timely detection of kidney injury, since NGAL rise occurs rapidly and is detectable within a few hours after the initial insult, anticipating by several hours the rise in serum creatinine. It has been reported that kidney epithelia express and excrete large quantities of NGAL into urine following acute injury, reaching up to 1000-fold induction of NGAL mRNA and protein in the most severe cases (33). There is a large body of literature to indicate that uNGAL increases during the first posttransplant week in renal transplant recipients with DGF, especially in the very early urine samples collected within six h postsurgery (34–36). Thus, the main potential advantage arising from this finding is the possibility to identify and stratify patients according to their risk of dialysis need after transplant, prior to the diagnosis of DGF. Most of the studies, including one from our group, concur to suggest that patients with higher uNGAL values in the early posttransplant phases are more prone to develop DGF and tend to maintain increased uNGAL levels, or even experience a further rise in the following days, different from patients with prompt function (25,34,37,38). However, contrasting results by Hollmen et al. (39), even if the higher initial uNGAL levels in DGF patients is confirmed, it showed a rapid decline on the following day, similar to transplant recipients with immediate recovery of graft function.

Plasma NGAL and DGF

The prognostic value of pNGAL after renal transplantation has been also extensively investigated. Recently, Pezeshgi et al. (40) reported that pNGAL, particularly 12 h after kidney transplant, appears to be a very sensitive and specific biomarker for predicting AKI. Comparing the changes in serum creatinine measured daily within the first week after transplant with pNGAL levels immediately before and at 6 and 12 h postsurgery, the authors found that pNGAL at 12 h was the most reliable predictor of AKI and graft rejection (sensitivity: 100%; specificity: 92%; cut-off value: 309 ng/mL), far better than the prognostic accuracy of corresponding serum creatinine (sensitivity: 66.7%; specificity: 61.9%).

The role of pNGAL as an early and accurate indicator of DGF and tacrolimus (Tac) toxicity and as a mediator of tissue regeneration in kidney transplant recipients from marginal donors was investigated by Cantaluppi et al. (41) The data confirmed previous evidence on the predictive value of plasma levels of NGAL in DGF group. Moreover in patients with no DGF, NGAL was able to discriminate between slow or immediate graft function. The rise in NGAL plasma concentration following Tac introduction seems to indicate a further role as marker of drug toxicity.

Which one between uNGAL or pNGAL might represent the best biomarker for graft outcome remains an open issue. In Table 1, we have summarized the main published studies performed in renal transplant recipients, where the predictivity of uNGAL and pNGAL in terms of cut-off levels, sensitivity and specificity were available (34,36,38,40–52).

NGAL as a perfusion marker in kidney preserved by hypothermic machine perfusion

NGAL has been also evaluated as a hypothermic machine perfusion biomarker for assessing organ quality in deceased donor kidney transplantation (53–55).

A first pilot ex vivo animal experiment was carried out by Jochmans at al. to evaluate the performance of biomarkers AST, H-FABP, and NGAL in the perfusates of 6 porcine kidneys exposed to incremental intervals of warm ischemia prior to a 22-h machine perfusion. The results reveled that all the selected biomarkers were detectable in the cold acellular perfusate and their release was in proportion to the degree of warm injury. In particular, NGAL increase in perfusate was directly related to

TABLE 1. *Literature review on predictivity of urinary NGAL and plasma NGAL on delayed graft function in renal transplant recipients*

