(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 September 2009 (17.09.2009)

PCT

(10) International Publication Number
WO 2009/114699 A2

(51) **International Patent Classification:**
G01N 33/53 (2006.01)

(21) **International Application Number:**
PCT/US2009/036972

(22) **International Filing Date:**
12 March 2009 (12.03.2009)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
61/035,841   12 March 2008 (12.03.2008)   US
61/057,076   29 May 2008 (29.05.2008)   US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title:** HIGH MOLECULAR WEIGHT NGAL AS A BIOMARKER FOR CHRONIC KIDNEY DISEASE

(57) **Abstract:** A high molecular weight form of Ngal is provided which can be used to diagnose chronic kidney disease. High molecular weight Ngal is about 75 kDa to about 350 kDa, and comprises non-Ngal proteins, such as polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain. Methods are disclosed for assessing high molecular weight Ngal in a diagnostic sample from a subject.

HIGH MOLECULAR WEIGHT NGAL AS A BIOMARKER FOR CHRONIC KIDNEY DISEASE

[0001] This application claims priority to U.S. Provisional Application No. 61/035,841 filed on March 12, 2008, and U.S. Provisional Application No. 61/057,076 filed on May 29, 2008, both of which are hereby incorporated by reference in their entireties.

[0002] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

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BACKGROUND

[0004] Chronic kidney disease (CKD), also known as chronic renal disease, is characterized by slow progressive loss of kidney function. Unlike acute kidney disease which may be reversible, CKD irreversibly progresses through five stages (Stages 1 – 5) of deteriorating kidney function. Stage 1 is slightly diminished kidney function. Gradual, permanent destruction of kidney tissue eventually leads to Stage 5 CKD, also called end-stage renal disease (ESRD), chronic kidney failure (CKF) or chronic renal failure (CRF), which requires kidney replacement therapies such as dialysis or transplantation, and ultimately leads to death.

[0005] CKD is a major worldwide public health problem. According to the National Kidney Foundation, 20 million Americans, or one in nine adults in the U.S., have CKD and another 20 million more are at increased risk of developing CKD. In the U.S., there is a rising incidence and prevalence of kidney failure. The number of patients enrolled in the ESRD Medicare-funded program has increased from approximately 10,000 beneficiaries in 1973 to 472,099 as of 2004. While recent trends show that the rate of increase of new cases has progressively flattened, the projected number of ESRD patients by the year 2010 has been

estimated to be 651,330. Early detection of CKD is critical in order to prevent or delay progression of CKD, and to provide proper treatment to CKD patients.

SUMMARY

[0006] A method is provided for determining whether a subject has chronic kidney disease, the method comprising detecting a protein complex, or one or more components thereof, in a sample from a subject, wherein the protein complex comprises neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the protein complex is from about 75 kDa to about 350 kDa.

[0007] A method is provided for determining whether a subject with kidney disease has chronic kidney disease or acute kidney disease, the method comprising detecting a protein complex, or one or more components thereof, in a sample from a subject, wherein the protein complex comprises neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the protein complex is from about 75 kDa to about 350 kDa, and wherein detection of the protein complex indicates that the subject has chronic kidney disease. The methods can be used to differentiate between chronic kidney disease and acute kidney injury or disease.

[0008] A method is provided for determining the efficacy of a therapy to treat chronic kidney disease in a subject, the method comprising detecting a protein complex, or one or more components thereof, in a sample from a subject, wherein the protein complex comprises neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the protein complex is from about 75 kDa to about 350 kDa, and wherein detection of the protein complex indicates that the subject should continue receiving the therapy.

[0009] In one embodiment, the complex further comprises polymeric immunoglobulin receptor. In one embodiment, the complex further comprises alpha-2-macroglobulin. In one embodiment, the complex further comprises immunoglobulin heavy chain. In one embodiment, the complex further comprises polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof. In one embodiment, the complex does not comprise matrix metalloproteinase-9 (MMP-9). In one embodiment, the complex consists essentially of Ngal, polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain. In one embodiment, the complex consists of Ngal, polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain.

[0010] In one embodiment, the detecting comprises detecting uncomplexed Ngal, uncomplexed polymeric immunoglobulin receptor, uncomplexed alpha-2-macroglobulin, uncomplexed immunoglobulin heavy chain, or any combination thereof. In one embodiment, the detecting comprises detecting a complex comprising, consisting of, or consisting essentially of Ngal and one or more proteins selected from the group consisting of: polymeric immunoglobulin receptor, alpha-2-macroglobulin, and immunoglobulin heavy chain. In one embodiment, the detecting comprises using an antibody, or fragment thereof. In another embodiment, the antibody comprises a detectable label. In another embodiment, the antibody, or fragment thereof, specifically binds Ngal, polymeric immunoglobulin receptor, alpha-2-macroglobulin or immunoglobulin heavy chain.

[0011] In one embodiment, the sample comprises a bodily fluid. In another embodiment, the bodily fluid is urine.

[0012] In one embodiment, the chronic kidney disease comprises Alport syndrome, analgesic nephropathy, glomerulonephritis, kidney stones, infection, obstructive uropathy, prostate disease, polycystic kidney disease, reflux nephropathy, diabetic nephropathy, hypertension nephrosclerosis, chronic interstitial nephritis, hypertension, heart disease, diabetes, immune system diseases, lupus, tumors, renal vascular abnormalities, renal artery stenosis, vasculitis, hereditary diseases of the kidney, or any combination thereof.

[0013] A method is provided for determining whether a subject has chronic kidney disease, the method comprising: (a) obtaining a sample from a subject; (b) separating the sample into a high molecular weight fraction and a low molecular weight fraction, wherein the high molecular weight fraction comprises proteins of at least about 75 kDa; and (c)   detecting an amount of neutrophil gelatinase-associated lipocalin (Ngal) in the high molecular weight fraction; wherein detection of Ngal indicates that the subject has chronic kidney disease. In one embodiment, the amount is a concentration value. In one embodiment, the detecting comprises using an antibody, or fragment thereof. In another embodiment, the antibody, or fragment thereof, specifically binds to Ngal. In one embodiment, the separating comprises using a molecular weight cutoff filter or a molecular weight cutoff membrane.

[0014] In one embodiment, the method further comprises: (d) comparing the amount in (c) with a predetermined cutoff value, wherein an amount above the cutoff value indicates that the subject has chronic kidney disease.

[0015] In one embodiment, the method further comprises: (d) detecting an amount of Ngal in the low molecular weight fraction; and (e) determining a ratio of the amount of Ngal in the high molecular weight fraction compared to the amount of Ngal in the low molecular weight fraction; wherein a ratio greater than 1 indicates that the subject has chronic kidney disease.

