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61/901,837 8 November 2013 (08.11.2013) US(71) Applicant: ICAHN SCHOOL OF MEDICINE AT
MOUNT SINAI [US/US]; One Gustave L. Levy Place,
Box 1030, New York, New York 10029 (US).(72) Inventor: BOTTINGER, Erwin P.; 12 Cottage Place,
White Plains, New York 10601 (US).(74) Agents: LODOVICE, Ian J. et al.; Fish & Richardson
P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022
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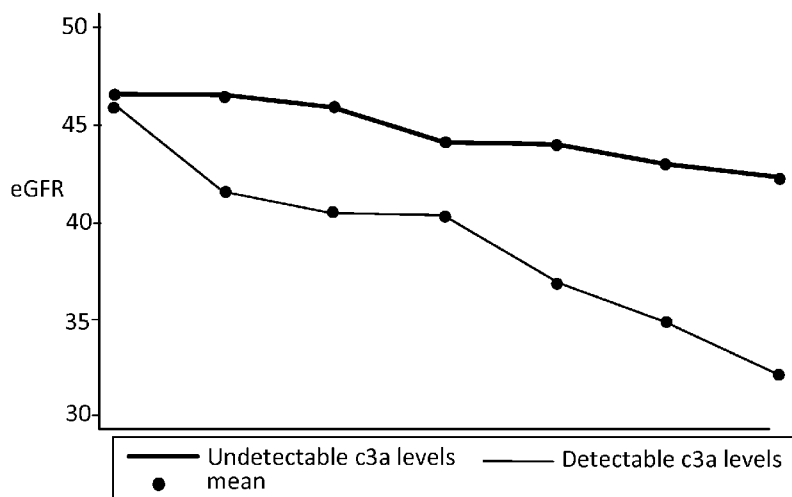


FIG. 1

(57) **Abstract:** Methods and kits for assessing risk of chronic kidney disease (CKD) progression using urine from a subject are described. In some aspects, the subject is a diabetic subject. In one aspect, the invention provides methods for monitoring, diagnosing or assessing the progression of chronic kidney disease (CKD) in a subject. The method comprising the steps of (a) obtaining a first urine sample from a human subject; (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample; (c) obtaining a second urine sample from the human subject, wherein the second sample is obtained following a predetermined time interval.

METHODS FOR MONITORING KIDNEY DYSFUNCTION

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/901,837, filed on November 8, 2013, the entire contents of which are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. DK085688 awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

This invention relates generally to the fields of molecular biology and medicine. More particularly, the present invention relates to methods for assessing the risk of chronic kidney disease progression, as well as methods of screening for molecular biomarkers useful for diagnosing the likelihood of chronic kidney disease progression.

BACKGROUND

Chronic kidney disease (CKD) is characterized by the progressive loss of kidney function over a period of months or years. The severity of CKD is often classified according to the reduction in glomerular filtration rate (GFR). Untreated CKD may further progress into end stage disease wherein endogenous kidney function is irreversibly lost, a condition which renders the patient dependent upon dialysis or kidney transplantation. Approximately 90% of end stage renal disease is attributed to untreated CKD.

CKD is increasing worldwide and is emerging as a major global health threat. The costs of treatment put an enormous burden on health care resources since renal replacement therapy represents one of the most expensive chronic therapies.

Progression of CKD to end-stage renal disease occurs only in a minority of CKD patients, indicating considerable heterogeneity in the risk of progressive decline of renal function in CKD. Because the Kidney Disease Outcomes Quality Initiative

(K/DOQI) of the National Kidney Foundation definition of CKD stages is based on glomerular filtration rate (GFR) alone, the relative risk of progression of patients within each stage is not characterized. As a simple method of risk assessment, family history of advanced CKD or end-stage renal disease and the extent of proteinuria are currently the best predictors of the risk to develop progressive CKD. However, the accuracy of these clinical markers is currently not sufficient to reliably predict the risk of CKD progression, or to guide preventive interventions.

Thus, one of the most important unmet needs in renal medicine is the identification and validation of predictive markers of CKD progression that facilitate targeted treatment of those at high risk, while avoiding unnecessary treatment and the attendant financial costs in low-risk patients. Consequently, there is a need for the identification of additional markers that allow for a risk stratification of patients with respect to acute renal events and/or chronic kidney disease (CKD), including end-stage renal disease and kidney transplant failure (i.e. kidney allograft loss). Thus, development of predictive markers that characterize the risk of CKD progression in individual patients is an important unmet need.

SUMMARY

Methods and kits for assessing risk of chronic kidney disease (CKD) progression using urine from a subject are described. In some aspects, the subject is a diabetic subject.

In one aspect, the invention provides methods for monitoring, diagnosing or assessing the progression of chronic kidney disease (CKD) in a subject. The method comprising the steps of (a) obtaining a first urine sample from a human subject; (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample; (c) obtaining a second urine sample from the human subject, wherein the second sample is obtained following a predetermined time interval; (d) measuring the level of the one or more complement C3 cleavage fragments in the second urine sample; (e) comparing the measured levels in the first urine sample with the measured levels in the second urine sample; and (f) identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of the subject having an increased likelihood (i.e., a high likelihood) of CKD progression, or identifying an

constant level or decrease in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of the subject having a low likelihood or decreased likelihood of kidney dysfunction, chronic kidney disease or diabetic kidney dysfunction progression.

In another aspect, the invention provides methods for monitoring, diagnosing or assessing the progression of kidney dysfunction, CKD or DKD in a subject. The methods comprising the steps of (a) obtaining a first urine sample from a human subject at a first time point; (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample; (c) obtaining a second urine sample from the human subject, wherein the second sample is obtained at a second time point; (d) measuring the level of the one or more complement C3 cleavage fragments in the second urine sample; (e) comparing the measured levels in the first urine sample with the measured levels in the second urine sample; and (f) identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of an increased likelihood (i.e., a high likelihood) of CKD progression, or identifying an constant level or decrease in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of the subject having a low likelihood or decreased likelihood of kidney dysfunction, chronic kidney disease or diabetic kidney dysfunction progression.

In some embodiments, the complement C3 cleavage fragment is C3a.

In some embodiments, the complement C3 cleavage fragment is C5b-9.

In some embodiments, the complement C3 cleavage fragment is iC3b.

In yet another aspect, the invention provides methods for monitoring, diagnosing or assessing the progression of kidney dysfunction, CKD or DKD in a subject, the methods comprising the steps of (a) obtaining a first urine sample from a human subject; (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample; (c) comparing the measured levels to one or more predetermined, statistically significant reference levels; and (d) identifying an increase in the measured levels of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a likelihood of kidney dysfunction,

chronic kidney disease or diabetic kidney dysfunction progression, or identifying an constant level or decrease in the measured levels of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a low likelihood or decreased likelihood of kidney dysfunction, chronic kidney disease or diabetic kidney dysfunction progression.

In some embodiments, a 2- to 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold increase in the level of the one or more complement C3 cleavage fragments in a second urine sample obtained at a second time point compared to the measured levels in a first urine sample taken at a first time point prior to the second time point is indicative of a likelihood of kidney dysfunction, CKD or DKD progression.

In some embodiments, a 2-fold to 10-fold increase in the level of the one or more complement C3 cleavage fragments in a second urine sample obtained at a second time point compared to the measured levels in a first urine sample taken at a first time point prior to the second time point is indicative of a likelihood of kidney dysfunction, CKD or DKD progression.

In some embodiments, the predetermined time interval is about 1 month to about 12 months, about 2 to about 10 months, about 3 to about 8 months, about 3 to about 7 months, about 4 to about 6 months, or about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, or about 12 months.

In some embodiments, the predetermined time interval is about 1 week to about 12 weeks, about 2 to about 10 weeks, about 3 to about 8 weeks, about 3 to about 7 weeks, about 4 to about 6 weeks, or about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, or about 12 weeks.

In some embodiments of any of the foregoing methods, the subject has diabetes. In some embodiments of any of the foregoing methods, the subject has hypertension.

The levels of the one or more complement C3 cleavage fragments can be measured using antibodies, wherein at least one antibody is capable of specifically binding to each of complement C3 cleavage fragments.

In some embodiments, the levels are measured by ELISA, immunoassays, enzymatic assays, mass spectrophotometry, colorimetry, or fluorometry.

According to some embodiments, obtaining the second urine sample from the human subject at a second time point is performed at about 1 month to about 12 months, about 2 to about 10 months, about 3 to about 8 months, about 3 to about 7 months, about 4 to about 6 months, or about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, or about 12 months after obtaining the first urine sample from the human subject at the first time point.

According to some embodiments, obtaining the second urine sample from the human subject at a second time point is performed at about 3 months, or about 6 months after obtaining the first urine sample from the human subject at the first time point.

In some embodiments, obtaining the second urine sample from the human subject at a second time point is performed at about 1 week to about 12 weeks, about 2 to about 10 weeks, about 3 to about 8 weeks, about 3 to about 7 weeks, about 4 to about 6 weeks, or about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, or about 12 weeks after obtaining the first urine sample from the human subject at the first time point.

In some aspects, the methods provided herein further comprises measuring the level of total protein in the first urine sample, second urine sample, or both; measuring the level of total creatinine in the first urine sample, second urine sample, or both; or determining the total protein to creatinine ratio (UPCR) in the first urine sample, second urine sample, or both.

In yet other aspects, the methods provided herein may further comprise administering to a subject having measured levels indicative of a likelihood of CKD progression a compound which inhibits the renin-angiotensin system selected from the group consisting of angiotensin-receptor-blockers selected from the group consisting of candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan, and angiotensin converting enzyme (ACE) inhibitors selected from the group consisting of tamipril, enalapril, lisinopril, and perindopril..

In some aspects, the methods described herein further comprise referring a subject having measured levels indicative of a likelihood of CKD progression to a renal specialist.

In some embodiments of any of the foregoing methods, the methods described herein further comprise modifying the clinical record of a subject having measured levels indicative of a likelihood of CKD progression to identify the subject having an increased likelihood of CKD progression.

In some aspects, the methods described herein further comprise instructing the insurance provider for a subject having measured levels indicative of a likelihood of CKD progression to approve payment for further treatment, specialist diagnostics or specialty care medical practices.

In some embodiments, the reference levels take into account one or more of the subject's age, sex, race, mean arterial blood pressure, body mass index, history of diabetes mellitus, fasting glucose level, hypertension, fasting insulin, blood pressure, glomerular filtration rate, urinary albumin to creatinine ratio (UACR), or urinary total protein to creatinine ratio (UPCR).

In some embodiments, the reference sample is obtained from the same subject. In some embodiments the reference sample is obtained from at least one individual not suffering from CKD. In some other embodiments, the reference sample is obtained from at least one individual previously diagnosed as having a CKD. In some embodiments, the reference sample comprises a predetermined, statistically significant reference levels of C3a, C5b-9 and/or iC3b.

In some embodiments, a 2-fold to 10-fold increase in the level of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a likelihood of CKD progression.

In some aspects, the disclosure provides methods intervening in a human subject's health care by, the methods comprising i) administering a renin-angiotensin compound to a human subject or instructing the human subject to self-administer a renin-angiotensin compound, or ii) referring a human subject to a renal specialist; provided that the human subject is selected for intervention if a urine sample from the subject tested according to any one of claims 1 to 3 is indicative of the subject having a likelihood of CKD progression.

In some aspects, the disclosure provides kits for diagnosing the risk of chronic kidney disease (CKD) progression, comprising one or more antibodies that bind to one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b; and a reagent useful for the detection of a binding between said antibody and said complement C3 cleavage fragment.