Marker	Number of patients	Cut-off level	Sensitivity (%)	Specificity (%)	Author (Ref.)
uNGAL	176 KTR	560 ng/mL (day 1)	68	73	Hollmen (39)
uNGAL	124 KTR	97 ng/mL (day 1)	71.8	100	Lacquaniti (42)
		105 ng/mL (day 1)	95.8	91.9	
uNGAL	123 KTR	521.7 ng/mL (4 h)	80	68.7	Cui (43)
		559.2 ng/mL (12 h)	80	68.7	
		688.3 ng/mL (24 h)	70	93.7	
		295.2 ng/mL (48 h)	80	96.9	
		297.4 ng/mL (72 h)	80	100	
uNGAL	91 KTR	45 ng/mL (day 1)	97	26	Hall (36)
		350 ng/mL (day 1)	77	74	
		800 ng/mL (day 1)	65	94	
uNGAL	79 KTR	>120 ng/mL (48 h)	75	71	Nieto-Ríos (44)
uNGAL	71 KTR	>33.1 μg/mmol sCr (24 h)	68	93	Pajek (45)
uNGAL	69 KTR	188.4 ng/mL (day 2)	64	8	Choi (46)
uNGAL	53 KTR (23 living donor, 30 deceased donor)	1000 ng/mg sCr (day 0)	90	83	Parikh (38)
uNGAL	40 KTR	479 ng/mL (3–6 h)	77	88	Fonseca (34)
		286 ng/mL (8–12 h)	100	76	
		277 ng/mL (day 2)	93	90	
		232 ng/mL (day 4)	93	95	
		63 ng/mL (day 7)	94	84	
uNGAL	38 KTR	128 ng/mL (day 1)	85.7	61.5	Kanter (47)
		124 ng/mL (day 3)	80	83	
pNGAL	176 KTR	423 ng/mL (day 1)	87	77	Hollmen (48)
pNGAL	67 KTR (39 living related, 1 brain dead, 27 postcardiac death donors)	500 ng/ml (day 1)	91	97	Kusaka (49)
		350 ng/ml (day 2)	86	90	
		300 ng/ml (day 3)	91	93	
pNGAL	59 KTR	233.3 ng/mL (day 1)	76.6	77.8	Lee (50)
pNGAL	50 KTR patients from ECD	532 ng/mL (day 1)	90.9	80.6	Cantaluppi (41)
pNGAL	41 KTR	>400 ng/mL (12 h)	93.3	88.5	Bataille (51)
pNGAL	37 KTR	309 ng/mL (12 h)	100	92	Pezeshgi (40)
pNGAL	27 KTR	174 ng/mL (day 1)	100	95.5	Rahimzadeh (52)

For each study, the optimal cut off levels achieving the best combination of sensitivity and specificity are reported.
ECD, extended criteria donors; KTR, kidney transplant recipients; pNGAL, plasma NGAL; sCr, serum creatinine; uNGAL, urinary NGAL.

the extent of graft ischemic damage, independent of neutrophil activation (53).

Successive clinical studies on patients who received a kidney from a donation after circulatory death donor showed that perfusate NGAL, but not kidney injury molecule 1 (KIM-1), correlated with some well-established donor risk factors for DGF, specifically donor age, serum creatinine, and cardiac cause of death (54).

Recently, a large multicenter cohort study by Parikh et al. investigated prospectively the associations of NGAL, KIM-1, interleukin-18 (IL-18) and liver-type fatty acid-binding protein (L-FABP) and pump parameters (resistance and flow) with DGF and estimated GFR (eGFR) at six months after kidney transplant. The results proved a release of all kidney injury biomarkers into perfusate and a rise over time in their concentration. However, one-hour flow was found to be associated with DGF, but no other independent correlations between the injury biomarkers and DGF were observed. In spite of the poor predictivity of the selected perfusate biomarkers for short-term graft outcomes, perfusate NGAL and L-FABP measured near the end of machine perfusion as well as pump parameters (resistance and flow) were modestly associated with six-month eGFR. Although the study found lacking or weak prognostic utility of the most known biomarkers of ischemia-reperfusion injury in the perfusate, additional candidate molecules involved in other pathways might deserve further research, especially in view of the growing organ shortage and the critical issue relative to kidney allocation acceptance or refusal decisions (55).

Urinary exosomal mRNA for NGAL and kidney transplant

The usefulness of NGAL in predicting AKI could be limited by its poor specificity, as several nonrenal diseases can also induce NGAL. In the last few years, mRNA extracted from urinary

exosomes has been proposed as a better source to identify novel biomarkers of kidney injury. Exosomes are small membrane-bound 50–130 nm diameter vesicles released into the urine from the kidney epithelium and their molecular composition feasibly mirrors the physiological or pathological status of the kidney. Urinary exosomes have acquired growing importance to predict DGF after renal transplantation, since they have proven to express increased levels of NGAL than the cellular fraction in DGF patients compared to those with an immediate recovery of their graft function.