[0016] In one embodiment, the detecting comprises an enzyme-linked immunosorbent assay (ELISA). In one embodiment, the high molecular weight fraction comprises a protein complex comprising Ngal, and wherein the size of the protein complex is from about 75 kDa to about 350 kDa. In another embodiment, the high molecular weight fraction comprises a protein complex comprising Ngal, and wherein the size of the protein complex is about 350 kDa.

[0017] The invention provides a high molecular weight Ngal composition comprising neutrophil gelatinase-associated lipocalin (Ngal), wherein the size of the protein complex is from about 75 kDa to about 350 kDa. In one embodiment, the composition further comprises polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof. In another embodiment, the composition comprises, consists of, or consists essentially of Ngal and one or more proteins selected from the group consisting of polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain.

BRIEF DESCRIPTION OF THE FIGURES

[0018] **Figure 1:** High molecular weight Ngal immunoreactivity in fractionated urine sample from patient on dialysis. See Example 1.

[0019] **Figure 2:** Different HMW Ngal compositions are found in different sample fractions. See Example 1.

[0020] **Figure 3:** Patterns of Ngal in different patient populations. See Example 3.

[0021] **Figure 4:** Patient cohort study described in Example 4.

DETAILED DESCRIPTION

[0022] Diagnosis and clinical follow-up of chronic kidney disease (CKD) are limited by the lack of reliable biomarkers that allow rapid analysis of renal function and damage. The existing diagnostic methods are elaborate and inaccurate. Disclosed herein is a newly

identified biomarker which displays elevated levels in CKD and distinguishes between chronic and acute kidney damage.

[0023] There is an ongoing, worldwide epidemic of CKD that over the next decade will double the incidence and prevalence of end-stage renal disease (ESRD). This demographic shift will cause a tremendous increase in the number of patients with ESRD, CKD related complications and excess mortality (1). Early detection of CKD and aggressive treatment of its most potent risk factors, diabetes, hypertension and proteinuria, can decrease the rate of progression of CKD to ESRD.

[0024] Kidney biopsy is the gold standard for diagnosis of chronic kidney disease (CKD); however, it is an invasive and expensive procedure. Novel biomarkers of kidney injury have emerged and can be correlated with kidney function. Current clinically used biomarkers of CKD progression are limited in their ability to accurately predict kidney function decline. Serum creatinine, a surrogate for glomerular filtration rate (GFR), is influenced by muscle mass, gender, race, and medications and may fall in advanced CKD secondary to extrarenal clearance (6, 7). Proteinuria, a good predictor of CKD progression; (8-12) is not present in many types of CKD (13). Kidney biopsy can predict the likelihood of progression to ESRD, regardless of the etiology of CKD. Nephron loss results in worsening kidney function through progressive interstitial fibrosis (IF) and tubular atrophy (TA) (14, 15). Furthermore, the degree of tubulointerstitial (TI) alteration on biopsy is more highly correlated with kidney function decline than severity of glomerular damage (16-25). However, kidney biopsy is an invasive procedure and impractical for routine clinical use in the monitoring of CKD progression. Therefore, in order to improve early detection of patients with CKD who will benefit from more aggressive CKD treatment, it is extraordinarily important to evaluate non-invasive biomarkers of CKD progression that are correlated to TI alteration on kidney biopsy (2, 3) (4, 5).

[0025] NGAL is a 25 kD lipocalin protein produced by the nephron in response to tubular epithelial damage (26). It has been well established that urine NGAL (uNGAL) levels rise in acute kidney injury (AKI) (27-30). Recent evidence suggest that uNGAL is elevated in CKD, regardless of etiologies, and that elevated uNGAL levels can predict CKD progression (31, 32).

[0026] The invention provides a high molecular weight (HMW) form of neutrophil gelatinase-associated lipocalin (Ngal, used interchangeably herein with NGAL) and uses

thereof as a biomarker for chronic kidney disease (CKD). A quantitative correlation was found between the presence of a high molecular weight Ngal complex in samples from patients with CKD (see Examples). Methods are provided for detecting a HMW Ngal composition to diagnose CKD in a subject or to determine the efficacy of treatment in a subject receiving treatment for CKD.

[0027] The invention provides a novel biomarker for CKD. The biomarker is a high molecular weight form of Ngal. High molecular weight Ngal composition comprises Ngal and has a molecular weight or size of from about 75 kDa to about 350 kDa. In one embodiment, the complex further comprises polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof. As described in Table 1A in the Examples, polymeric immunoglobulin receptor has a molecular weight of about 83 kDa, alpha-2-macroglobulin has a molecular weight of about 163 kDa, and immunoglobulin heavy chain has a molecular weight of about 45 kDa. In another embodiment, the HMW Ngal composition comprises, consists of, or consists essentially of Ngal and one or more proteins selected from the group consisting of polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain. HMW Ngal may comprise a protein complex between Ngal and polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof as components of the complex. The components of a HMW Ngal complex may be held together covalently or non-covalently or both. One or more of the components of the complex may comprise post-translation protein modification (for example, phosphorylation or glycosylation), where the component was modified before or after formation of the complex.

[0028] Ngal is generated when there is a change in renal function due to acute renal injury. Ngal was identified as a reproducible marker that is upregulated after acute kidney damage such as post-transplant rejection, ischemic injury, drug nephrotoxicity. This allows for easy diagnosis and clinical follow-up. See, for example, Schmidt-Ott KM et al. (2007) Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol. 18:407-13; Mori K and Nakao K. (2007) Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage. Kidney International 71:967-970; Wagener G et al. (2008) Increased incidence of acute kidney injury with aprotinin use during cardiac surgery detected with urinary NGAL. Am J Nephrol. 28:576-582. Ngal has also been shown to be a marker of CKD in children (Mitsnefes MM et al. (2006) Serum neutrophil gelatinase-associated

lipocalin as a marker of renal function in children with chronic kidney disease. Pediatr Nephrol. 22:101-8).

[0029] Two examples of measures of renal function that are currently used to diagnose and classify CKD are (1) albuminurea (albumin found in the urine) and (2) estimates of glomerular filtration rate (GFR). If the kidney fails, albumin and other proteins will leak into the urine while it normally is retained by the body. The GFR is the total volume of plasma filtered by the kidneys. This can be calculated precisely by injecting tracers and following their excretion. Since method is cumbersome, endogenous markers (e.g. creatinine) are used to estimate GFR (estimated GFR = eGFR). These methods based on albuminurea and eGFR have at least the following major disadvantages: (i) they are inaccurate, especially in late disease stages and specific patient populations (children, unusual muscle or fat mass, pregnant women and specific ethnic groups); (ii) eGFR often requires 24 hour urine collection; (iii) creatinine levels (required to estimate GFR) can be falsely elevated; and (iv) these tests do not distinguish between acute and chronic kidney damage.