In some aspects, the disclosure provides kits for assessing the risk of chronic kidney disease (CKD) progression, comprising (a) one or more reagents suitable for performing an ELISA or mass spectrometry analysis on a urine sample from a human subject to measure a level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b; and (b) instructions to measure the levels of the one or more complement C3 cleavage fragments from a first urine sample collected at a first time point and from a second urine sample collected at a second time point; (c) optionally, one or more control samples; (d) instructions or software for comparing the measured levels at the first time point to the measured levels at the second time point; and (e) instructions or software for identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of a high or increased likelihood of CKD progression, or for identifying a constant level or decrease in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of a low or decreased likelihood of CKD progression,.

In some aspects, the disclosure provides kits for assessing the risk of chronic kidney disease (CKD) progression, comprising (a) one or more reagents suitable for performing an ELISA or mass spectrometry analysis on a urine sample from a human subject to measure a level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b; (b) one or more reference samples predetermined reference levels of the one or more complement C3 cleavage fragments; (c) instructions or software for comparing the measured levels to one or more predetermined reference levels for the same complement C3 cleavage fragments; and (d) instructions or software for identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a likelihood of CKD progression, of for identifying an unchanged or decrease in measured levels of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a likelihood of CKD progression.

As used herein, “obtain” or “obtaining” can be any means whereby one comes into possession of the sample by “direct” or “indirect” means. Directly obtaining a sample means performing a process (e.g., performing a physical method such as extraction) to obtain the sample. Indirectly obtaining a sample refers to receiving the

sample from another party or source (e.g., a third party laboratory that directly acquired the sample). Directly obtaining a sample includes performing a process that includes a physical change in a physical substance, e.g., a starting material, such as a blood, e.g., blood that was previously isolated from a patient. Thus, obtain is used to mean collection and/or removal of the sample from the subject. Furthermore, “obtain” is also used to mean where one receives the sample from another who was in possession of the sample previously.

As used herein, the term “one or more” includes at least one, more suitably, one, two, three, four, five, ten, twenty, fifty, one-hundred, five-hundred, etc., of the item to which “one or more” refers.

The terms “first” and “second” are used in this disclosure in their relative sense only. It will be understood that, unless otherwise noted, those terms are used merely as a matter of convenience in the description of one or more of the embodiments. The terms “first” and “second” are only used to distinguish one element from another element, and the scope of the rights of the disclosed technology should not be limited by these terms. For example, a first element may be designated as a second element, and similarly the second element may be designated as the first element.

The term “subject” refers to an animal or human, or to one or more cells derived from an animal or human. Preferably, the subject is a human. Subjects can also include non-human primates. Cells may be in any form, including but not limited to cells retained in tissue, cell clusters, immortalized, transfected or transformed cells, and cells derived from an animal that has been physically or phenotypically altered. A human subject can be known as an “individual” or as a “patient.”

The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. When definitions of terms in incorporated references appear to differ from the definitions provided in the present teachings, the definition provided in the present teachings shall control. It will be appreciated that there is an implied “about” prior to metrics such as temperatures, concentrations, and times discussed in the present teachings, such that slight and insubstantial deviations are within the scope of the present teachings herein. In this application, the use of the singular includes the plural unless specifically stated otherwise. Also, the use of “comprise,” “comprises,” “comprising,” “contain,” “contains,” “containing,” “include,” “includes,” and “including” are not

intended to be limiting. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention. The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1 is a graph showing the change in eGFR over time stratified by detectable c3a levels.

Figure 2 is a graph eGFR's at different time points stratified by c3a tertiles in patients with detectable levels.

Figure 3 is a graph showing the change in eGFR over time stratified by detectable c5b9 levels.

Figure 4 is a graph showing eGFR's at different time points stratified by c5b9 tertiles in patients with detectable levels.

Figure 5 is a graph showing the change in eGFR over time stratified by detectable ic3b levels.

Figure 6 is a graph showing the change in eGFR over time stratified by ic3b tertiles.

Figure 7 is a graph showing LogC3a/Cr; LogC5b-9/Cr; and LogiC3b/Cr ($\mu\text{g}/\text{mg}$) values obtained from urine samples obtained from control patients, patients with abnormal kidney function, and patients with chronic kidney disease ($\text{C3a}/\text{c} = \text{LogC3a}/\text{Cr}$ (ng/mg); $\text{C5b-9}/\text{c} = \text{LogC5b-9}/\text{Cr}$ (ng/mg); $\text{iC3b}/\text{c} = \text{LogiC3b}/\text{Cr}$ ($\mu\text{g}/\text{mg}$) (average $\pm$ S.E.M.))

Figure 8 is a graph showing the percent of patient samples having detectable levels of C3a, C5b-9 and iC3b (control; Abn Kid Fct = abnormal kidney function; CKD = chronic kidney disease).

Figure 9 is a graph showing the fraction of patients with detectable fragments (a = T2D-Ctrl (N=41); b - T2D-Abn Kid Fct (N=96); c = T2D-CKD Non-Prog (N=24); d = T2D-CKD Prog (N=54)).

Figure 10 is a graph showing the ratio of C3a to creatinine (C3a/Cr) obtained from urine samples (pg/mg) (a = control; b = abnormal kidney function; c = non-progressive CKD; d = progressive CKD).

Figure 11 is a graph showing the ratio of C5b-9 to creatinine (C5b-9) obtained from urine samples (pg/mg) (a = control; b = abnormal kidney function; c = non-progressive CKD; d = progressive CKD).

Figure 12 is a graph showing the ratio of iC3b to creatinine (C5b-9) obtained from urine samples (pg/mg) (a = control; b = abnormal kidney function; c = non-progressive CKD; d = progressive CKD).

DETAILED DESCRIPTION

To begin to overcome these challenges to the development of urgently needed predictive markers of CKD progression, the present disclosure provides biomarkers for Chronic Kidney Diseases (CKD) and kits thereof, as well as methods of screening for biomarkers, and methods of assessing the risk of progression of CKD.

Method and kits for assessing risk of kidney dysfunction, chronic kidney disease (CKD) or diabetic kidney disease (DKD) using urine from a subject are described. In some aspects, the subject is a diabetic subject. The inventive methods comprise assaying a urine sample obtained from a subject, for example a diabetic or hypertensive human subject, for complement C3a cleavage fragments differentially produced in urine from subjects deteriorating (e.g., progressing) CKD.

In some aspects, the present invention provides methods for assaying complement C3 fragments C3a, C5B9, iC3b in urine, and kits useful in performing said methods.

The complement system is part of an innate system that provides an organism a natural defense against microbial agents and infection without the need for a specific antibody. The complement system consists of a number of small plasma proteins, generally synthesized by the liver, and normally circulating as inactive precursors (pro-proteins). When stimulated by one of several triggers (e.g., microbial polysaccharides or lipids, gram-negative bacterial lipopolysaccharides, and surface determinants present on some viruses, parasites, virally-infected mammalian cells, and cancer cells), proteases in the complement system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The end-result of this activation cascade is massive amplification of the response and activation of the cell-killing membrane attack complex (MAC). Over 25 proteins and protein fragments make up the complement system.

Numerous studies indicate that the complement system is a fundamental element of normal host defense mechanisms. As a consequence, complement activation is commonly associated with a variety of pathological states. Because of these correlations clinical laboratory methods that detect complement activation are useful in diagnosing certain disease conditions.

Complement activation can occur by either of two primary modes known as the “classical” pathway and the “alternative” pathway, respectively. These different pathways are generally distinguished according to the process which initiates complement activation. Activation via the classical pathway is usually associated with an immunologic stimulus whereas activation via the alternative pathway is most commonly associated with non-immunologic stimuli. A third pathway, the “lectin pathway” is activated by the binding of acute phase reactant mannose-binding protein (MBP; or mannose-binding lectin, MBL) to a complex carbohydrate. Regardless of the initiating stimulus each of the pathways converge, followed by the conversion of the C3 component of complement into its C3a and C3b fragments via a C3 convertase enzyme. The C3b fragment may then be further cleaved, via alternate pathways, to yield C5b-9 and iC3b.

The results provided herein introduce the novel finding that complement activation products C3a, C5b-9 and iC3b are excreted in the urine in association with deteriorating kidney disease (i.e., CKD progression).

For the purposes of the present disclosure, the methods and kits provided herein for assessing the progression of CKD are inclusive of methods for assessing the progression of diabetic kidney disease (DKD) in an subject, i.e. a human subject, with diabetes, of methods for assessing the progression of chronic kidney disease (CKD) in an subject, i.e. a human subject, with hypertension, and/or kidney transplant failure in a subject.

The term “determining the progression of chronic kidney disease (CKD)” or “assessing the progression of chronic kidney disease (CKD)” as used herein generally refers to the assessment of kidney function in a subject including the assessment of end-stage disease. In particular, it refers to the assessment of a subject’s kidney functions and/or how severe a subject’s kidney disease is. Standard parameters that are routinely used for diagnosing kidney disease in a patient are well known in the art and familiar to the skilled person. In the present context, the term “end-stage disease” is equivalently used to kidney and/or renal failure. The term “determining kidney transplant failure” as used herein generally refers to the assessment of kidney transplant rejection including the risk that a kidney transplant that has before been implanted into a subject in need thereof will be rejected and/or is not well accepted by the patient's organism. In the context of the present invention, the term “kidney transplant failure” also refers to and is equally used for kidney allograft loss. Therefore, preferably, the method of the invention also includes assessing the risk of chronic kidney disease progression and/or assessing the risk of kidney transplant failure (i.e. kidney allograft loss). Accordingly, in a preferred embodiment, the method of the invention further comprises determining the risk of chronic kidney disease (CKD) progression and/or the risk of kidney transplant failure in a patient.

The term “kidney disease” is used herein to refer to any condition that greatly reduces the function of the kidneys in removing waste products and excess fluid from the body. Symptoms of a kidney disease may include burning or difficulty during urination, an increase in the frequency of urination, passage of blood in the urine, volume retention (e.g. puffiness around the eyes, swelling of the hands and feet), pain in the small of the back just below the ribs, high blood pressure, a reduction in the glomerular filtration rate (GFR), and albuminuria or proteinuria.

By “chronic kidney disease” (CKD) it is meant a kidney disease in which the decline in kidney function is slow and progressive. There are five stages of CKD. Stage 1 CKD is slightly diminished function, observed as a normal or relatively high glomerular filtration rate (GFR) (>90 mL/min/1.73 m²) and kidney damage defined as pathological abnormalities or markers of damage, including abnormalities in blood or urine test or imaging studies. Stage 2 CKD (mild CKD) presents as a mild reduction in GFR (60-89 mL/min/1.73 m²) with evidence of kidney damage defined as above. Stage 3 CKD (moderate CKD) is observed as a moderate reduction in GFR (30-59 mL/min/1.73 m²). Stage 4 CKD (severe CKD) is observed as a severe reduction in GFR (15-29 mL/min/1.73 m²). Stage 5 CKD, also known as End Stage Renal Disease (ESRD), is established kidney failure (GFR <15 mL/min/1.73 m² and requires permanent renal replacement therapy (RRT, including maintenance dialysis or renal transplantation). Although chronic kidney disease (CKD) is common, only a fraction of CKD patients progress to end-stage renal disease.

In some aspects, the progression of chronic kidney disease (CKD) in a patient according to the present invention also includes end-stage renal disease.