Studies on mRNA expression in urinary exosomes of NGAL, IL-18, KIM-1, and cystatin C revealed that, while the concentrations of all the corresponding urinary proteins increase at 24 and 168 hours after kidney transplantation and correlate with the day 7 creatinine reduction ratio (CRR), exosomal mRNA for NGAL, IL-18, and cystatin C show no association with the day 7 CRR, or urinary biomarker concentrations at any time after transplantation. These results might indicate that, even if mRNA for these biomarkers is detectable in urinary exosomes, their levels do not seem to reproduce or predict urinary protein levels or the CRR. A possible explanation might lie in the fact that the incorporation of mRNA into exosomes is a selective process, not necessarily representative of mRNA in the parent cells responsible for biomarker production (56).

NGAL and other candidate biomarkers in kidney transplant setting: benefits and limitations

In renal transplant settings, the ideal biomarker with a noninvasive, safe and low-cost measurement, and able to reflect allograft injury with 100% sensitivity and 100% specificity has not been identified yet. Whether urinary or plasma biomarkers are more reliable predictors of graft outcome is also matter of debate.

In the past years, besides NGAL, several AKI and DGF biomarkers have been extensively investigated, including urinary KIM-1, IL-18, heat shock protein 72 (uHsp72), L-FABP, calprotectin, CXCL9, CXCL10, CCL2, IL-18, cystatin C, T-cell immunoglobulin, and mucin domain-3 (Tim-3), tissue inhibitor of metalloproteinase 2 (TIMP-2), insulin-like growth factor-binding protein 7 (IGFBP-7) (57–63).

However, every single molecule has advantages and limitations. At present, none of the studied biomarkers is being employed worldwide for diagnostic use in the routine clinical practice, with some local exceptions: NGAL, approved by the CE (Conformité Européene) and currently pending Food and Drug Administration (FDA) approval in the USA, L-FABP in Japan, a combination of TIMP-2 and IGFBP-7 in some jurisdictions of the USA (63,64). Moreover, urinary KIM-1, albumin, clusterin, trefoil factor-3 (TFF3), total proteins, cystatin C, ß2-microglobulin have been approved by the US FDA, European Medicines Agency and Pharmaceuticals and Medical Devices Agency for preclinical drug development in acute rodent toxicity models (65).

There are currently three CE-marked and launched tests for diagnostic use in Europe for a timely (10 to 35 min) determination of NGAL in blood or urines. The Triage assay (Alere Triage NGAL test, Alere Inc., San Diego, CA, USA) is a blood point-of-care immunoassay, the ARCHITECT (ARCHITECT analyzer, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL, USA) is a chemiluminescent microparticle immunoassay for the quantitative determination of NGAL in urine, and the NGAL Rapid ELISA Kit 037CE (BioPorto Diagnostics A/S, Gentofte, Denmark) is a particle-enhanced turbidimetric immunoassay for the measurement of both urine and blood NGAL.

Based on manufacturers' and literature information the NGAL assay is more expensive (ranging from £24 to £27 per test) than assaying serum creatinine alone by the Jaffe method (around £2/test). The average cost per test for NGAL is estimated according to the prices of the individual components (NGAL Test Reagent kit: £1770; calibrator kit: £213; control kit: £417), to be used for about 100 patients (66).

In a nutshell, even if NGAL is the most studied and seems to emerge as an intriguing troponin-like biomarker in the plasma and urine to assess DGF risk after renal transplant, there are still some limitations mainly related to its poor specificity.

CONCLUSIONS

At the moment, it is not possible to draw any firm conclusion about the best predictive performance for delayed graft function between NGAL elevation in serum or urine. Considering that pNGAL levels result from release into circulation by organs other than the kidney, theoretically uNGAL might be expected to be more specific and representative of kidney injury than sNGAL. However, beside the obvious inapplicability of uNGAL in case of anuric patients, this concept is not fully

corroborated by the currently available literature data (Table 1), showing a large variability of sensitivity and specificity for sNGAL versus uNGAL, maybe related to the different cut-off levels, study designs and patient sample sizes.

So, the challenge is currently open for ongoing biomarker discovery studies in the fields of proteomics and metabolomics, aimed at the identification of patterns of reliable markers rather than a single standalone molecule.

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