NGAL (Lcn2) monomer is associated with tubulointerstitial damage in chronic kidney disease

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The type and the extent of tissue damage inform the prognosis of chronic kidney disease (CKD), but kidney biopsy is not a routine test. Urinary tests that correlate with specific histological findings might serve as surrogates for the kidney biopsy. We used immunoblots and ARCHITECT-NGAL assays to define the immunoreactivity of urinary neutrophil gelatinase–associated lipocalin (NGAL) in CKD, and we used mass spectroscopy to identify associated proteins. We analyzed kidney biopsies to determine whether specific pathological characteristics associated with the monomeric NGAL species. Advanced CKD urine contained the NGAL monomer as well as novel complexes of NGAL. When these species were separated, we found a significant correlation between the NGAL monomer and glomerular filtration rate ($r = -0.53$, $P < 0.001$), interstitial fibrosis (mild vs. severe disease; mean 54 vs. 167 μg uNGAL/g Cr, $P < 0.01$), and tubular atrophy (mild vs. severe disease; mean 54 vs. 164 μg uNGAL/g Cr, $P < 0.01$). Monospecific assays of the NGAL monomer demonstrated a correlation with histology that typifies progressive, severe CKD.

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Over the next decade, the number of patients reaching end stage renal disease (ESRD) will double as a result of the progression of chronic diseases of the kidney (CKD).[1] The rate of progression however is difficult to predict by currently available tests. Serum creatinine measurements are confounded by age, muscle mass, gender, race, medications, hydration status, and extrarenal clearance.[2] Proteinuria is a critical cause of disease progression, yet progression may occur even in its absence.[3] Tubulointerstitial disease perhaps best presages worsening CKD,[3] however, kidney biopsy is not practical for routine use and generally does not sample medullary tubules. Here, we evaluated relationships between a urinary protein and biopsy findings in a CKD cohort.

Neutrophil gelatinase–associated lipocalin (NGAL) gene product (Lcn2, Siderocalin) is a 'monomeric' protein (23–26 kDa)[4] that is induced by the triggers of acute kidney injury (AKI).[4–6] In contrast, NGAL is only marginally increased during periods of slowly progressive CKD,[4,7]whereas progressive disease,[8] advancing HIVAN,[9] diabetes,[10] and end-stage[11–13] upregulate NGAL in the absence of an acute event. It is important to determine whether a specific molecular form of urinary NGAL identifies a specific histological characteristic, implying not only that it may serve as a surrogate marker, but also that it participates in a pathway modulating the growth of damaged tubules[12,14] and defense of the urinary tract by scavenging iron.[15]

RESULTS

High-molecular weight NGAL species identified with cation-exchange and gel filtration

To determine the molecular form of NGAL, we analyzed the urine of 99 patients by immunoblot and found a recurrent pattern of immunoreactivities. The majority of immunoreactive NGAL was 'monomeric', but additional reactivities were found at >250, 125, 75 kDa in non-reducing gels. The

75-kDa species was potentially trimeric NGAL, and the 125-kDa species was potentially NGAL-MMP-9[16] but the >250-kDa immunoreactive band was a novel complex. To identify this protein, we fractionated the urine of a stage 5 patient using cation-exchange and gel filtration. Mass methodology identified the >250-kDa complex as the secretory component of the polymeric immunoglobulin receptor (peptides: LVSLTLNLVTR; ILLNPQDKDGSFSVVITGLR; and QGHFYGETAAVYVAVEER) together with α2-macroglobulin. Upon reduction, monomeric NGAL dissociated from the complex (Figure 1).

As the monomeric form of NGAL has been specifically associated with epithelial stress,[4,5] we decided to quantify this species with ARCHITECT-NGAL (Abbott) assays,[7] which correlated with the detection of the monomer by immunoblots ($r=0.69$, $P<0.001$), demonstrating the same rank order and producing nearly identical statistical differences (analysis of variance) across different diagnoses (<0.05, ARCHITECT-NGAL and <0.01, immunoblot). The ARCHITECT-NGAL demonstrated 3.8% (s.d. 7.7%) of total immunoreactivity was >100 kDa, but with variable contribution (0–41.9%).