[0030] C reactive protein (CRP) has emerged as an early marker of renal dysfunction. CRP however is a very aspecific marker that will be increased in all forms of infection. Homocysteine, asymmetric dimethyl arginine, brain natriuretic peptide and troponin T are all studied but their value in CDK still requires clinical support form specifically designed intervention studies. However, these markers are also up-regulated in other disease (e.g. cardiovascular).

[0031] As described herein, HMW Ngal can be used as a biomarker for CKD in at least four non-limiting applications: (1) as a screening tool to detect preclinical renal failure in the general population and risk groups; (2) as a diagnostic tool to follow disease progression in CKD patients; (3) to determine efficacy of CKD treatment; and (4) as a prognostic tool to determine the type or stage of CKD. If the up-regulation of this high molecular weight form plays a role in the origin or progression of renal failure, therapeutic approaches based on this molecule and its biology may be useful in the treatment of CKD.

[0032] Early detection of CKD is important and allows for early commencement of proper therapies to slow the progression of kidney deterioration. Early detection is particularly important in patients who are at high risk of developing CKD. Examples of high risk groups that may be screened for early detection of CKD include patients with a history of diabetes,

hypertension, cardiovascular disease, smoking, obesity, nephrotoxic drugs, malignancy, or family history of CKD.

[0033] The present discovery is based on the observation of a correlation between CKD and a HMW form of Ngal. The HMW form of Ngal is at least 75 kDa, at least 150 kDa, or at least 350 kDa, and comprises non-Ngal proteins, such as polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain. In one embodiment, the HMW form of Ngal is about 350 kDa. The HMW form of Ngal may be a large protein complex that comprises component complexes, for example, (i) Ngal bound to polymeric immunoglobulin receptor; (ii) Ngal bound to alpha-2-macroglobulin; (iii) Ngal bound to immunoglobulin heavy chain; (iv) Ngal bound to polymeric immunoglobulin receptor and alpha-2-macroglobulin; (v) Ngal bound to polymeric immunoglobulin receptor and immunoglobulin heavy chain; (vi) Ngal bound to alpha-2-macroglobulin and immunoglobulin heavy chain; (vii) Ngal bound to polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain. In embodiments of the methods provided herein, any one or more component complexes may be detected to assess the presence of HMW Ngal in a sample. In other embodiments, individual uncomplexed protein components of HMW Ngal may be detected to assess the presence of HMW Ngal in a sample. For example, uncomplexed or free Ngal, polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof.

[0034] A 125 kDa complex between Ngal and matrix metalloproteinase-9 (MMP-9) has been previously reported (U.S. Patent No. 7,153,660; Yan L et al. (2001) The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL)).

[0035] Methods are provided for determining an amount of HMW Ngal in a sample. For example, a patient sample can be subjected to a molecular weight filter to separate a high molecular weight fraction of the sample (comprising any HMW Ngal present in the sample) and a low molecular weight fraction. Separating the fractions will separate any HMW Ngal away from any uncomplexed or free Ngal in the sample, thus eliminating potential cross-detection between HMW Ngal and uncomplexed or free Ngal. To determine the presence of HMW Ngal in the sample, protein detection methods known in the art can be used to detect the Ngal complex, component complexes thereof, or individual protein components of the complex. For example, antibody detection of Ngal can be used, for example, enzyme-linked immunosorbent assay (ELISA). Antibodies that specifically bind Ngal are available from

commercial vendors, such as Santa Cruz Biotechnology. Ngal ELISA kits are available from commercial vendors, such as R&D Systems and BioProto.

EXAMPLES

EXAMPLE 1

NGAL IMMUNOREACTIVITY IS FOUND AT DIFFERENT MOLECULAR WEIGHTS

[0036] Urine from a patient on dialysis was fractionated on an anion exchange column in Buffer (MES 20 mM, pH 6.0) and salt eluted (Buffer A + NaCl, 0.5M). As shown in Figure 1, fractions were immunoblotted for NGAL and high molecular weight immunoreactivity was identified in early fractions from the column. The same samples were then reduced with beta-mercaptoethanol resulting in dissolution of the high molecular weight complexes and the appearance of 'monomeric' glycosylated NGAL at 25 KDa. Recombinant NGAL was added to the gel as an internal maker (21 KDa). Note that monomeric NGAL might derive from any of the high molecular bands.

[0037] NGAL complexes were isolated first by anion exchange chromatography (Buffer system =MES 20 mM pH 6.0---see above) and fractions containing high molecular weight NGAL immunoreacitvity were pooled and then further fractionated by gel filtration by Superdex200 (see Figure 2). A number of molecular weights were found on non-reducing gels including 350, 125, 75, 37, 25 KDa, and these forms were located in different fractions. Fractions 9-12 had only the 350 KDa reactive band. This fraction was pooled and then reduced. After reduction, the only immunoreactive molecule was 25 KDa NGAL. These data show that NGAL was associated with proteins totaling 350 KDa. The 350 KDa band was sequenced (see Example 2).

EXAMPLE 2

IDENTIFICATION OF PROTEINS IN HIGH MOLECULAR WEIGHT NGAL COMPLEX

[0038] Samples of a HMW Ngal composition (350 kDa) were subjected to tryptic digest. To obtain a mass fingerprint of the proteins in the sample, 100% of the peptides generated by the digest were subjected to a micro-clean-up procedure using 2 µl bed-volume of Poros 50 R2 (PerSpective Biosystems) reversed-phase (RP) beads, packed in an Eppendorf gel-loading tip. Mass spectrometry (MALDI-ReTOF) was conducted on two peptide pools (16 and 30% MeCN) recovered from the RP-microtip column using a Bruker Ultraflex TOF/TOF instrument with delayed extraction.

[0039] For peptide mass fingerprinting (PMF), experimental masses (m/z) combined from both MALDI-ReTOF experiments were used to search a non-redundant human protein database (NR; ~148,041 entries), using the PeptideSearch (M. Mann, Max-Planck Institute for Biochemistry, Martinsried 82152, Germany) algorithm. A molecular weight range twice the predicted weight was covered, with a mass accuracy restriction better than 40 ppm, and maximum one missed cleavage site allowed per peptide.