By “glomerular filtration rate” or GFR it is meant the flow rate of filtered fluid through the kidney. In other words, it is the volume of fluid filtered from the renal (kidney) glomerular capillaries into the Bowman's capsule per unit time. GFR may be determined by a number of different techniques. For example, inulin or the inulin-analogon sinistrin may be injected into the plasma and its excretion in urine measured. As another example, GFR may be approximated based on determined (CCr) or estimated (eCCr) rate of creatinine clearance from the body using any convenient methodology. GFR in a normally functioning kidney is typically above 90 mL/min/1.73 m² and no proteinuria

By “proteinuria” it is meant the presence of excessive amounts of serum protein in the urine. Proteinuria is a characteristic symptom of either renal (kidney), urinary, pancreatic distress, nephrotic syndromes (i.e., proteinuria larger than 3.5 grams per day), eclampsia, toxic lesions of kidneys, and it is frequently a symptom of diabetes mellitus. With severe proteinuria general hypoproteinemia can develop and it results in diminished oncotic pressure (ascites, edema, hydrothorax). Nonlimiting examples of methods for detecting proteinuria include a urinalysis for protein, e.g. a quantitative protein determination in a timed urine collection or the ratio of protein

levels relative to creatinine levels in a random urine collection, or by a foamy appearance or excessive frothing of the urine.

“Diabetic kidney disease” and “Diabetic nephropathy” are used interchangeably herein to mean a chronic kidney disease caused by or associated with diabetes. Symptoms of diabetic kidney disease include the occurrence of microalbuminuria or macroalbuminuria, or the progressive decline of GFR in a normoalbuminuric individual with any form of diabetes.

By “early stage” diabetic kidney disease, or “early stage” diabetic nephropathy it is meant diabetic kidney disease with normoalbuminuria or microalbuminuria and normal or high GFR, i.e. a GFR of 90 mL/min/1.73 m² or more.

By “progressive” diabetic kidney disease or “progressive” diabetic nephropathy it is meant diabetic kidney disease with macroalbuminuria or with stage 2 chronic kidney disease (CKD) or worse, i.e., a glomerular filtration rate (GFR) of less than 90 cc/min/1.73 m².

As used herein, the term “diagnosing” refers to identifying a subject as having a particular disease or disorder or identifying a subject as having a risk of disease or disorder progression based on numerous criteria including, but not limited to, complement C3a fragments levels in urine, protein expression level, subject symptoms, and/or family history.

As used herein, the phrase “risk of CKD progression” refers to the probability of CKD progression in subjects based on one or more of numerous criteria including, but not limited to, complement C3a fragment levels in urine, protein expression level, subject symptoms, and/or family history. In some embodiments described herein, the risk of CKD progression is based on levels of complement C3a fragments in urine, as disclosed herein.

The terms “decrease”, “decreased”, “reduced”, “reduction” or “down-regulated” are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, “reduced”, “reduction”, “decreased” or “decrease” means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level, or at least about a 0.5-fold, or at

least about a 1.0-fold, or at least about a 1.2-fold, or at least about a 1.5-fold, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold decrease, or any decrease between 1.0-fold and 10-fold or greater as compared to a reference level.

Unless specifically stated or obvious from context, as used herein, the term “about” is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless obvious from context, all numerical values provided herein can be understood to be modified by the term about.

The terms “increased”, “increase” or “up-regulated” are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms “increased” or “increase” means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 0.5-fold, or at least about a 1.0-fold, or at least about a 1.2-fold, or at least about a 1.5-fold, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 1.0-fold and 10-fold or greater as compared to a reference level.

Thus, an increase or decrease compared to a control reference sample is understood as having a level an analyte (e.g., a complement C3a fragment) is statistically different than a sample from a normal, untreated, or control sample. Methods to select and test control samples are within the ability of those in the art.

As used herein, levels refer to the amount or concentration of an analyte in a sample (e.g., a urine sample) or subject. Whereas, occurrence refers to the presence or absence of a detectable analyte in a sample. Thus, level is a continuous indicator of amount, whereas occurrence is a binary indicator of an analyte. In some cases, an occurrence may be determined using a threshold level above which a biomarker is present and below which a biomarker is absent.

The term “determining the amount of each analyte” as used herein refers to determining at least one characteristic feature of the at least one analyte (e.g., a complement C3a fragment) comprised by the sample referred to herein. Characteristic

features in accordance with the present invention are features which characterize the physical and/or chemical properties including biochemical properties of an analyte. Such properties include, e.g., molecular weight, viscosity, density, electrical charge, spin, optical activity, elementary composition, chemical structure, capability to react with other compounds, capability to elicit a response in a biological read out system (e.g., induction of a reporter gene) and the like. Values for said properties may serve as characteristic features and can be determined by techniques well known in the art. Moreover, the characteristic feature may be any feature which is derived from the values of the physical and/or chemical properties of an analyte by standard operations, e.g., mathematical calculations such as multiplication, division or logarithmic calculus. Most preferably, the at least one characteristic feature allows the determination and/or chemical identification of the said complement C3 cleavage fragments.

The analyte (e.g., complement C3 cleavage fragments selected from C3a, C5b-9 and iC3b) comprised by the urine sample may be determined in accordance with the present invention quantitatively or qualitatively. For qualitative determination, the presence or absence of the analyte will be determined by a suitable technique. Moreover, qualitative determination may, preferably, include determination of the chemical structure or composition of the analyte. For quantitative determination, either the precise amount of the analyte(s) present in the sample will be determined or the relative amount of the analyte(s) will be determined, preferably, based on the value determined for the characteristic feature(s) referred to herein above. The relative amount may be determined in a case where the precise amount of an analyte can or shall not be determined. In said case, it can be determined whether the amount in which the analyte(s) is present is enlarged or diminished with respect to a second sample comprising said analyte(s) in a second amount. Quantitatively analyzing an analyte(s), thus, also includes what is sometimes referred to as semi-quantitative analysis of a metabolite.

The levels of the complement C3a fragment(s) for a subject can be obtained by any art recognized method. According to the embodiments provided herein, the level is “determined” or “measured” by measuring the complement C3a fragment in urine. The level can be determined by any method known in the art, e.g., ELISA, immunoassays, enzymatic assays, spectrophotometry, colorimetry, fluorometry,

bacterial assays, compound separation techniques, or other known techniques for determining the presence and/or quantity of an analyte.

For some methods contemplated herein, the levels of the one or more complement C3 cleavage fragments can be measured using one or more antibodies capable of specifically binding to at least one complement C3 cleavage fragment as described herein. The term “antibody” is used in the broadest sense and includes fully assembled antibodies, monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), antibody fragments that can bind antigen (e.g., Fab', F'(ab)₂, Fv, single chain antibodies, diabodies), and recombinant peptides comprising the forgoing as long as they exhibit the desired biological activity.

Compound separation techniques yield a time resolved separation of the analytes comprised by the sample. Suitable techniques for separation to be used include, for example, all chromatographic separation techniques such as liquid chromatography (LC), high performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography, size exclusion or affinity chromatography. These techniques are well known in the art and can be applied by the person skilled in the art. In some embodiments, the methods utilize LC and/or GC chromatographic techniques including, for example, gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), direct infusion mass spectrometry or Fourier transform ion-cyclotron-resonance mass spectrometry (FT-ICR-MS), capillary electrophoresis mass spectrometry (CE-MS), high-performance liquid chromatography coupled mass spectrometry (HPLC-MS), quadrupole mass spectrometry, any sequentially coupled mass spectrometry, such as MS-MS or MS-MS-MS, inductively coupled plasma mass spectrometry (ICP-MS), pyrolysis mass spectrometry (Py-MS), ion mobility mass spectrometry or time of flight mass spectrometry (TOF). In some embodiments, LC-MS and/or GC-MS. As an alternative or in addition to mass spectrometry techniques, the following techniques may be used for compound determination: nuclear magnetic resonance (NMR), magnetic resonance imaging (MRI), Fourier transform infrared analysis (FT-IR), ultra violet (UV) spectroscopy, refraction index (RI), fluorescent detection, radiochemical detection, electrochemical detection, light scattering (LS), dispersive Raman spectroscopy or flame ionization detection (FID). These techniques are well known to the person skilled in the art and can be applied without further ado. In some

embodiments, the methods disclosed herein shall be, optionally, assisted by automation. For example sample processing or pre-treatment can be automated by robotics. Data processing and comparison can be assisted by suitable computer programs and databases. Automation as described herein before allows using the method of the present invention in high-throughput approaches.

Conventional “determining” or “measuring” methods include sending a clinical sample(s) to a commercial laboratory for measurement or the use of commercially available assay kits. Commercially available assay kits are known in the art. For example, Quidel’s MicroVue C3a Plus EIA, MicroVue iC3b EIA and MicroVue SC5b 9 Plus EIA are exemplary suppliers of such assays. Exemplary kits and suppliers will be apparent to the skilled artisan.

In some cases, the methods disclosed herein involve comparing levels or occurrences to a reference. The reference can take on a variety of forms. In some cases, the reference comprises predetermined values for the plurality of analytes (e.g., complement C3a fragments C3a, C5b-9 and/or iC3b). The predetermined value can take a variety of forms. It can be a level or occurrence of an analyte obtained from a subject known to have CKD (e.g., a symptomatic subject), or obtained from a subject known not to suffer from CKD (e.g., an asymptomatic subject). It can be a level or occurrence of an analyte obtained from a subject having no previous history of kidney disease. It can be a level or occurrence in the same subject, e.g., at a different time point. A predetermined value that represent a level(s) of an analyte is referred to herein as a predetermined level. A predetermined level can be single cut-off value, such as a median or mean. It can be a range of cut-off (or threshold) values, such as a confidence interval. It can be established based upon comparative groups, such as where the risk in one defined group is a fold higher, or lower, (e.g., approximately 2-fold, 4-fold, 8-fold, 16-fold or more) than the risk in another defined group. It can be a range, for example, where a population of subjects (e.g., control subjects) is divided equally (or unequally) into groups, such as a low-risk group, a medium- risk group and a high-risk group, or into quartiles, the lowest quartile being subjects with the lowest risk and the highest quartile being subjects with the highest risk, or into n-quantiles (i.e., n regularly spaced intervals) the lowest of the n-quantiles being subjects with the lowest risk and the highest of the n-quantiles being subjects with the highest risk. Moreover, the reference could be a calculated reference, most preferably the average or median, for the relative or absolute amount of an analyte of a

population of individuals comprising the subject to be investigated. The absolute or relative amounts of the analytes of said individuals of the population can be determined as specified elsewhere herein. How to calculate a suitable reference value, preferably, the average or median, is well known in the art. The population of subjects referred to before shall comprise a plurality of subjects, preferably, at least 5, 10, 50, 100, 1,000 or 10,000 subjects. It is to be understood that the subject to be diagnosed by the method of the present invention and the subjects of the said plurality of subjects are of the same species.

Subjects associated with predetermined values are typically referred to as control subjects (or controls). A control subject may or may not have suffer from CKD. In some cases it may be desirable that control subject is a symptomatic subject, and in other cases it may be desirable that a control subject is an asymptomatic subject. Thus, in some cases the level of an analyte in a subject being greater than or equal to the level of the analyte in a control subject is indicative of a clinical status (e.g., indicative of CKD progression). In other cases the level of an analyte in a subject being less than or equal to the level of the analyte in a control subject is indicative of a clinical status. The amount of the greater than and the amount of the less than is usually of a sufficient magnitude to, for example, facilitate distinguishing a subject from a control subject using the disclosed methods. Typically, the greater than, or the less than, that is sufficient to distinguish a subject from a control subject is a statistically significant greater than, or a statistically significant less than. In cases where the level of an analyte in a subject being equal to the level of the metabolite in a control subject is indicative of a clinical status, the “being equal” refers to being approximately equal (e.g., not statistically different).

The predetermined value can depend upon a particular population of subjects (e.g., human subjects) selected. For example, an apparently healthy population will have a different ‘normal’ range of a complement C3a fragment levels in urine than will a population of subjects which have, or are likely to have, CKD. Accordingly, the predetermined values selected may take into account the category (e.g., healthy, at risk, diseased) in which a subject (e.g., human subject) falls. Appropriate ranges and categories can be selected with no more than routine experimentation by those of ordinary skill in the art. In some cases a predetermined value of a biomarker is a value that is the average for a population of healthy subjects (human subjects) (e.g., human subjects who have no apparent signs and symptoms of CKD). The predetermined

value will depend, of course, on the particular analyte (biomarker) selected and even upon the characteristics of the population in which the subject lies. In characterizing likelihood, or risk, numerous predetermined values can be established.