Correlations between NGAL levels and histological diagnoses

Accurate quantification of the monomer permitted a comparison with clinical and pathological characteristics at the time of the biopsy (Supplementary Table S1 online). Rapidly progressive glomerulonephritis ($n=3$), dialysis ($n=10$), and AKI were excluded at the time of enrollment. Six percent of our cohort had been on steroids before biopsy. On an average, the cohort was male (70%), age 52.2 years (s.d. 16.8) with a glomerular filtration rate (GFR) 57.7 ml/min (s.d. 34.5); hypertension was common (69.7%, mean 138.7 mm Hg s.d. 22.0/81.9 mm Hg s.d. 11.3) but diabetes was present in only 18.2%. Pathological diagnoses were diverse: nephrotic syndromes (49.5%: membranous, focal segmental glomerulosclerosis, minimal change disease, and amyloidosis), nephritic syndromes (31%: immunoglobulin (Ig)A and lupus nephropathies, membranoproliferative and mesangial proliferative glomerulonephritides, and fibrillary and immunotactoid nephritis), diabetic nephropathy (10%), and other diagnoses (9%: nephroangiosclerosis, ESRD, light-chain nephritis, myeloma, and isolated chronic tubulointerstitial nephritis).

Although the monomer appeared most elevated in diabetics (Supplementary Table S1 online), statistical significance was demonstrated with measures of disease chronicity, rather than specific diagnoses. For example, the monomer correlated inversely with GFR ($r=-0.53$, $P<0.001$) and directly with the chronicity index ($P=0.003$) as reflected by a dose-dependent relationship (Figure 2). Additionally, the monomer specifically associated with both tubular atrophy ($P=0.002$) and interstitial fibrosis ($P=0.006$). Moderate or severe fibrosis or atrophy were associated with three- to four-fold higher levels of NGAL monomer (each $P<0.01$) compared with mild or absent fibrosis or atrophy (Table 1, Figure 3). Similarly, fibrous crescents ($P=0.001$) and global glomerulosclerosis ($P=0.037$) were highly correlated with the monomer, both suggestive of

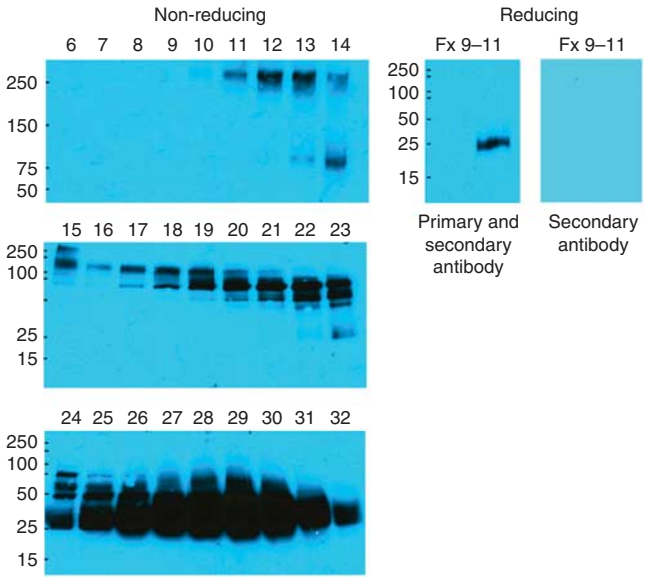

Figure 1 | Urine was fractionated by cation exchange chromatography and fractions containing immunoreactive neutrophil gelatinase–associated lipocalin (NGAL) species were then separated by filtration chromatography (left panels). Note that the monomer (23–26 kDa, fractions (Fxs) 24–32) comprised the majority of immunoreactive NGAL, but additional species can be found at 75 kDa (fractions 19–23), 125 kDa (fractions 15–18), and >250 kDa (fractions 9–11). When the latter were pooled and reduced, the only immunoreactive species was the monomer (right). These data show that in advanced chronic kidney disease (CKD), a proportion of NGAL is associated with other proteins.