[0040] Mass spectrometric sequencing (MALDI-TOF-MS/MS) of selected peptides from the partially fractionated pools was done on a Bruker Ultrflex TOF/TOF instrument in 'Lift' mode, and the fragment ion spectra taken to search human databases using the MASCOT MS/MS Ion Search program (Matrix Science).

[0041] The experiment described above identified the following proteins in the sample of HMW Ngal: polymeric immunoglobulin receptor, alpha-2-macroglobulin and immunoglobulin heavy chain (see Table 1A).

Table 1A: Proteins identified as components of a HMW form of Ngal			
Name	Size	NCBI #	Sequence
Polymeric immunoglobulin receptor	~83 kDa	31377806	LVSLTLNLVTR; (SEQ ID NO:1) ILLNPQDKDGSFSVVITGLR; (SEQ ID NO:2) QGHFYGETAAVYVAVEER; (SEQ ID NO:3)
α-2-macroglobulin	~163 kDa	25303946	HYDGSYSTFGER; (SEQ ID NO:4) QGIPFFGQVR; (SEQ ID NO:5)
Immunoglobulin heavy chain	~45 kDa	9367869	WLQGSQELPR; (SEQ ID NO:6) YLTWASR; (SEQ ID NO:7) QEPSQGTTTFAVTSILR; (SEQ ID NO:8)

EXAMPLE 3

PATTERNS OF HIGH MOLECULAR WEIGHT NGAL IN PATIENT POPULATIONS

[0042] NGAL Western Blots from four previous clinical studies were analyzed for the presence of HMW NGAL. Results are shown in Figure 3; patients were grouped according to renal diagnosis as follows:

1. Those patients who displayed stable eGFR > 60ml/min were labeled "Controls".

2. Very Low Birthweight Infants (<1500 grams) who attained a clinical diagnosis of Acute Renal Failure were labeled "Acute Neonatal".

3. Patients with baseline eGFR <60ml/min that was stable either over 3 day hospital stay or stable over 3 months were denoted "CKD".

4. Patients who demonstrated a baseline eGFR <60ml/min and who demonstrated either a new-onset and sustained 1.5X increase in creatinine or a new onset and sustained 25% decrease in eGFR (satisfying minimal RIFLE (*r*isk for kidney dysfunction, *i*njury to the kidney, *f*ailure of kidney function, *l*oss of kidney function, and *e*nd-stage kidney disease) criteria) over the course of hospitalization were denoted "Acute on Chronic".

5. Patients with dialysis dependence prior to urine sampling were labeled "ESRD".

[0043] As shown in Figure 3, high molecular weight forms of NGAL correlate with different types of CKD.

EXAMPLE 4

PROSPECTIVE COHORT STUDY TO DETERMINE THE RELATIONSHIP BETWEEN NOVEL BIOMARKERS AND KIDNEY BIOPSY FINDINGS IN A BROAD SPECTRUM OF PATIENTS WITH CKD, INCLUDING PRIMARY AND SECONDARY GLOMERULAR, TUBULOINTERSTITIAL (TI), AND VASCULAR DISEASES

[0044] This example presents results of a biopsy cohort of patients with CKD from a spectrum of etiologies. This study includes the important discovery of a high molecular weight (HMW) NGAL complex that can be measured by ELISA. HMW NGAL accounted for a small proportion of total NGAL measured. The results (described below) also show that uNGAL levels are elevated in the setting of severe TI damage and when glomerular injury is co-prevalent with TI damage. A strong inverse correlation between GFR and uNGAL is also demonstrated by both ELISA and Western Blot and a weak correlation between proteinuria and uNGAL measured by ELISA. Some differences were found in uNGAL measurements based on the type of NGAL assay used, but the relationships between uNGAL and either biopsy findings or GFR were not altered.

[0045] uNGAL accurately discriminates AKI from stable CKD (27), suggesting that non-progressive CKD does not induce tubular production of NGAL to the same extent as AKI. However, multiple studies have demonstrated that uNGAL is a marker of CKD severity and progression (34, 35, 32, 36, 31, 37). Furthermore, the fractional urinary excretion of NGAL is increased in the setting of CKD, suggesting active tubular production in response to ongoing tubular injury (36). A renal tubular source of uNGAL was confirmed by immunohistochemical staining in Lee grade III IgA nephropathy patients (32). These observations, and the results of this study, suggest that in the setting of CKD, uNGAL represents a marker of the degree of onging, active kidney damage, as proposed by Mori and Nakao (37).

[0046] While the sample size of this study was small and individual primary etiologies of CKD were not evaluated, histological characteristics associated with NGAL expression were found that were independent of underlying disease. Furthermore, the inclusion of patients with comorbidities makes these results generalizable. Since there was no long-term follow-up data for patients, uNGAL values could not be correlated with disease progression. Larger scale studies with long follow-up periods can be conducted.

[0047] As shown below, NGAL, a well-established marker of AKI, is elevated in patients with TI damage on kidney biopsy. uNGAL is elevated in patients with severe TA and IF, known predictors of progressive disease. Measurement of uNGAL may be a non-invasive way to gain insight into pathophysiologic changes within the kidney and help identify patients who are will progress to end stage disease.

Results

[0048] Of 88 patients who underwent kidney biopsy, 14 were excluded for reasons indicated in Figure 4. Of the remaining 74 patients, 78% were male and mean age was 51.5 years (SD, 17.2). Diagnostic groups based on histology are presented in Figure 4. uNGAL, measured by immunoblot or ELISA, was not significantly different among these groups (Table 1B).

Table 1B. Mean uNGAL, GFR and Proteinuria Among Diagnostic Groups of CKD								
Diagnostic Group	Mean uNGAL western (SD)	P-value	Mean uNGAL ELISA (SD)	P-value	Mean GFR (SD)	P-Value	Mean Proteinuria (SD)	P-value
Nephrotic (n=34)	89 (213)		109 (179)		58 (31)		7.6 (6.4)	
Nephritic (n=21)	36 (76)	0.381	33 (52)	0.110	63 (31)	0.041	7.2 (4.0)	<0.001
Diabetic (n=7)	80 (95)		367 (745)		53 (31)		2.7 (1.9)	
Other (n=12)	81 (106)		133 (214)		38 (33)		3.1 (2.6)	

[0049] TI damage was defined as the degree of TA or IF on biopsy. Patients with severe TI damage had uNGAL levels that were significantly 3-5 fold greater than for those patients with mild degrees of TI damage (Table 2A). uNGAL levels were also significantly greater for those patients with global glomerulosclerosis (Table 2B). It is interesting to note that in these 51 patients with global glomerulosclerosis all had some degree of TA with 39% having severe TA. Furthermore, 50 of these patients with global glomerulosclerosis had some degree of IF, with 38% having severe IF. GFR was significantly lower in patients with severe TI damage and global glomerulosclerosis (Tables 2A and 2B). Proteinuria levels were not associated with any biopsy findings.