A level, in some embodiments, may itself be a relative level that reflects a comparison of levels between two states. Relative levels that reflect a comparison (e.g., ratio, difference, logarithmic difference, percentage change, etc.) between two states (e.g., healthy and diseased) may be referred to as delta values. The use of relative levels is beneficial in some cases because, to an extent, they exclude measurement related variations (e.g., laboratory personnel, laboratories, measurements devices, reagent lots/preparations, assay kits, etc.). However, the invention is not so limited.

Analyte levels (e.g., complement C3 cleavage fragments) and/or reference levels of complement C3 cleavage fragments may be stored in a suitable data storage medium (e.g., a database) and are, thus, also available for future diagnoses. This also allows efficiently diagnosing prevalence for a disease because suitable reference results can be identified in the database once it has been confirmed (in the future) that the subject from which the corresponding reference sample was obtained did have CKD. As used herein a “database” comprises data collected (e.g., analyte and/or reference level information and /or patient information)) on a suitable storage medium. Moreover, the database, may further comprises a database management system. The database management system is, preferably, a network-based, hierarchical or object-oriented database management system. Furthermore, the database may be a federal or integrated database. More preferably, the database will be implemented as a distributed (federal) system, e.g. as a Client-Server-System. More preferably, the database is structured as to allow a search algorithm to compare a test data set with the data sets comprised by the data collection. Specifically, by using such an algorithm, the database can be searched for similar or identical data sets being indicative of CKD (e.g. a query search). Thus, if an identical or similar data set can be identified in the data collection, the test data set will be associated CKD. Consequently, the information obtained from the data collection can be used to diagnose the progression of CKD or based on a test data set obtained from a subject. More preferably, the data collection comprises characteristic values of all analytes comprised by any one of the groups recited above.

In some embodiments, the methods disclosed herein further comprise modifying the subject's clinical record to indicate the measured levels of the analyte(s) or to identify the subject as having progressing CKD. The clinical record maybe be stored in any suitable data storage medium (e.g., a computer readable medium).

The invention also provides kits for evaluating analyte biomarkers in a subject. The kits of the invention can take on a variety of forms. Typically, the kits will include reagents suitable for determining levels of the analyte(s) (e.g., those disclosed herein) in a sample. Optionally, the kits may contain, one or more control samples. Typically, a comparison between the levels of the analyte(s) in the subject and levels of the analyte(s) in the control samples is indicative of a clinical status (e.g., CKD progression). Also, the kits, in some cases, will include written information (indicia) providing a reference (e.g., predetermined values), wherein a comparison between the levels of the analyte(s) in the subject and the reference (predetermined values) is indicative of a clinical status. In some cases, the kits comprise software useful for comparing analyte levels or occurrences with a reference (e.g., a prediction model). Usually the software will be provided in a computer readable format such as a compact disc, but it also may be available for downloading via the internet. However, the kits are not so limited and other variations with will apparent to one of ordinary skill in the art.

The present methods can also be used for selecting a treatment and/or determining a treatment plan for a subject, based on the occurrence or levels of certain analytes relevant to CKD. In some embodiments, using the method disclosed herein, a health care provider (e.g., a physician) identifies a subject as having or being at risk of having for having CKD progression and, based on this identification the health care provider determines an adequate treatment plan for the subject. In some embodiments, the methods further include administering the treatment to the subject.

In some embodiments, the invention relates to identifying subjects who are likely to have successful treatment with a particular drug dose, formulation and/or administration modality. Other embodiments include evaluating the efficacy of a drug using the methods of the present invention. In some embodiments, the methods are useful for identifying subjects who are likely to have successful treatment with a particular drug or therapeutic regiment. For example, during a study (e.g., a clinical study) of a drug or treatment, subjects who have CKD may respond well to the drug

or treatment, and others may not. Disparity in treatment efficacy is associated with numerous variables, for example genetic variations among the subjects. In some embodiments, subjects in a population are stratified based on the methods disclosed herein. In some embodiments, resulting strata are further evaluated based on various epidemiological, and or clinical factors (e.g., response to a specific treatment). In some embodiments, stratum, identified based on a metabolic profile, reflect a subpopulation of subjects that response predictably (e.g., have a predetermined response) to certain treatments. In further embodiments, samples are obtained from subjects who have been subjected to the drug being tested and who have a predetermined response to the treatment. In some cases, a reference can be established from all or a portion of the analytes from these samples, for example, to provide a reference metabolic profile. A sample to be tested can then be evaluated (e.g., using a prediction model) against the reference and classified on the basis of whether treatment would be successful or unsuccessful. A company and/or person testing a treatment (e.g., compound, drug, and life-style change) could discern more accurate information regarding the types or subtypes of CKD for which a treatment is most useful. This information also aids a healthcare provider in determining the best treatment plan for a subject.

In some embodiments, treatment for the CKD is to administer to the subject a composition comprising an effective amount of at therapeutic agent and/or to instruct the subject to adopt at least one therapeutic lifestyle change (e.g., change in diet or exercise). Therapeutic compounds suitable for treating (e.g., slowing the progression of) CKD are well known in the art and some are disclosed herein. Non-limiting examples include compounds which inhibits the renin-angiotensin system (e.g., an angiotensin-receptor-blocker, an ACE-inhibitor, or a vasopeptidase inhibitor). Appropriate lifestyle changes to improve a subjects overall health and well-being are also well known in the art. Non-limiting examples include increased physical activity, caloric intake restriction, nutritional meal planning, and weight reduction. However, the invention is not so limited and other appropriate treatments will be apparent to one of ordinary skill in the art.

In one aspect, the methods provided herein comprise administering a compound which inhibits the renin-angiotensin system selected from the group consisting of angiotensin-receptor-blockers and angiotensin converting enzyme (ACE) inhibitors. Angiotensin receptor-blockers include, for example, compounds

selected from the group consisting of candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan. Angiotensin converting enzyme (ACE) inhibitors include, for example, compounds selected from the group consisting of tamipril, enalapril, lisinopril, and perindopril, and vasopeptidase inhibitors.

When a therapeutic agent or other treatment is administered, it is administered in an amount effective to treat CKD or delay the progression of CKD. An effective amount is a dosage of the therapeutic agent sufficient to provide a medically desirable result. The effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration and the like factors within the knowledge and expertise of the health care practitioner. For example, an effective amount can depend upon the degree to which a subject has abnormal levels of certain analytes (e.g., analytes as described herein) that are indicative of progressing CKD. It will be recognized when the therapeutic agent is used in acute circumstances, it is used to prevent one or more medically undesirable results that typically flow from such adverse events. Methods for selecting a suitable treatment and an appropriate dose thereof will be apparent to one of ordinary skill in the art

The invention further provides for the communication of assay results or diagnoses or both to technicians, physicians or patients, for example. In certain embodiments, computers will be used to communicate assay results or diagnoses or both to interested parties, e.g., physicians and their patients.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

The following materials and methods outlined in Examples 1-3 were used in the experiments described in Example 4. The following materials and methods outlined in Examples 1-3 and 5 were used in the experiments described in Examples 6-8.

Example 1: Development of modified SOP for detection of C3a in human urine adapting Quidel C3a Sandwich ELISA kit.

The level of the complement fragment C3a in urine was assessed using a Quidel MicroVue C3a Plus Enzyme Immunoassay ("EIA") kit ("the Quidel C3a kit"), with the following modifications. As indicated in the manufacturer's manual, the

Quidel C3a kit has only been validated for detection of C3a in human plasma or serum. As described below, significant modifications were developed by the inventor to achieve satisfactory assay performance for C3a detection in human urine

C3a is generated during the activation of the complement system via the classical or alternative pathway. C3 convertase cleaves the Complement component C3 to C3a and C3b. The anaphylatoxin C3a itself is very short lived and in serum is cleaved rapidly to the more stable C3a-desArg. C3a, the subject of this ELISA kit is a 77 amino acid protein which is rapidly stabilized by the serum enzyme carboxypeptidase N to a more stable, 76 amino acid form C3a des-Arg. Therefore, quantitation of C3a-desArg allows reliable conclusions about the level of complement activation in a test sample. For convenience, we refer to both forms as C3a.

The Quidel C3a kit is a three step procedure utilizing (1) a microassay plate coated with a murine monoclonal antibody specific for a neo-epitope on human C3a, (2) an HRP-conjugated polyclonal antibody to the C3a region of C3, and (3) a chromogenic substrate.

In Step 1, Standards, Controls and diluted test specimens are added to the assay wells coated with a murine monoclonal antibody to C3a. The monoclonal antibody binds to C3a in the Standards, Controls or specimens. After the incubation period, a wash cycle removes any unbound material.

In Step 2, horseradish peroxidase (HRP)- conjugated anti-C3(C3a) is added to each assay well. The enzyme conjugated anti-C3(C3a) binds to the immobilized C3a captured in the first step. After the incubation period, a wash cycle removes any unbound conjugate.

In Step 3, 3,3',5,5' tetramethylbenzidine (TMB), a ready-to-use, chromogenic substrate solution, is added to the assay wells. The bound HRP reacts with the substrate, forming a blue color. After the incubation period, the reaction is stopped chemically, which results in a color change from blue to yellow, confirming that the reaction has taken place. The color intensity is measured spectrophotometrically at A₄₅₀. The color intensity of the reaction mixture is proportional to the concentration of C3a present in the Standards, Controls and diluted test specimens. Results are calculated from the generated standard curve using 4-parameter analysis.

Reagents Provided in the Quidel C3a kit:

- C3a Plus Standards (5.4, 2.46, 0.52, 0.21, and 0.05 ng/ml)

- ELISA plate coated with a mouse monoclonal antibody specific for a neo-epitope on human C3a.
- Horseradish Peroxidase-conjugated polyclonal antibody to the C3a region of C3
- TMB Substrate. Ready to use. Contains 3,3',5,5'-tetramethylbenzidine (TMB) and
- Hydrogen Peroxide
- 20 x Wash Solution concentrate (1 x PBS 0.05% Tween-20, Proclin 300)
- Stop Solution (1N HCl)

Modified ELISA Assay

Urine samples are received within 10 hours of collection from 4°C storage. 1 ml aliquots are made and placed at -80°C until required. For an assay a 1 ml aliquot is thawed in room temperature water for quick thaw and subsequently stored on ice until used up (sufficient for eight assays). When thawed mix, brief vortex agitation until urine vortex reaches bottom of tube followed by brief centrifuge ~ 10 rcf (Benchtop centrifuge at 13 krpm) for 5 minutes.

C3a Plus Standards (5.4, 2.46, 0.52, 0.21, and 0.05 ng/ml) are supplied by the manufacturer ready to use. Standard curves with the C3a Plus Standards were prepared according to the manufactures instructions.

Loading the urine analyte

The Quidel kit C3a assay plate was prepared by adding ~300 µl of wash buffer to each well and incubating for 2 min at room temp. A template plate was prepared by adding 120 µl aliquots of undiluted urine analyte and protein standards into the wells of a Polypropylene template plate, followed by loading of the ELISA plate. The ELISA plate is covered with an adhesive plate sealer, and shaken on the Bellco orbital shaker at setting 7 (about 80 revolutions of the table per minute) for 1 h at room temperature (18-25°C).

Following incubation of the samples a room temperature, samples are aspirated and the urine and standards are discarded. 200 µl of Wash Solution (PBS or Wash Solution) was added to each well, and incubated wash for 1 min. Aspirate the solution with the multichannel pipette and perform three additional washes.