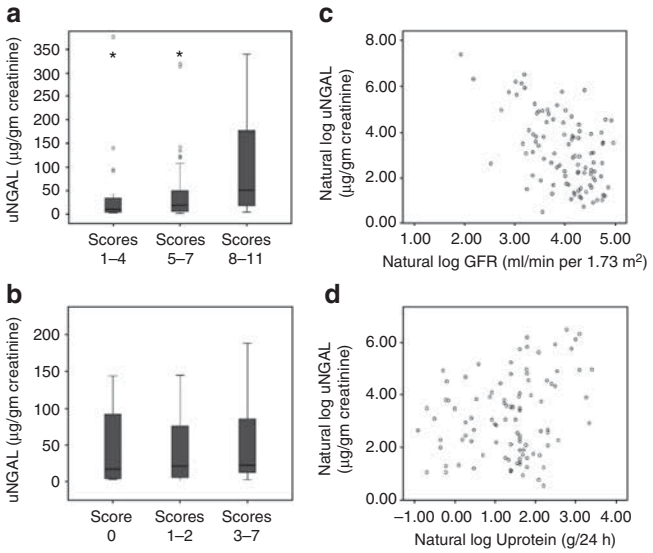

Figure 2 | uNGAL associations in chronicity, activity indices, GFR, and proteinuria. Urinary neutrophil gelatinase–associated lipocalin (uNGAL) monomer according to (**a**) chronicity and (**b**) activity indices of kidney biopsies concurrent with the urine samples. *$P<0.01$ compared with scores 8–11. uNGAL monomer according to (**c**) glomerular filtration rate (GFR) and (**d**) proteinuria.

Table 1 | Relationship between clinical characteristics, GFR, proteinuria, NGAL monomer, and chronic histological characteristics

	Global glomerulosclerosis		Atherosclerosis		Interstitial fibrosis		Tubular atrophy	
	None (n=32)	Present (n=67)	None mild (n=42)	Moderate severe (n=57)	None mild (n=68)	Moderate severe (n=31)	None mild (n=68)	Moderate severe (n=31)
Female (%)	34.4	26.9	35.7	24.6	32.4	22.6	33.8	19.4
Age (years)	48.1 (17.9)	52.4 (16.3)	46.0 (16.9)	55.9 (15.8)	49.6 (17.3)	56.2 (15.4)	48.8 (16.7)	58.1 (15.7)
HTN (%)	50*	74.6	52.4*	77.2	54.4**	93.5	55.9**	90.3
Diabetes (%)	12.5	16.4	7.1	21.1	7.4**	32.3	7.4**	32.3
PCreatinine (mg/dl)	1.1 (0.5)**	1.9 (1.3)	1.2 (0.5)**	2.0 (1.4)	1.3 (0.6)**	2.5 (1.5)	1.3 (0.7)**	2.4 (1.6)
eGFR (ml/min)	81.7 (31.6)**	53.2 (29.1)	78.6 (31.5)**	50.1 (28.2)	73.4 (30.3)**	38.4 (23.5)	72.1 (31.5)**	41.3 (24.2)
Proteinuria (g/24 h)	7.0 (6.3)	5.6 (5.8)	5.9 (6.0)	6.2 (6.0)	5.5 (5.0)	7.3 (7.8)	5.7 (5.8)	6.8 (6.5)
Mean uNGAL (μg/g creatinine)	53.4 (101.1)*	105.8 (232.0)	49.9 (80.1)	117.8 (252.3)	54.1 (111.0)**	167.1 (309.8)	54.1 (112.0)**	163.6 (305.2)

Abbreviations: eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; HTN, hypertension; NGAL, neutrophil gelatinase–associated lipocalin; PCreatinine, plasma creatinine; uNGAL, urinary NGAL.
*$P<0.05$ compared with present.
**$P<0.01$ compared with present.

chronic damage. For example, 96% of patients with fibrous crescents had some degree of tubulointerstitial disease, 35% moderate or severe. In all, 99% of patients with global glomerulosclerosis demonstrated tubulointerstitial disease, 44% of which had moderate or severe levels. Diabetics may have somewhat higher levels of NGAL because 75% demonstrated moderate-to-severe interstitial fibrosis (vs. nephrotic syndrome (26%), $P=0.002$; vs. nephritic syndrome (9%), $P=0.002$) and tubular atrophy (vs. nephrotic syndrome (28%), $P=0.001$; vs. nephritic syndrome (16%), $P=0.002$) in excess of other forms of CKD (without significant difference in GFR). The monomer was also associated with proteinuria ($r=0.27$, $P=0.008$), albeit this association weakened further when adjusted for GFR ($r=0.20$, $P=0.06$).