[0050] An inverse relationship was found between uNGAL and GFR (r= -0.605, p < 0.001 and r = -0.491, p<0.001 for Immunoblot and ELISA, respectively.) Although there was no significant correlation between proteinuria and uNGAL when measured by immunoblot (r=0.222 p=0.06), we identified a weak, statistically significant relationship between ELISA and proteinuria, (r = 0.297, p = 0.01), accounting for approximately 7% of the variability in the ELISA measurement.

[0051] In the determination of high molecular weight forms of NGAL, 10 samples were excluded because insufficient urine volume remained, 18 were excluded because *NGAL loss* (described below) was > 50% of the total value measured by ELISA. 48 patients had measurement of NGAL in the filtrate (MW < 100kDa) and retentate (MW > 100kDa). A higher molecular weight NGAL complex was found in the retentate that could be measured by ELISA. Analysis of the amount of NGAL in the retentate revealed that high molecular weight forms accounted for a mean 3.8%, SD 7.7%, range (0 – 41.9%) of the entire NGAL measurement.

Methods

[0052] <u>Patients:</u> Patients were 18 years and older undergoing kidney biopsy at the University of Parma as part of routine care from January 2005 – April 2008. Urine and fasting blood samples were collected at the time of biopsy. Patients were excluded if kidney biopsy findings were consistent with AKI or if insufficient urine volume was collected to perform both biomarker assays. At the time of biopsy routine clinical and laboratory information were obtained (see Table 3).

Table 3: Baseline characteristics

Parameter	All Patients Mean (SD)
Gender (% female)	32.4
Age	51.5 (17.2)
Systolic BP (mmHg)	139 (22.5)
Diastolic BP (mmHg)	82 (11.1)
Diabetes (%)	16.2
Hypertension (%)	71.6
GFR (mL/min/1.73m2)	55.9 (31.7)
Serum Creatinine (mg/dl)	1.86 (1.24)
Proteinuria (g/24h)	5.46 (5.21)
Serum Protein (g/dl)	5.44 (1.29)
Albumin (g/dl)	1.86 (1.24)
Cholesterol (mg/dl)	248 (71.4)
uNGAL Elisa (μg/g creatinine)	115 (275)
uNGAL Western (μg/g creatinine)	71.6 (158)
Diagnostic Categories	
Nephrotic (%)	45.9
Nephritic (%)	28.4
Diabetic (%)	9.5
Other (%)	16.2
1. N= 67 2. N=64 3. N=62	

[0053] GFR was estimated using the MDRD formula (33). Participants were 74 Caucasian patients undergoing kidney biopsy as a part of routine care. 78% were men, 46% nephrotic syndrome, 14% nephritic syndrome, 10% diabetic CKD, 16% other. Diagnosis of CKD was based on the consensus definition developed by the Kidney Disease Outcomes Quality Initiative and assigned by researchers who were blinded to experimental measurements. Kidney biopsy scores for individual pathologic findings were determined by two blinded pathologists. An activity and chronicity index were calculated. Kidney function was evaluated by serum creatinine measurement, glomerular filtration rate (GFR) calculation and 24-hour urine protein measurement, other routine serum chemistries were measured (see below). Urinary NGAL was measured by immunoblot and enzyme-linked immunesorbent assay, urine creatinine by spectrophotometry, and serum creatinine by Jaffe kinetic reaction (see below). Experimental measurements were not available to treating physicians.

[0054] Urine Ngal measurements:　1mL of urine was centrifuged at 12,000rpm for 10 minutes and stored it at -80°C. uNGAL (10uL) was quantified by immunoblots with nonreducing 4% to 15% gradient polyacrylamide gels (Bio-Rad Laboratories) and monoclonal antibodies (1:1000; Antibody Shop, BioPorto Diagnostics) with standards (0.2 to

10 ng) of human recombinant NGAL protein (32, 33). The measurement was reproducible to 0.4 ng/lane. The immunoblotting procedure was selected to authenticate monomeric NGAL at 25kD. Urinary NGAL was also measured using enzyme-linked immunosorbant assay (ELISA) (Architect, Abbott).

[0055] HMW Ngal measurments: To determine the potential contribution of high molecular weight NGAL complexes that may be derived from the serum to the ELISA measurement, 400ul of urine was filtered with a Microcon Ultracel YM-100 100 kDa filter (Millipore Corporation) and centrifuged until nearly dry. PBS was added to the retentate to bring the volume to a minimum of 100ul. Total volumes were determined and NGAL was measured in the original sample, the flow through and retentate using the Abbott Architect NGAL Assay. *NGAL loss* was calculated by subtracting the amount of NGAL measured in the flow through and retentate from the total NGAL concentration.

[0056] Creatinine measurements: Urinary creatinine was measured by using a QuantiChrom Creatinine Assay Kit (BioAssay Systems). Serum creatinine was measured by Jaffe reaction. Urine protein was measured by nephelometry.

[0057] Histological evaluation: Formalin-fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin/eosin, sliver methenamine and periodic-acid Schiff. IgG, IgM, IgA, C3 and fibrinogen deposition were detected by immunoflourescence staining. Glomerular and mesangial proliferation were graded from 0-3. Grade 0, 1, 2, 3 represented normal, <1/3, 1/3-2/3, >2/3 of the glomerulus involved respectively. TI lesions were also scored as 0, absent; 1, mild; 2, moderate; 3, severe.

[0058] Statistical analysis: Statistical analysis was performed with SPSS v16.0 (SPSS, Chicago, Illinois). Continuous variables were log-transformed and compared by Student's t-test for unequal variances or ANOVA. Categorical variables were compared by c^2. The null hypothesis was rejected at $p<0.05$. Data were represented as the mean $\pm$ standard deviation (SD). Pearson Correlations were run between urine NGAL and proteinuria, urine NGAL and GFR to determine if a linear relationship existed.

[0059] Although the invention has been described and illustrated in the foregoing illustrative embodiments, it is understood that the present disclosure has been made only by way of example, and that numerous changes in the details of implementation of the invention can be made without departing from the spirit and scope of the invention, which is limited only by

the claims that follow. Features of the disclosed embodiments can be combined and rearranged in various ways within the scope and spirit of the invention.