50 µl of the C3a conjugate was dispensed into each well with the multichannel pipette and incubate with shaking for 30 min at room temp with shaking. Wash the plate five times (200 µl of PBS or Wash Solution).

100 µl of the TMB Substrate into each well with the multichannel pipette, and incubated in a closed, empty drawer for 15 min with occasional very gentle tapping to

mix the reaction components and disperse the color. At $t = 15$ minutes, 100 μl /well of Stop Solution was added to each well to stop development.

Data Capture and analysis

The color intensity (A_{450}) of the urine and standards were assessed using a Perkin Elmer 1420 Multilabel Counter and plate reader to determine concentrations.

Quality control of the data

Coefficient of Variation (CV) is derived from the ratio of the standard deviation to the non-zero mean of duplicate concentration values, i.e. Coefficient of Variation $CV = \text{Standard Deviation} / \text{Mean}$. CV is expressed as percentage by multiplying CV by 100. For any sampled assay, a %CV value of more than 20% is a threshold for removal of the value and scheduling for repeat measurement of this sample in a subsequent assay.

Example 2: Development of modified SOP for detection of C5b-9 in human urine adapting Quidel C5b-9 Sandwich ELISA kit.

The level of the complement fragment C5b-9 in urine was assessed using Quidel MicroVue Complement SC5b-9 Plus EIA kit (“the Quidel SC5b-9 kit”), with the following modifications. As indicated in the manufacturer’s manual, the Quidel SC5b-9 kit measures the amount of the SC5b-9 complex present in human plasma or serum specimens. As described below, significant modifications were developed by the inventor to achieve satisfactory assay performance for SC5b-9 detection in human urine

As the kit instructions indicates the Terminal Complement Complex (TCC, SC5b-9) is generated by the assembly of C5 through C9 as a consequence of activation of the complement system by either the classical, lectin or alternative pathway. The membrane attack complex (MAC), a form of TCC, is a stable complex that mediates the irreversible target cell membrane damage associated with complement activation. For purposes of this document, we refer to all forms of stable Terminal Complement Complex interchangeably as TCC and SC5b-9, recognizing that other complement regulatory proteins, like Clusterin, also form these stable complexes and are detectable in the (“the Quidel SC5b-9 kit).

This ELISA kit uses a mouse monoclonal antibody specific for the C9 ring of human SC5b-9 coated to wells of a 96-well plate to capture the complex. The trapped SC5b-9 is subsequently detected with horseradish peroxidase-conjugated (Goat) antibodies to antigens of SC5b-

The Quidel SC5b-9 kit is a three-step procedure utilizing (1) a microassay plate coated with a mouse monoclonal antibody that binds specifically to the C9 ring of SC5b-9, (2) HRP-conjugated antibodies to antigens of SC5b-9, and (3) a chromogenic substrate.

In the first step, standards, controls, and test specimens are added to microassay wells precoated with an anti-SC5b-9 specific monoclonal antibody. SC5b-9 present in the standards, controls, or specimens will bind to the immobilized anti-SC5b-9. After incubation, a wash cycle removes unbound material. Constituent proteins of the TCC, including C9, do not bind to this antibody and are washed away during the wash cycle.

In the second step, horseradish peroxidase (HRP)-conjugated antibodies to antigens on SC5b-9 are added to each test well. The enzyme-conjugated antibodies bind to

SC5b-9 that was captured by the monoclonal anti-SC5b-9 bound on the surface of the microassay wells. After incubation, a wash cycle removes unbound conjugate.

In the third step, TMB is added to each microassay well. The bound HRP-conjugate reacts with the substrate forming a blue color. After incubation, a reagent is added to stop color development, resulting in a yellow color. The standard, control, and test specimen absorbances (A_{450} values) are measured spectrophotometrically. The color intensity of the reaction mixture is proportional to the concentration of SC5b-9 (TCC) present in the test specimens, standards, and controls.

Reagents provided in the Quidel SC5b-9 kit:

- SC5b-9 Plus Standards (198, 128, 49, 13, 0 ng/ml)
- ELISA plate coated with a mouse monoclonal antibody specific for the C9 ring of SC5b-9
- Horseradish Peroxidase-conjugated (Goat) antibodies to antigens of SC5b-9
- TMB Substrate. Ready to use.
- 20 x Wash Solution concentrate (1 x PBS 0.05% Tween-20, Proclin 300)
- Stop Solution (2N H₂SO₄)

Modified ELISA Assay

Urine samples are received within 10 hours of collection from 4°C storage. 1 ml aliquots are made and placed at -80°C until required. For an assay a 1 ml aliquot is thawed in room temperature water for quick thaw and subsequently stored on ice until used up (sufficient for eight assays). When thawed mix, brief vortex agitation

until urine vortex reaches bottom of tube followed by brief centrifuge ~ 10 rcf (Benchtop centrifuge at 13 krpm) for 5 minutes.

SC5b-9 Plus Standards (198, 128, 49, 13, 0 ng/ml) are supplied by the manufacturer ready to use. Standard curves with the SC5b-9 Plus Standards were prepared according to the manufactures instructions.

Loading the urine analyte

The Quidel SC5b-9 assay plate was prepared by adding ~300 µl of wash buffer to each well and incubating for 2 min at room temp. A template plate was prepared by adding 120 µl aliquots of undiluted urine analyte and protein standards into the wells of a polypropylene template plate, followed by loading of the ELISA plate. The ELISA plate is covered with an adhesive plate sealer, and shaken on the Bellco orbital shaker at setting 7 (about 80 revolutions of the table per minute) for 1 h at room temperature (18-25°C).

Following incubation of the samples a room temperature, samples are aspirated and the urine and standards are discarded. 200 µl of Wash Solution (PBS or Wash Solution) was added to each well, and incubated wash for 1 min. Aspirate the solution with the multichannel pipette and perform three additional washes.

Immediately after the last wash, 50 µl of the SC5b-9 conjugate was dispensed into each well with the multichannel pipette, and incubated with shaking for 30 min at room temp with shaking. Wash the plate five times (200 µl of PBS or Wash Solution).

100 µl of the TMB Substrate into each well with the multichannel pipette, and incubated in a closed, empty drawer for 15 min with occasional very gentle tapping to mix the reaction components and disperse the color. At t = 15 minutes, 100 µl/well of Stop Solution was added to each well to stop development.

Data Capture and analysis

The color intensity of the urine and standards were assessed using a Perkin Elmer 1420 Multilabel Counter and plate reader to determine concentrations.

Quality control of the data

Coefficient of Variation (CV) is derived from the ratio of the standard deviation to the non-zero mean of duplicate concentration values, i.e. Coefficient of Variation $CV = \text{Standard Deviation} / \text{Mean}$. CV is expressed as percentage by multiplying CV by 100. For any sampled assay, a %CV value of more than 20% is a threshold for removal of the value and scheduling for repeat measurement of this sample in a subsequent assay.

Example 3: Development of modified SOP for detection of iC3b in human urine adapting Quidel iC3b Sandwich ELISA kit.

The level of the complement fragment iC3b in urine was assessed using a Quidel MicroVue Complement iC3b Plus EIA kit (“the Quidel iC3b kit”), with the following modifications. If used according to the manufacturer’s instructions, the Quidel iC3b kit is designed to measure the amount of iC3b present in human plasma, serum and other biological or experimental samples. Levels of iC3b are indicative of the amount of C3 cleavage (hence total complement activation) in the sample.

iC3b is generated during the activation of the complement system via the classical or alternative pathway. When a convertase enzyme cleaves C3, C3a and C3b are released in parallel. *In vivo*, the C3b molecule has a very short half-life; C3b is rapidly cleaved to iC3b which can serve as a marker for complement activation by the alternative or classical pathway. This ELISA kit uses immobilized anti-human iC3b antibody coated wells of a 96-well plate to capture iC3b from human bodily fluids. The trapped iC3b is subsequently detected with horseradish peroxidase-conjugated (Goat) antibody to another iC3b epitope.

The Quidel iC3b kit for the quantitation of iC3b in human serum or plasma provides a three step procedure utilizing (1) a microassay plate coated with a monoclonal anti-human iC3b, (2) HRP-conjugated anti-human iC3b, and (3) a chromogenic substrate.

In the first step, standards, controls, and test specimens are added to microassays wells precoated with an anti-iC3b monoclonal antibody. The anti-iC3b monoclonal antibody is specific for iC3b and will not bind to C3, C3b, nor any other smaller C3b degradation fragment. iC3b present in the standards, controls, or specimens will bind the immobilized anti-iC3b. After incubation, a wash cycle removes unbound material.

In the second step, horseradish peroxidase (HRP)-conjugated goat anti-human iC3b is added to each test well. In this step, the enzyme conjugated anti-iC3b binds to the iC3b that was captured by the monoclonal anti-iC3 on the surface of the microassay wells. After incubation, a wash cycle removes unbound, excess conjugate.

In the third step, a chromogenic enzyme substrate is added to each microassay well. The bound HRP-conjugate reacts with the substrate forming a green color. After incubation, the enzyme reaction is stopped chemically, and the color intensity is

measured spectrophotometrically at 405 nm. The color intensity of the reaction mixture is proportional to the concentration of iC3b present in the test specimens, standards, and controls.

As described below, significant modifications were developed by the inventor to achieve satisfactory assay performance for iC3b detection in human urine

Reagents provided in the Quidel iC3a kits:

- iC3b standards (1.29, 0.59, 0.14 µg/ml).
- ELISA plate coated with a mouse anti-human iC3b monoclonal antibody.
- Hydrating reagent for solubilization of standards: 0.035% ProClin® 300.
- Peroxidase-conjugated (Goat) anti-human iC3b in PBS, stabilizers and 0.01% Thimerosal.
- Substrate Diluent: 0.1 M citrate buffer and 0.05% peroxide.
- Substrate contains 0.7% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).
- 20 x Wash Solution concentrate (1 x PBS 0.05% Tween-20, 0.01% Thimerosal)
- Stop Solution (250mM oxalic acid)

Modified ELISA Assay

Urine samples are received within 10 hours of collection from 4°C storage. 1 ml aliquots are made and placed at -80°C until required. For an assay a 1 ml aliquot is thawed in room temperature water for quick thaw and subsequently stored on ice until used up (sufficient for eight assays). When thawed mix, brief vortex agitation until urine vortex reaches bottom of tube followed by brief centrifuge ~ 10 rcf (Benchtop centrifuge at 13 krpm) for 5 minutes.

The standards are supplied by the manufacturer in lyophilized form and are solubilized in hydrating solution as per manufacturer. To extend the range of standards to detect lower concentrations of iC3b the lowest concentration of protein standard (0.14 µg/ml) was further diluted 1:1 in hydrating solution to give: 0.07, 0.035, 0.0175 and 0.00875 µg/ml.

A template plate was set up by adding 120 µl aliquots of undiluted urine analyte and protein standards into the wells of a polypropylene template, followed by loading of the ELISA plate. The ELISA plate is covered with an adhesive plate sealer, and shaken on the Bellco orbital shaker at setting 7 (about 80 revolutions of the table per minute) for 1 h at room temperature (18-25°C).

Following incubation of the samples at room temperature, samples are aspirated and the urine and standards are discarded. 200 µl of Wash Solution (PBS or Wash Solution) was added to each well, and incubated wash for 1 min. Aspirate the solution with the multichannel pipette and perform three additional washes.

Immediately after the last wash, 50 µl of the iC3b conjugate was dispensed into each well with the multichannel pipette, and incubated with shaking for 30 min at room temp with shaking. Wash the plate five times (200 µl of PBS or Wash Solution).