In contrast, the monomer was either associated with activity indices of glomerular disease ($P=0.847$, Figure 2), particularly cellular changes such as glomerular leukocyte infiltrates ($P=0.34$), mesangial proliferation ($P=0.616$), cellular crescents ($P=0.569$), fibrinoid necrosis ($P=0.168$), nor strongly with monocellular-predominant interstitial infiltrates ($P=0.056$), while there was an association with mesangial matrix expansion ($P=0.004$) implying that the monomer was better associated with chronic rather than acute cellular changes.

Neutrophils and macrophages may be present in the interstitium and contribute to NGAL but our pathological analysis failed to associate the monomer with interstitial cellular infiltrates including the dominant lympho-monocytic interstitial infiltrate. Additionally, isoelectric point analysis of dithiothreitol-reduced samples demonstrated similar isoelectric points for monomeric NGAL in patients with AKI or CKD (pI: 7.1, 8.2, 8.5, and 8.8–9.2), whereas neutrophil NGAL demonstrated a different pattern of alkaline-shifted species (pI: 8.5–9.2). Urinary myeloperoxidase, a protein released from neutrophils, also did not correlate with NGAL monomer in the current ($R^2=0.087$) or

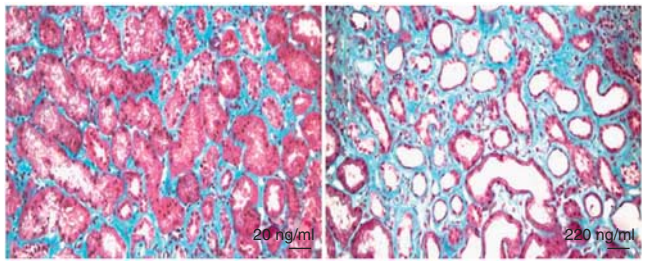

Figure 3 | Illustrative kidney biopsies of two patients with membranous nephropathy. Urinary neutrophil gelatinase–associated lipocalin (NGAL) levels are indicated. Note the widened interstitial compartments, tubular flattening, and tubular dilation in the more advanced cases that expresses a 10-fold higher level of NGAL. Mason trichrome stain. Bar = 50 μm.

in a previously identified cohort ($R^2=0.0092$) whereas NGAL and myeloperoxidase were highly correlated in isolated blood neutrophils ($R^2=0.99$).

DISCUSSION

In summary, although the monomer of NGAL was the predominant, if not the only form expressed in AKI, CKD urine consistently demonstrated additional immunoreactivities including the presence of known and novel HMW complexes formed by disulfide linkages. The latter might form in thick ascending limb epithelia or in the urinary space because both the extracellular secretory component of polymeric IgR (68 kDa) and NGAL are secreted from the luminal membrane of the TAL.[17] α-2-Macroglobulin can also be found in CKD urine[18] whereas its receptor LRP1/CD91 is found in the tubulointerstitial compartment where it may concentrate and endocytose α2-macroglobulin.[19] Perhaps these transport proteins provide a clearance mechanism, rather than directly depicting epithelial damage. In this light, determining the site of complex formation will help identify their clinical significance, as recently demonstrated by the

identification of the dimer in neutrophils and the monomer in epithelia.[20]

Most importantly, this study showed that monomer reflects the severity of tubulointerstitial, rather than glomerular-specific pathologies.[3] Tubulointerstitial genes (*Scara5*, *Col6a*$_{1-3}$, *Nfix*, *Acvrl1*, and myogenic proteins; J. Barasch, unpublished) induced by NGAL in rat kidney mesenchyme are compatible with this hypothesis. These data are novel because they suggest that the monomer is expressed in many common forms of advanced CKD, whereas previously characterized patterns of NGAL expression were disease-restricted including the HIVAN microcyst,[9] and the TAL and α-intercalated cell in AKI.[5] Consequently, we propose that the association of NGAL with CKD may be better appreciated by specific measurements of the monomer, as NGAL complexes >100 kDa contribute variably to total immunoreactivity and potentially involve proteins derived from non-renal cell types. The same type of molecular analysis might apply to other urinary 'biomarkers.'