Table 2A. Tubulointerstitial Findings and mean uNGAL, proteinuria and GFR

	Number of Patients	Mean uNGAL western (SD) (μg/g creatinine)			p value	Mean uNGAL ELISA (SD) (μg/g creatinine)			p value	Mean proteinuria (SD)			p value	Mean GFR (SD)			p value
Interstitial Mononuclear cell infiltration	45	23 (48)	103 (194)		0.005	55 (134)	154 (332)		0.049	6.2 (6.3)	5.0 (4.4)		0.749	73 (31)	45 (27)		<0.00
		Absent	Mild	Severe		Absent	Present			Absent	Present			Absent	Present		
Atherosclerosis	Present, 83	14 (18)	52 (170)		0.030	36 (72)	129 (296)		0.054	5.7 (6.2)	5.4 (5.1)		0.777	75 (36)	63 (32)		0.002
		Absent	Mild	Severe		Absent	Mild	Severe		Absent	Mild	Severe		Absent	Mild	Severe	
Interstitial Fibrosis	Absent 7 Mild 44 Severe 23	48 (91)	30 (83)	159 (248)	<0.001	41 (253)	51 (104)	233 (435)	0.018	3.2 (7.0)	4.9 (4.7)	5.7 (5.5)	0.429	70 (39)	69 (29)	32 (25)	<0.001
		Absent	Mild	Severe		Absent	Mild	Severe		Absent	Mild	Severe		Absent	Mild	Severe	
Tubular Atrophy	Absent 3 Mild 47 Severe 24	9 (11)	31 (66)	159 (234)	<0.001	20 (4)	59 (49)	219 (424)	0.010	5.3 (4.6)	5.2 (5.0)	5.9 (5.6)	0.754	98 (18)	85 (29)	34 (25)	<0.001

†p<0.05, * p<0.01, ‡ p<0.001
Interstitial Mononuclear cell infiltration and atherosclerosis, were analyzed as either absent (biopsy score 0) or present (biopsy score 1-3).
Interstitial fibrosis and tubular atrophy were analyzed as none = grade 0, mild = grade 1, and severe = grade 2 or 3.

Table 2B. Glomerular Findings and Mean uNGAL, proteinuria and GFR

	Number with biopsy finding present	Mean uNGAL western (SD) (μg/g creatinine)		p-value	Mean uNGAL ELISA (SD) (μg/g creatinine)		p value	Mean proteinuria (SD)		p value	Mean GFR (SD)		p value
		Absent	Present		Absent	Present		Absent	Present		Absent	Present	
Global Glomerulosclerosis	51	29 (54)	91 (185)	0.045	57 (149)	142 (314)	0.036	6.8 (6.6)	4.9 (4.4)	0.421	69 (29)	56 (29)	0.037
Glomerular Leukocyte Infiltration	3	70 (164)	62 (111)	0.186	122 (289)	83 (78)	0.441	5.7 (6.4)	3.9 (3.3)	0.366	67 (33)	47 (22)	0.582
Capillary Wall Thrombosis	28	97 (193)	46 (99)	0.037	126 (319)	99 (189)	0.780	5.2 (6.3)	5.8 (5.2)	0.479	62 (33)	62 (29)	0.194
Mesangial Cell Proliferation	17	70 (171)	81 (108)	0.815	122 (289)	92 (185)	0.491	6.0 (6.5)	3.7 (3.6)	0.063	65 (32)	60 (30)	0.219
Mesangial Matrix Expansion	66	45 (88)	45 (88)	0.826	98 (162)	109 (303)	0.673	6.2 (6.3)	5.4 (5.2)	0.562	63 (63)	54 (31)	0.584
Cellular Crescents	12	78 (171)	38 (66)	0.523	83 (163)	233 (581)	0.532	4.8 (5.7)	8.7 (9.5)	0.587	64 (31)	67 (32)	0.126
Fibrous Crescents	21	55 (149)	112 (176)	0.101	77 (152)	213 (452)	0.258	5.5 (4.7)	5.4 (5.4)	0.480	59 (32)	49 (30)	0.180

All glomerular findings were analyzed as either absence (grade 0) or presence (grade 1-3).

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What is claimed is:

1. A method for determining whether a subject has chronic kidney disease, the method comprising detecting a protein complex, or one or more components thereof, in a sample from a subject, wherein the protein complex comprises neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the protein complex is from about 75 kDa to about 350 kDa.

2. The method of claim 1, wherein the complex further comprises polymeric immunoglobulin receptor.

3. The method of claim 1, wherein the complex further comprises alpha-2-macroglobulin.

4. The method of claim 1, wherein the complex further comprises immunoglobulin heavy chain.

5. The method of claim 1, wherein the complex further comprises polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof.

6. The method of claim 1, wherein the detecting comprises detecting uncomplexed Ngal, uncomplexed polymeric immunoglobulin receptor, uncomplexed alpha-2-macroglobulin, uncomplexed immunoglobulin heavy chain, or any combination thereof.

7. The method of claim 1, wherein the detecting comprises detecting a complex comprising Ngal and polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof.

8. The method of claim 1, wherein the complex does not comprise matrix metalloproteinase-9 (MMP-9).

9. The method of claim 1, wherein the sample comprises a bodily fluid.

10. The method of claim 9, wherein the bodily fluid is urine.

11. The method of claim 1, wherein the detecting comprises using an antibody, or fragment thereof.

12. The method of claim 11, wherein the antibody comprises a detectable label.

13. The method of claim 11, wherein the antibody, or fragment thereof, specifically binds to Ngal, polymeric immunoglobulin receptor, alpha-2-macroglobulin or immunoglobulin heavy chain.

14. The method of claim 1, wherein the chronic kidney disease comprises Alport syndrome, analgesic nephropathy, glomerulonephritis, kidney stones, infection, obstructive uropathy, prostate disease, polycystic kidney disease, reflux nephropathy, diabetic nephropathy, hypertension nephrosclerosis, chronic interstitial nephritis, hypertension, heart disease, diabetes, immune system diseases, lupus, tumors, renal vascular abnormalities, renal artery stenosis, vasculitis, hereditary diseases of the kidney, or any combination thereof.

15. A method for determining whether a subject with kidney disease has chronic kidney disease or acute kidney disease, the method comprising detecting a protein complex, or one or more components thereof, in a sample from a subject, wherein the protein complex comprises neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the protein complex is from about 75 kDa to about 350 kDa, and wherein detection of the protein complex indicates that the subject has chronic kidney disease.

16. A method for determining the efficacy of a therapy to treat chronic kidney disease in a subject, the method comprising detecting a protein complex, or one or more components thereof, in a sample from a subject, wherein the protein complex comprises neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the protein complex is from about 75 kDa to about 350 kDa, and wherein detection of the protein complex indicates that the subject should continue receiving the therapy.