100 µl of the TMB Substrate into each well with the multichannel pipette, and incubated in a closed, empty drawer for 15 min with occasional very gentle tapping to mix the reaction components and disperse the color. At t = 15 minutes, 100 µl/well of Stop Solution was added to each well to stop development.

Data Capture and analysis

The color intensity (A_{405}) of the urine and standards were assessed using a Perkin Elmer 1420 Multilabel Counter and plate reader to determine concentrations.

Quality control of the data

Coefficient of Variation (CV) is derived from the ratio of the standard deviation to the non-zero mean of duplicate concentration values, i.e. Coefficient of Variation $CV = \text{Standard Deviation} / \text{Mean}$. CV is expressed as percentage by multiplying CV by 100. For any sampled assay, a %CV value of more than 20% is a threshold for removal of the value and scheduling for repeat measurement of this sample in a subsequent assay.

Example 4. Cross-Sectional Analysis of C3a, C5b-9, iC3b Candidate Biomarkers

Urine samples were obtained from 569 patients as part of routine clinical care from February to May 2012. Clinical (demographic information/blood pressure) and laboratory data were extracted in a de-identified manner from the comprehensive Mount Sinai Data Warehouse. Intact C3, as well as C3a, C3c, C3d, C3b, iC3b, and C5b-9 fragments were screened as potential CKD biomarkers in a phase I exploratory study. C3a, C5b-9, iC3b satisfied exploratory study criteria. Descriptive and summary statistics were used for the demographics of the participants (Table 1)

Table 1.

Demographic and Clinical Features	Control* (N=96)	Abn Kid Fct** (N=232)	*P	Confirmed CKD*** (N=241)	*P
Age (Mean(SD))	44(13.9)	57(16)	<0.01	65(14.6)	<0.01
Male Gender n (%)	40(41.7)	106(46)	0.5	118(49)	0.26
Diabetes Mellitus n (%)	41(42.7)	96(41)	0.82	132(53)	0.04
Hypertension	45(47)	143(62)	0.01	215(89)	<0.01

n (%)					
Diabetes and Hypertension n (%)	26(27)	81(35)	0.16	126(52.3)	<0.01
Mean Arterial BP (Mean (SD) mmHg)	91(9.6)	89(14)	0.62	93(10.8)	0.11
UPCR (median mg/mg [IQR])	0.064 [0.041]	0.086 [0.207]	<0.01	0.297 [1.213]	<0.01
eGFR in ml/min/1.73 m ² Median[IQR]	107 [21]	75 [18]	<0.01	39 [21]	<0.01

* = healthy control

** = Abn Kid Fct – Abn Kid Fct = Abnormal Kidney Function: GFR =>60<90 ml/min/1.72m² and/or UPCR >0.2 g/mg (N=232);

*** = CKD = chronic kidney disease with confirmed diagnosis (N=241)

C3a, C5b-9, iC3b ELISAs (for plasma/serum) were purchased from Quidel® Corp and extensively modified as described above (Examples 1-3) for optimal performance in human urine samples. Association between detectable C3a, C5b-9, or iC3b and abnormal kidney function, CKD, or eGFR was tested using multivariable logistic regression (Table 2, Figures 7-9). Association between detectable complement cleavage fragments and tertiles of eGFR using multivariable linear regression modeling in a cross-sectional study design (Table 3, Figures 10-12).

Table 2. Multivariable Logistic Regression (OR Compared to Controls)

	Abn Kid Fct (eGFR ≥60<90 and/or UPCR >0.2 g/mg)		CKD (III-V) eGFR loss/yr <3ml/min/1.73m ²)		CKD (III-V) eGFR loss/yr ≥3ml/min/1.73m ²)	
Detectable	*OR (95% CI)	P	*OR (95% CI)	P	*OR (95% CI)	P
C3a	2.16(1.18-3.95)	0.01	5.15(1.50-17.7)	<0.01	2.63(1.13-6.10)	0.02
C5b9	3.49(1.61-7.50)	<0.01	10.87(2.55-46.30)	<0.01	9.77(3.22-29.6)	<0.01
iC3b	2.48(1.31-4.70)	<0.01	10.11(2.51-40.72)	<0.01	10.20(3.81-27.19)	<0.01

* Adjusted for age, sex, race, mean arterial BP, history of diabetes mellitus, and history of hypertension.

Table 3. delta eGFR (ml/min/1.732) by Tertiles of C3a/Cr, C5b-9, iC3b/Cr

	C3a/Cr	C5b-9	iC3b/Cr
Undetectable	Reference	Reference	Reference
Tertile 1	0.1	1.2	-4.5
Tertile 2	3.2	-2.5	-7.9**
Tertile 3	-14.2**	-5.5	-12.3**

* Adjusted for age, sex, race, proteinuria, and history of diabetes mellitus.

** p < 0.05

In summary, urine complement C3a, C5b-9, and iC3b are associated with abnormal kidney function and CKD Stage III or higher. And, importantly, C3a, C5b-9, and iC3b levels are inversely associated with eGFR independent of proteinuria.

Example 5. Longitudinal Complement Fragment Analysis: Methods:

The following materials and methods were used in the experiments described in Examples 6-8.

Data Collection:

The initial cohort consisted of 620 participants for whom urine complement fragment levels were measured either in February or May 2012. Using the Mount Sinai DataMart, All available estimated glomerular filtration rates (eGFRs) were extracted from the Mount Sinai DataMart electronic health record for these participants along with the date and time of results. These values were then manually reviewed by a physician (GNN). Patients were included if they had 3 values at roughly 6 month intervals (both before and after the eGFR at time of urinary biomarker measurement). eGFR values within a month of the 6 monthly intervals were considered to be acceptable. If there was more than one value during this period, then the mean of the available values were used. All other information was extracted electronically from the EHR

Statistical Analysis:

Descriptive and summary statistics were used for the demographics of the participants (Table 4). The primary outcome variables of interest were the change in the eGFR (delta eGFR) at 6, 12 and 18 months from the eGFR at time of complement measurement. The delta eGFR was assessed both as a continuous variable as well as a percentage change from time of sample collection. The primary predictor variables of interest were the concentrations of urinary complement fragments (viz. c3a, c5b and ic3b). These were assessed both as a categorical variable (detectable) and continuous

(urinary concentration in ng/mg of creatinine). For continuous variables, we then used tertiles to assess relationship between tertiles and change in eGFR.

Missing values were imputed based on independent variables primarily prior eGFR measurements. The accuracy of imputation was confirmed by imputing in participants with confirmed values and then cross validating.

The relationship between the urinary biomarker levels and eGFR was assessed separately for each biomarker using multivariable regression. Confounding variables were selected depending on their significance in univariable analysis as well as their role in the confounding pathway. P values of <0.05 were considered as significant. All analyses were performed using STATA 12 SE, College Station, TX.

Results:

We had data on 151 participants. Demographic characteristics are presented in Table 4. The initial eGFR 18 months prior to and at time of sample collection was 46 and 42 ml/min/1.73m² respectively.

Table 4: Demographic characteristics of participants (N= 151)

Age Mean(SD)	65(14)
Sex n (%) Male	79(52)
Race n (%) White African American Hispanic Other/Unknown/Missing	40(27) 45(30) 26(17) 40(26)
Hypertension n (%)	133(88.1)
Diabetes n (%)	81(54)
Diabetes and Hypertension n (%)	77(51)
Urine protein creatinine ratio Mean(SD)	1(1.8)
Estimated Glomerular filtration rate in ml/min Mean(SD)	
18 months prior	46(14)
12 months prior	44(12)
6 months prior	43(12)
At time of sample collection	42(12)
6 months after	41(12.6)
12 months after	38(14)
18 months after	38(15)

Example 6: Longitudinal Complement Fragment Analysis C3A BIOMARKER.

Out of 151, 77 (51%) had detectable levels of c3a. The mean level of c3a was 1.38 (SD 4) ng/mg of creatinine. The demographic characteristics of participants stratified by detectable c3a levels are shown in Table 5.

Though the eGFR 18 months prior to sample collection was similar, the subsequent eGFR's were significantly lower in participants with detectable c3a levels as compared to those without (bolded) in Table 5. Figure 1 shows the change in eGFR over time stratified by detectable c3a levels.

Table 5: Demographic characteristics of participants (N= 151) stratified by detectable c3a levels

	Undetectable c3a	Detectable c3a	P
Age Mean(SD)	66(13.5)	65(15)	0.77
Sex n (%) Male	41(55)	38(49)	0.46
Race n (%) White African American Hispanic Other/Unknown/Missing	20(27) 28(38) 10(13) 16(22)	20(26) 17(22) 16(21) 25(31)	0.13
Hypertension n (%)	66(89.2)	67(87)	0.68
Diabetes n (%)	36(49)	45(58)	0.23
Diabetes and Hypertension n (%)	36(49)	41(53)	0.57
Urine protein creatinine ratio Mean(SD)	0.36(0.08)	1.63(0.27)	<0.01
Estimated Glomerular filtration rate in ml/min Mean(SD)			
18 months prior	46.5(9.5)	46(17)	0.83
12 months prior	46(9.6)	42(13.7)	0.03
6 months prior	46(9.4)	40(13.4)	0.01
At time of sample collection	44(9.7)	40(13.5)	0.04
6 months after	44(10.5)	37(14.5)	0.01
12 months after	43(11)	35(15.3)	0.01
18 months after	42(12.3)	32(15.9)	0.01

In patients with detectable c3a levels we then made tertiles of concentrations in ng/mg. The demographic characteristics of participants stratified by tertiles of c3a levels are shown in Table 6. The significantly different characteristics are bolded. Figure 2 shows the change in eGFR over time stratified by c3a tertiles

Table 6: Demographic characteristics of participants (N= 77) stratified by detectable c3a levels

	Lowest tertile	Middle tertile	Highest tertile
Age Mean(SD)	64(14)	68(11)	64(19)
Sex n (%)			
Male	13(52)	16(64)	7(29)
Race n(%)			
White	10(40)	5(20)	3(12.5)
African American	5(20)	6(24)	6(25)
Hispanic	4(16)	8(32)	4(17)
Other/Unknown/Missing	6(24)	6(24)	11(46)
Hypertension n (%)	21(84)	20(80)	24(100)
Diabetes n (%)	16(64)	14(56)	15(63)
Diabetes and Hypertension n (%)	14(56)	12(48)	15(63)
Urine protein creatinine ratio Mean(SD)	0.21(0.62)	1.1(1.5)	2.34(2.90)
Estimated Glomerular filtration rate in ml/min Mean(SD)			
18 months prior	48(13)	47(17)	43(21)
12 months prior	43(12)	43(11)	38(17)
6 months prior	43(12)	41(11.5)	36(16)
At time of sample collection	43.4(12)	42(12)	33.4(15)
6 months after	39(13)	39(12)	30(16)
12 months after	38.2(13.2)	36(14)	27(16)
18 months after	35(14)	33.5(15)	25(16)

6 month change in eGFR from time of sample collection:

The mean change at 6 months in participants with detectable levels was higher than those with undetectable levels (-3.4 vs. -0.24 ml/min; $p < 0.01$). In multivariable logistic regression, detectable c3a levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -2.4; $p = 0.01$) even after adjusting for proteinuria with the change with detectable c3a levels being higher than with proteinuria (-2.4 vs. -0.7). (Table 7).

A similar result was seen with the outcome variable being the *percent* change in eGFR from time of sample collection (Table 8) with the change with detectable c3a levels being higher than with proteinuria (-6.2 vs. -2.8).

In an ancillary analysis, we also analyzed whether detectable levels of c3a predicted change in eGFR at 6 months in patients without proteinuria. As shown in Tables 9 and 10, in participants without proteinuria, detectable c3a levels were

predictive of a higher change in eGFR (-2.02; p=0.05) and percent eGFR (-8; p=0.02) even after adjusting for covariates.