MATERIALS AND METHODS

Urine collection and NGAL measurement

The University of Parma and Columbia approved the study; informed consent was obtained. Patients (1/2005–4/2008) were >18 years old. Urine was centrifuged (12,000 r.p.m., ×10 min), stored (between 2 months and 3 years at −80°C) and analyzed in batch with non-reducing 4–15% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and monoclonals (1:1000; Antibody-Shop, Gentofte, Denmark) using standards (0.3–3 ng) of human NGAL by workers blinded to biopsy data. ARCHITECT-NGAL assays were performed at the Cincinnati Children's Hospital.

High-molecular weight NGAL evaluation

High-molecular weight immunoreactivity was evaluated by filtering the urine (Microcon Ultracel YM-100, 100 kDa filter; Millipore, Billerica, MA), followed by MiniS (Mes 20 mM, pH6.0 + gradient of NaCl, 0.5 M) and Superdex200 (PBS) chromatography. NGAL-containing complexes were digested with trypsin, batch fractionated on a Poros 50 R2 RP micro-tip, and the resulting peptide pools were analyzed by MALDI-reTOF, using a BRUKER UltraFlex TOF/TOF instrument (Bruker Daltonics; Bremen, Germany).[21] Selected experimental masses (*m*/*z*) were taken to search human non-redundant protein database (NCBI) utilizing the Mascot Peptide Mass Fingerprint,[22] version 2.3.01, with a mass accuracy restriction better than 35 p.p.m., and maximum allowed one cleavage site missed per peptide. To confirm PMF results with scores <40, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) reanalysis, using the UltraFlex instrument in 'LIFT' mode. Fragment ion spectra were taken to search NR using Mascot MS/MS Ion Search program.[22] Isoelectric focusing (Kendrick Labs, Madison, WI)[23] utilized polyvinylidene difluoride immunoblotting.

Pathological analysis of biopsy specimens

Kidney biopsies (obtained as part of routine care in all subjects at the time of urine sampling) were formalin-fixed, paraffin-embedded, and sections stained with hematoxylin/eosin, Masson's trichrome stain, silver methenamine, and periodic acid–Schiff. IgG, IgM, IgA, C3, and fibrinogen deposition were detected by immunoflourescence. Biopsies were graded by two independent pathologists blinded to both diagnosis and NGAL level. Glomerular, tubular, interstitial, and vascular lesions were scored as 0, absent; 1, mild; 2, moderate; and 3, severe.

Measurement of other biomarkers

The sensitivity of myeloperoxidase enzyme-linked immunosorbent assay (Alpco, Salem, NH) was determined using serial dilutions of neutrophils isolated from citrated blood.[24] Although the enzyme-linked immunosorbent assay was sensitive to a 0.01× dilution, immunoblot detection of NGAL in the same sample was consistent only to 0.1×, indicating that these assays favored detection of myeloperoxidase compared with NGAL.

GFR was estimated by the MDRD formula,[25] urinary creatinine measured by QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA), serum creatinine by Jaffe, and urinary protein by nephelometry.

Statistical analysis

Statistical analysis (SPSS v16.0, Chicago, IL): continuous variables were log-transformed and compared by Student's *t*-test for unequal variances or analysis of variance. Categorical variables were compared by χ^2. The null hypothesis was rejected at $P<0.05$. Data were represented as the mean + s.d..

DISCLOSURE

The funding sources had no role in study design, data collection, analysis, interpretation or presentation. College of Physicians and Surgeons of Columbia University, New York, New York, and Cincinnati Children's Hospital, Cincinnati, Ohio have licensed NGAL to Abbott Labs and to Biosite-Alere. PD has received lecture honoraria from Abbott and Biosite-Alere and TLN has a consultation agreement with Abbott.

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SUPPLEMENTARY MATERIAL

Table S1. Mean values and cohort baseline characteristics by etiology of CKD, including urinary NGAL monomer.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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