17. A method for determining whether a subject has chronic kidney disease, the method comprising:

(a) obtaining a sample from a subject;

(b) separating the sample into a high molecular weight fraction and a low molecular weight fraction, wherein the high molecular weight fraction comprises proteins of at least about 75 kDa; and

(c) detecting an amount of neutrophil gelatinase-associated lipocalin (Ngal) in the high molecular weight fraction; wherein detection of Ngal indicates that the subject has chronic kidney disease.

18. The method of claim 17, wherein the amount is a concentration value.

19. The method of claim 17, wherein the detecting comprises using an antibody, or fragment thereof.

20. The method of claim 17, wherein the antibody, or fragment thereof, specifically binds to Ngal, polymeric immunoglobulin receptor, alpha-2-macroglobulin or immunoglobulin heavy chain.

21. The method of claim 17, wherein the separating comprises using a molecular weight cutoff filter or a molecular weight cutoff membrane.

22. The method of claim 17, further comprising:

(d) comparing the amount in (c) with a predetermined cutoff value, wherein an amount above the cutoff value indicates that the subject has chronic kidney disease.

23. The method of claim 17, further comprising:

(d) detecting an amount of Ngal in the low molecular weight fraction; and

(e) determining a ratio of the amount of Ngal in the high molecular weight fraction compared to the amount of Ngal in the low molecular weight fraction; wherein a ratio greater than 1 indicates that the subject has chronic kidney disease.

24. The method of claim 17, wherein the detecting comprises an enzyme-linked immunosorbent assay (ELISA).

25. The method of claim 17, wherein the high molecular weight fraction comprises a protein complex comprising Ngal, and wherein the size of the protein complex is from about 75 kDa to about 350 kDa.

26. The method of claim 17, wherein the high molecular weight fraction comprises a protein complex comprising Ngal, and wherein the size of the protein complex is about 350 kDa.

27. A high molecular weight Ngal composition, comprising neutrophil gelatinase-associated lipocalin (Ngal), and wherein the size of the composition is from about 75 kDa to about 350 kDa.