The inventors also analyzed whether the change eGFR at 6 months differed between tertiles of detectable c3a. Though significant when proteinuria was not included as a covariate especially with the highest tertile, it became insignificant when proteinuria was included. This was likely due to the fact that patients with the highest tertile of detectable c3a also had proteinuria two times that of remaining tertiles along with the fact that patients in the highest tertile had a much lower starting eGFR than those in the lowest tertiles

Table 7: Multivariable regression of change in eGFR at 6 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-2.42(-4.14 to -0.70)	<0.01
Proteinuria > 0.5	-0.71(-1.17 to -0.25)	<0.01
Male sex	0.02(-1.65 to 1.70)	0.98
Age	0.02(-0.03 to 0.08)	0.34
Race		
White	Ref	-
African American	1.26(-0.95 to 3.48)	0.26
Hispanic	2.15(-0.41 to 4.72)	0.10
Other/Unknown/Missing	3.16(0.88 to 5.44)	0.01
Diabetes	-1.73(-3.42 to -0.04)	0.04
Hypertension	-1.95(-4.59 to 0.68)	0.15

Table 8: Multivariable regression of percentage change in eGFR at 6 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-6.13(-10.5 to -1.84)	<0.01
Proteinuria > 0.5	-2.79(-3.97 to -1.65)	<0.01
Male sex	0.89(-3.45 to 5.12)	0.70
Age	0.10(-0.04 to 0.25)	0.16
Race		
White	Ref	-
African American	0.78(-4.88 to 6.43)	0.79
Hispanic	3.93(-2.62 to 10.5)	0.24
Other/Unknown/Missing	6.42(0.60 to 12.2)	0.03
Diabetes	-3.65(-7.96 to 0.67)	0.09
Hypertension	-7.52(14.2 to -0.85)	0.03

Table 9: Multivariable regression of change in eGFR at 6 months from time of sample collection in patients without proteinuria (n=90)

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-2.02(-4.14 to -0.3)	0.05
Male sex	1.42(-0.68 to 0.05)	NS

Age	-0.03(-0.11 to 0.05)	NS
Race		NS
White	Ref	
African American	0.83(-1.89 to 3.55)	
Hispanic	1.87(-1.26 to 4.99)	
Other/Unknown/Missing	2.39(-0.58 to 5.36)	
Diabetes	-0.022(-2.45 to 2.01)	NS
Hypertension	-1.92(-4.89 to 1.05)	NS

Table 10: Multivariable regression of *percent* change in eGFR at 6 months from time of sample collection in patients without proteinuria (n=90)

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-8.00(-15.1 to -0.92)	0.02
Male sex	2.16(-.32 to 0.22)	NS
Age	-0.03(-0.11 to 0.05)	NS
Race		NS
White	Ref	
African American	-2.72(-11.8 to 6.41)	
Hispanic	2.32(-8.15 to 12.7)	
Other/Unknown/Missing	1.58(-8.37 to 11.5)	
Diabetes	-5.48(-13 to 2.02)	NS
Hypertension	-3.5(-13.5 to 6.43)	NS

12 month change in eGFR from time of sample collection:

The mean change at 12 months in participants with detectable levels was higher than those with undetectable levels (-5.25 vs. -2.38 ml/min; p=0.01).

In multivariable logistic regression, detectable c3a levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -2.15;p=0.06) and mean percent change(-8.3;p=0.01). However, this association was not significant when proteinuria was adjusted for. There was no significant difference between the tertiles. Also detectable c3a levels were not predictive of higher change in eGFR (Data not tabled)

18 month change in eGFR from time of sample collection:

The mean change at 18 months in participants with detectable levels was higher than those with undetectable levels (-8.1 vs. -1.5 ml/min; p=0.01).

In multivariable logistic regression, detectable c3a levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -3.28;p=0.01) even after adjusting for proteinuria (Table 11). A similar result was seen with the outcome variable being the *percent* change in eGFR from time of

sample collection (Table 10) (Mean percent change -8.1 ; $p=0.02$) even after adjusting for proteinuria.

The inventors also analyzed whether detectable levels of c3a predicted change in eGFR at 6 months in patients without proteinuria. As shown in Tables 13 and 14, in participants without proteinuria, detectable c3a levels were predictive of a higher change in eGFR (-2.02 ; $p=0.05$) and percent eGFR (-8 ; $p=0.02$) even after adjusting for covariates.

In summation, detectable c3a levels is a predictor of change in eGFR at 6, 12 and 18 months independent of proteinuria (except at 12 months) and in participants without proteinuria ($n=90$). Within the detectable range, there does not seem to be any added change in prediction between tertiles.

Table 11: Multivariable regression of change in eGFR at 18 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-3.28(-15.1 to -1.10)	0.02
Proteinuria > 0.5	-6.90(-9.77 to -4.02)	<0.01
Male sex	-.092(-3.57 to 1.73)	0.49
Age	0.11(0.02 to 0.20)	0.02
Race		NS
White		
African American	-0.66(-4.30 to 2.99)	
Hispanic	-0.06(-4.27 to 4.14)	
Other/Unknown/Missing	0.92(-2.78 to 4.63)	
Diabetes	-3.32(-6.06 to -0.60)	0.02
Hypertension	-3.35(-7.56 to 0.87)	0.12

Table 12: Multivariable regression of percentage change in eGFR at 18 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-8.10(-15.1 to 1.08)	<0.01
Proteinuria > 0.5	-21.3(-28.5 to -14.1)	<0.01
Male sex	0.89(-3.45 to 5.12)	0.70
Age	0.29(0.06 to 0.52)	0.01
Race		NS
White	Ref	
African American	0.78(-4.88 to 6.43)	
Hispanic	3.93(-2.62 to 10.5)	
Other/Unknown/Missing	6.42(0.60 to 12.2)	
Diabetes	-6.38(-13.2 to 0.50)	0.06
Hypertension	-9.92(-20.5 to 0.65)	0.06

Table 13: Multivariable regression of change in eGFR at 18 months from time of sample collection in patients without proteinuria (n=90)

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	-3.56(-6.67 to -0.49)	0.02
Male sex	1.13(-1.96 to 4.21)	NS
Age	0.009(-0.11 to 1.13)	NS
Race		NS
White	Ref	
African American	-0.07(-4.09 to 3.94)	
Hispanic	0.66(-3.94 to 5.27)	
Other/Unknown/Missing	1.18(-3.20 to 5.56)	
Diabetes	2.29(-5.58 to 1.01)	0.17
Hypertension	-2.37(-6.76 to 2.01)	0.28

Table 14: Multivariable regression of percentage change in eGFR at 18 months from time of sample collection in patients without proteinuria (n=90)

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c3a levels	--8.00(-15.1 to -0.92)	0.02
Male sex	-0.05(-0.32 to 0.22)	NS
Age	2.16(-4.85 to 9.17)	NS
Race		NS
White	Ref	
African American	-2.72(-11.84 to 6.40)	
Hispanic	2.32(-8.15 to 12.80)	
Other/Unknown/Missing	1.58(-8.37 to 11.53)	
Diabetes	-5.48(-13 to 2.01)	NS
Hypertension	-3.53(-13.5 to 6.43)	NS

Example 7: Longitudinal Complement Fragment Analysis C5B9 BIOMARKER.

Out of 151, 58(38%) had detectable levels of c5b9. The mean level of c5b9 was 18.4 ng/mg of creatinine. The demographic characteristics of participants stratified by detectable c5b9 levels are shown in Table 15. The significant characteristics are bolded.

Though the eGFR 18 and 12 months prior to sample collection was similar, the subsequent eGFR's were significantly lower in participants with detectable c5b9 levels as compared to those without (bolded) in Table 15. Figure 3 shows the change in eGFR over time stratified by detectable c5b9 levels.

Table 15: Demographic characteristics of participants (N= 151) stratified by detectable c5b-9 levels

	Undetectable c5b9	Detectable c5b9	P
Age Mean(SD)	66.5(12.8)	64(16.1)	NS
Sex n (%)			
Male	58(62.4)	21(36.2)	<0.01
Race n (%)			NS
White	28(30)	12(21)	
African American	29(31)	16(28)	
Hispanic	17(18)	9(15)	
Other/Unknown/Missing	19(20)	21(36)	
Hypertension n (%)	79(85)	54(93)	NS
Diabetes n (%)	47(50.5)	34(59)	NS
Diabetes and Hypertension n (%)	44(47)	33(57)	NS
Urine protein creatinine ratio Mean(SD)	0.59(1.43)	1.68(2.20)	<0.01
Estimated Glomerular filtration rate in ml/min Mean(SD)			
18 months prior	46.2(9.40)	46.3(19.4)	NS
12 months prior	45.3(10.2)	43(14)	NS
6 months prior	44.8(10.7)	40.3(13.3)	0.03
At time of sample collection	44(10.6)	40(13.4)	0.04
6 months after	42.5(11.6)	37(14.7)	0.01
12 months after	416(12.3)	34.4(15.5)	0.01
18 months after	40(13.5)	32.3(16.3)	0.01

In patients with detectable c5b9 levels we then made tertiles of concentrations in ng/mg. The demographic characteristics of participants stratified by tertiles of c5b9 levels are shown in Table 16. The significantly different characteristics are bolded. Figure 4 shows the change in eGFR over time stratified by c5b9 tertiles

Table 16: Demographic characteristics of participants (N= 58) stratified by detectable c5b9 levels

	Lowest tertile	Middle tertile	Highest tertile
Age Mean(SD)	60.5(15.5)	68.9(12)	61(19)
Sex n (%) Male	9(45)	5(26)	7(37)
Race n (%) White	4(20)	4(21)	4(21)
African American	8(40)	6(32)	2(11)
Hispanic	3(15)	2(10)	4(21)
Other/Unknown/Missing	5(25)	7(37)	9(47)
Hypertension n (%)	20(100)	16(84)	18(95)
Diabetes n (%)	12(60)	8(42)	14(74)
Diabetes and Hypertension n (%)	12(60)	8(42)	13(68)
Urine protein creatinine ratio Mean(SD)	0.54(0.58)	1.36(1.55)	3.19(2.92)
Estimated Glomerular filtration rate in ml/min Mean(SD)			
18 months prior	44.3(16.2)	43(13.4)	43.5(14.4)
12 months prior	41.1(14)	43(14.3)	38(17)
6 months prior	40.1(13.5)	40.5(13.7)	36.8(15.2)
At time of sample collection	40.1(14)	40(14)	38.5(13.4)
6 months after	38.4(14.4)	36.8(15.9)	33.3(14.5)
12 months after	37.7(15.2)	33.5(15.9)	32(16.6)
18 months after	34.9(15.2)	37.5(15.2)	33.1(17.7)

6 month change in eGFR from time of sample collection:

The mean change at 6 months in participants with detectable levels was higher than those with undetectable levels (-2.95 vs. -1.28 ml/min; $p=0.08$) with a trend towards significance. However, the percent change from sample collection was much significantly higher (-9.1 vs. -2.8; $p<0.01$).

In multivariable logistic regression, detectable c5b9 levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -1.61; $p=0.08$) [Table 17] with a trend towards significance; it became non-significant when proteinuria was adjusted for. A similar result was seen with the outcome variable being the *percent* change in eGFR from time of sample collection (Mean percent change -5.779; $p=0.02$) [Table 18]; it became non-significant when proteinuria was adjusted for.

Detectable c5b9 not was significantly predictive of change in eGFR or percent eGFR at 6 months in patients without proteinuria.