28. The composition of claim 27, further comprising polymeric immunoglobulin receptor.

29. The composition of claim 27, further comprising alpha-2-macroglobulin.

30. The composition of claim 27, further comprising immunoglobulin heavy chain.

31. The composition of claim 27, further comprising polymeric immunoglobulin receptor, alpha-2-macroglobulin, immunoglobulin heavy chain, or any combination thereof.
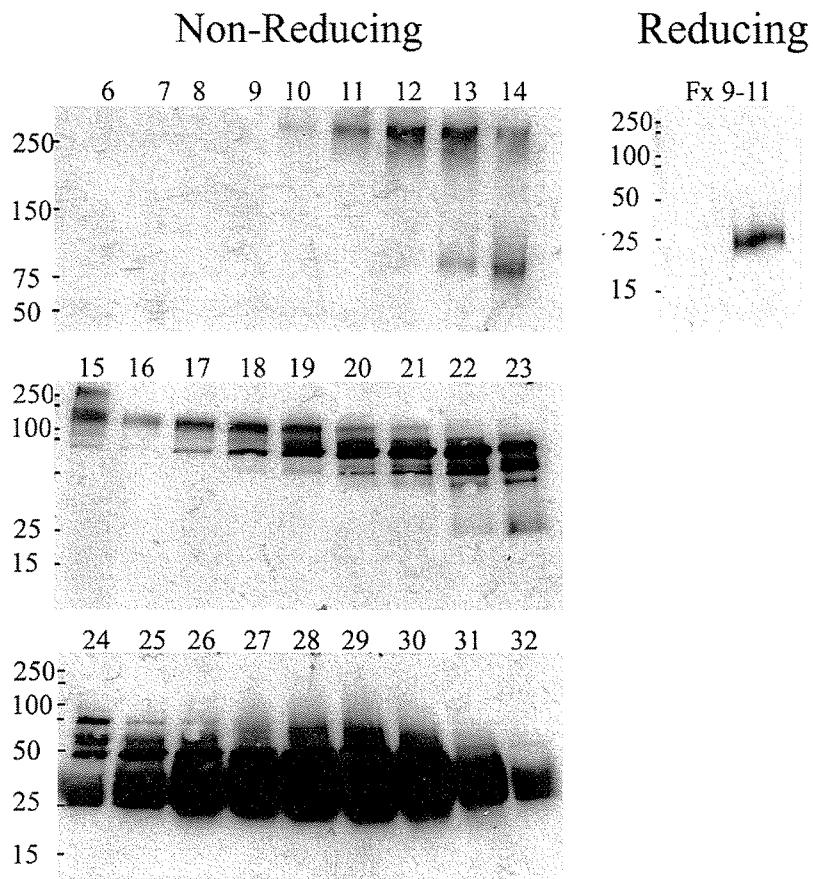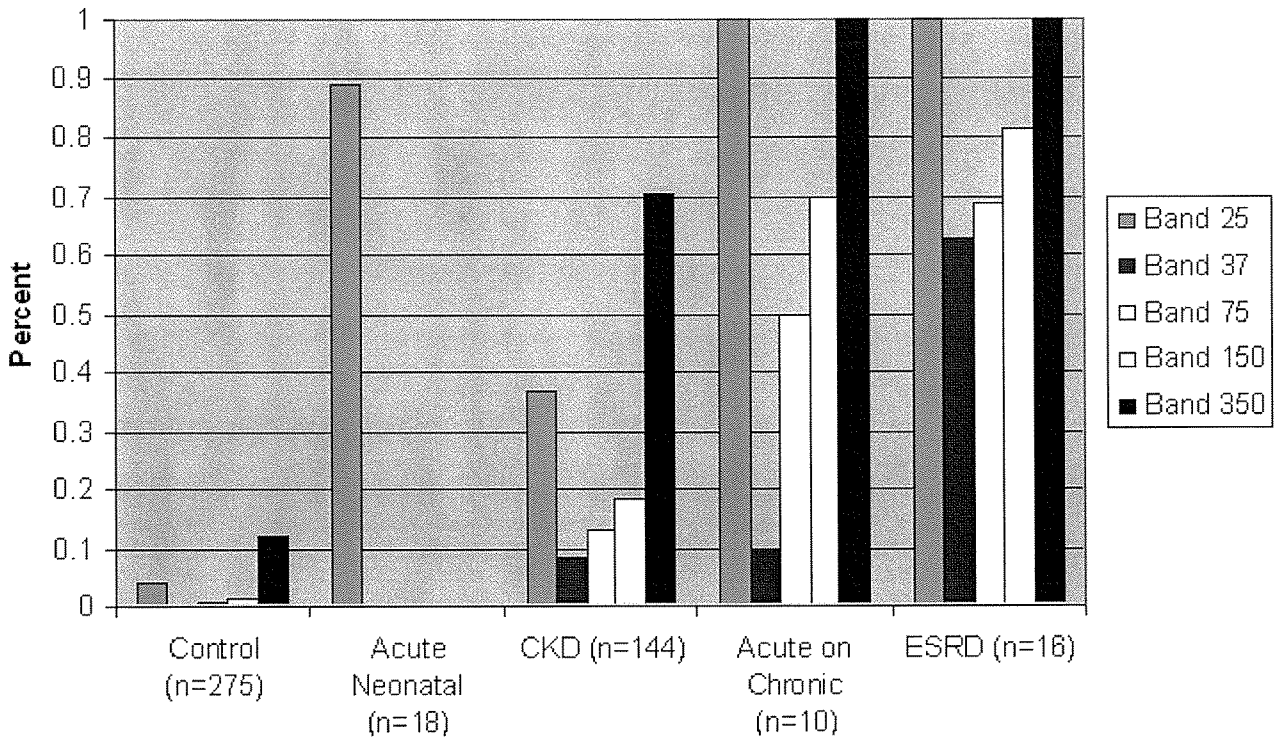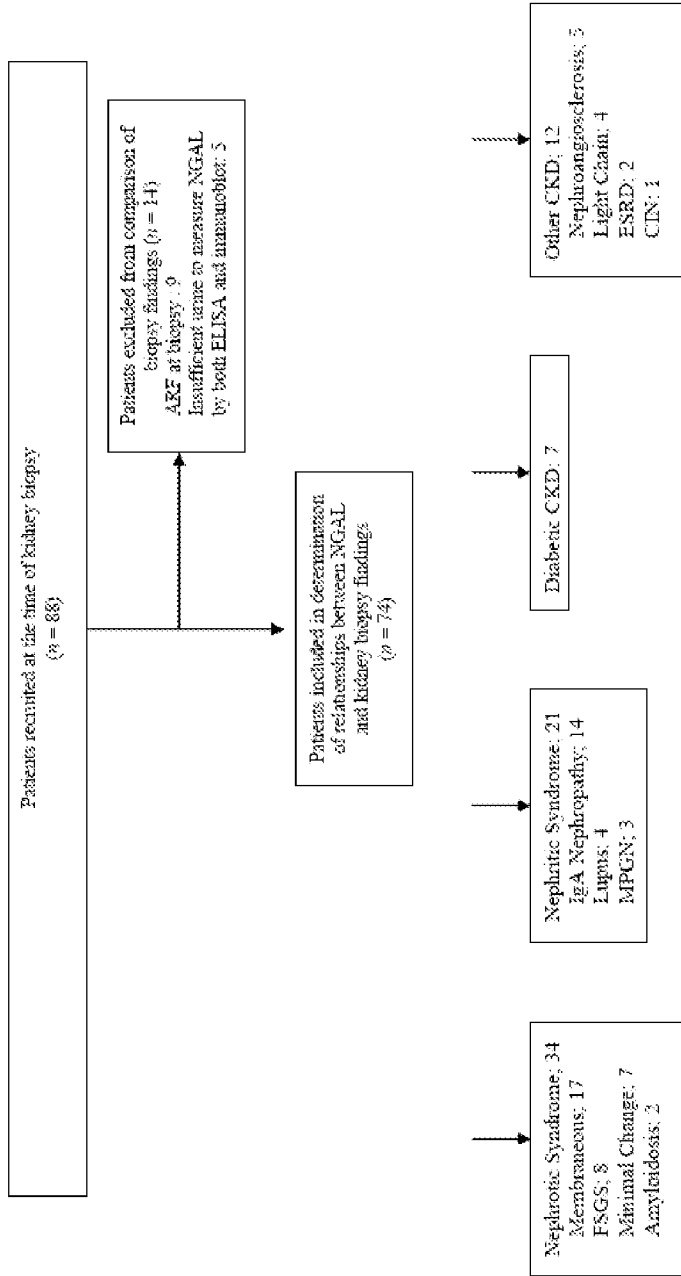
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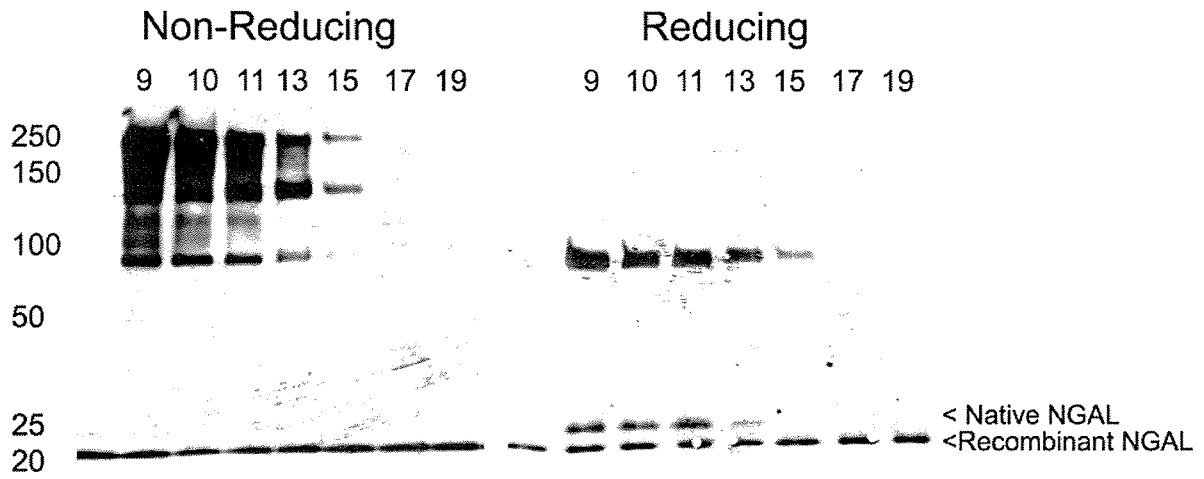
Figure 1

Figure 2

Figure 3

Figure 4

NGAL = Neutrophil Gelatinase associated Lipocalin, ELISA = enzyme-linked immunosorbant assay, FSGS = focal segmental glomerulosclerosis, MPGN = membranoproliferative glomerulonephritis, CKD = chronic kidney disease, ESRD = end-stage renal disease, CIN = chronic intersititial nephritis.