There was no difference in the tertiles of c5b9 in patients with detectable biomarker and change in eGFR/percent eGFR. (Data not tabled)

Table 17: Multivariable regression of change in eGFR at 6 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c5b9 levels	-1.61(-3.46 to 0.24)	0.08
Male sex	0.003(-1.84 to 1.85)	NS
Age	0.04(-0.02 to 0.10)	NS
Race		
White	Ref	-
African American	1.26(-0.95 to 3.48)	0.26
Hispanic	2.15(-0.41 to 4.72)	0.10
Other/Unknown/Missing	2.94(0.43 to 5.44)	0.02
Diabetes	-2.18(-4.02 to -0.33)	0.02
Hypertension	-2.15(-4.99 to 0.69)	0.14

Table 18: Multivariable regression of percentage change in eGFR at 6 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c5b9 levels	-5.78(-10.7 to -0.92)	0.02
Male sex	0.76(-4.10 to 5.62)	NS
Age	0.10(-0.07 to 0.26)	NS
Race		
White	Ref	
African American	2.21(-4.28 to 8.70)	NS
Hispanic	2.39(-5.10 to 9.86)	NS
Other/Unknown/Missing	6.22(-0.39 to 12.8)	0.06
Diabetes	-4.39(-9.26 to 0.49)	0.07
Hypertension	-5.99(-13.5 to 1.51)	0.12

12 month change in eGFR from time of sample collection:

The mean change at 12 months in participants with detectable levels was higher than those with undetectable levels (-7.94 vs. -2.51 ml/min; p=0.02). The mean percent change was also significantly higher in those with detectable levels (-15.6 vs. -5.166; p<0.01)

In multivariable logistic regression, detectable c5b9 levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -2.57;p=0.03)[Table 19]; it became non-significant when proteinuria was adjusted for. A similar result was seen with the outcome variable being the *percent* change in

eGFR from time of sample collection (Mean percent change -8.91; $p < 0.01$) [Table 20]; it became non-significant when proteinuria was adjusted for. (Data not tabled)

Detectable c5b9 not was significantly predictive of change in eGFR or percent eGFR at 12 months in patients without proteinuria.

In patients with detectable levels, higher tertiles of c5b9 were predictive of higher percent changes in eGFR (-12; $p = 0.07$ for tertile 2 and -13.1 for tertile 3; $p = 0.04$ compared to tertile 1) after adjusting for confounders.

Table 19: Multivariable regression of change in eGFR at 12 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c5b9 levels	-2.57(-4.99 to -0.15)	0.03
Male sex	-0.23(0.02 to 0.18)	NS
Age	0.10(0.02 to 0.18)	0.02
Race		
White	Ref	-
African American	2.14(-1.09 to 5.36)	NS
Hispanic	0.86(-2.85 to 4.58)	NS
Other/Unknown/Missing	1.78(-1.51 to 5.07)	NS
Diabetes	-2.85(-5.28 to -0.43)	0.02
Hypertension	-2.63(-6.36 to 1.10)	NS

Table 20: Multivariable regression of percentage change in eGFR at 12 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c5b9 levels	-8.91(-15.37 to -2.45)	<0.01
Male sex	0.26(0.06 to 0.49)	NS
Age	0.28(0.06 to 0.49)	0.01
Race		
White	Ref	
African American	3.96(-4.65 to 12.6)	NS
Hispanic	-0.51(-10.4 to 9.42)	NS
Other/Unknown/Missing	4.63(-4.14 to 13.4)	NS
Diabetes	-5.73(-12.2 to 0.74)	0.08
Hypertension	-8.81(18.8 to 1.13)	0.08

18 month change in eGFR from time of sample collection:

The mean change at 18 months in participants with detectable levels was higher than those with undetectable levels (-7.94 vs. -2.5 ml/min; $p = 0.01$).

In multivariable logistic regression, detectable c5b9 levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -3.03; $p = 0.05$) [Table 21]. A similar result was seen with the outcome variable being the *percent* change in eGFR from time of sample collection (Mean

percent change -10.5 ; $p=0.01$) [Table 22]. However, these associations became insignificant after adjusting for proteinuria in the model

Detectable c5b9 not was significantly predictive of change in eGFR or percent eGFR at 18-months in patients without proteinuria.

In patients with detectable levels, higher tertiles of c5b9 were predictive of higher percent changes in eGFR at 18 months (-11 ; $p=0.07$ for tertile 2 and -18 for tertile 3; $p=0.01$ compared to tertile 1) after adjusting for confounders.

Table 21: Multivariable regression of change in eGFR at 18 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c5b9 levels	-3.04(-6.11 to 0.03)	0.05
Male sex	-0.95(-4.01 to 2.16)	NS
Age	0.13(0.03 to 0.24)	0.01
Race		
White	Ref	-
African American	0.17(-3.92 to 4.27)	NS
Hispanic	-0.07(-4.78 to 4.65)	NS
Other/Unknown/Missing	0.44(-3.73 to 4.61)	NS
Diabetes	-4.21(-7.29 to -1.14)	0.01
Hypertension	-4.05(-8.78 to 0.67)	0.09

Table 22: Multivariable regression of percentage change in eGFR at 18 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable c5b9 levels	-10.5(-18.4 to -2.54)	0.01
Male sex	-1.78(-9.70 to 0.62)	NS
Age	0.35(0.08 to 0.62)	0.66
Race		
White	Ref	
African American	-1.01(-11.6 to 9.58)	NS
Hispanic	-3.12(-15.3 to 9.06)	NS
Other/Unknown/Missing	0.36(-10.4 to 11.1)	NS
Diabetes	-8.91(-16.8 to -0.97)	0.02
Hypertension	-12(-24 to 0.21)	NS

Example 8: Longitudinal Complement Fragment Analysis IC3B BIOMARKER:

Out of 151, 80(53%) had detectable levels of ic3b. The mean level of ic3b was 347 ng/mg of creatinine. The demographic characteristics of participants stratified by detectable ic3b levels are shown in Table 23. The significant characteristics are bolded.

Table 23: Demographic characteristics of participants (N= 151) stratified by detectable ic3b levels

	Undetectable ic3b	Detectable ic3b	P
Age Mean(SD)	66.1(13)	64.7(15)	0.56
Sex n (%) Male	40(56.3)	39(48.8)	0.35
Race n (%) White African American Hispanic Other/Unknown/Missing	19(27) 23(32) 15(21) 14(20)	21(26) 22(28) 11(14) 26(32)	0.13
Hypertension n (%)	60(84.5)	73(91)	0.20
Diabetes n (%)	38(53.5)	43(53.8)	0.98
Diabetes and Hypertension n (%)	37(52)	40(50)	0.57
Urine protein creatinine ratio Mean(SD)	0.21(0.04)	1.71(2.29)	<0.01
Estimated Glomerular filtration rate in ml/min Mean(SD)			
18 months prior	47.5(10.5)	45.2(16.6)	0.38
12 months prior	46(10.4)	42.5(13)	0.11
6 months prior	46(9.4)	40.6(13)	<0.01
At time of sample collection	44.7(10.8)	39.9(13)	0.01
6 months after	44.2(11.3)	36.4(13.8)	<0.01
12 months after	43.9(11)	34(14.5)	<0.01
18 months after	43.7(12.1)	31(15.1)	<0.01

Though the eGFR 18 and 12 months prior to sample collection was similar, the subsequent eGFR's were significantly lower in participants with detectable ic3b levels as compared to those without (bolded) in Table 23. Figure 5 shows the change in eGFR over time stratified by detectable ic3b levels.

In patients with detectable ic3b levels we then made tertiles of concentrations in ng/mg. The demographic characteristics of participants stratified by tertiles of ic3b levels are shown in Table 24. The significantly different characteristics are bolded. Figure 6 shows the change in eGFR over time stratified by ic3b tertiles

Table 24: Demographic characteristics of participants (N= 58) stratified by detectable ic3b levels

	Lowest tertile	Middle tertile	Highest tertile
Age Mean(SD)	68.6(14)	66(13.1)	59(17)
Sex n (%) Male	14(52)	16(59)	9(35)
Race n (%) White	7(26)	11(40)	3(12)
African American	11(41)	7(26)	4(15)
Hispanic	3(11)	4(15)	4(15)
Other/Unknown/Missing	6(22)	5(19)	15(58)
Hypertension n (%)	25(93)	22(81)	26(100)
Diabetes n (%)	13(48)	15(56)	15(58)
Diabetes and Hypertension n (%)	12(44)	13(49)	15(58)
Urine protein creatinine ratio Mean(SD)	1.34(2.29)	1.38(1.63)	2.44(2.73)
Estimated Glomerular filtration rate in ml/min Mean(SD)			
18 months prior	42.4(11)	45(11.5)	48(24)
12 months prior	41.8(11.3)	43.7(12.8)	41.6(15.2)
6 months prior	41.7(12.6)	41.6(13.5)	38.1(13.5)
At time of sample collection	40.5(11.1)	40.7(14.2)	38.7(13.9)
6 months after	38.2(11.9)	37(14.5)	33.8(15.1)
12 months after	36.4(13.3)	35.9(15)	29.6(14.8)
18 months after	33.5(14.4)	34(15.3)	26.1(14)

6 month change in eGFR from time of sample collection:

The mean change at 6 months in participants with detectable levels was higher than those with undetectable levels (-3.83 vs. 0.36 ml/min; $p < 0.01$) with a trend towards significance. However, the percent change from sample collection was much significantly higher (-10.2 vs. 0.44; $p < 0.01$).

In multivariable logistic regression, detectable ic3b levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -2.20; $p < 0.02$) [Table 25] even after adjusting for proteinuria > 0.5 . A similar result was seen with the outcome variable being the *percent* change in eGFR from time of sample collection (Mean percent change -5.88; $p = 0.02$) [Table 26] even after adjusting for proteinuria > 0.5 .

Detectable ic3b was predictive of change in eGFR at 6 months (-2.04; p=0.06) as well of percent change in eGFR (-5.18; p=0.05) in patients without proteinuria (n=90) [Tables 27 and 28]

There was a difference in the tertiles of ic3b in patients with the highest tertile having higher eGFR change (-2.5 vs -0.73) compared to the lower tertile as well as higher percent change in eGFR (-8.65 vs. -2.45) even after adjusting for proteinuria.

Table 25: Multivariable regression of change in eGFR at 6 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-2.20(-4.11 to -0.29)	0.02
Proteinuria	-2.85(-4.82 to -0.89)	<0.01
Male sex	-0.06(-1.72 to 1.59)	NS
Age	0.03(-0.03 to 0.09)	NS
Race		
White	Ref	-
African American	1.32(-0.96 to 3.60)	NS
Hispanic	1.65(-0.98 to 4.27)	NS
Other/Unknown/Missing	3.07(0.76 to 5.39)	0.01
Diabetes	-1.96(-3.69 to -0.26)	0.02
Hypertension	-1.47(-4.10 to 1.17)	0.27

Table 26: Multivariable regression of percentage change in eGFR at 6 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-5.88(-10.8 to -0.94)	0.02
Proteinuria	-9.13(-14.2 to -4.06)	<0.01
Male sex	0.81(-3.47 to 5.10)	NS
Age	0.07(-0.08 to 0.22)	NS
Race		
White	Ref	-
African American	1.07(-4.82 to 6.96)	NS
Hispanic	1.12(-5.68 to 7.91)	NS
Other/Unknown/Missing	6.54(0.56 to 12.5)	0.03
Diabetes	-3.75(-8.19 to 0.70)	0.03
Hypertension	-4.11(-10.9 to 2.71)	NS

Table 27: Multivariable regression of change in eGFR at 6 months from time of sample collection in patients without proteinuria (n=90)

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-2.04(-4.19 to 0.12)	0.06
Male sex	1.34(-0.11 to 0.05)	NS
Age	-0.03(-0.11 to 0.05)	NS
Race		
White	Ref	-
African American	0.83(-1.89 to 3.56)	NS
Hispanic	1.26(-1.92 to 4.45)	NS
Other/Unknown/Missing	2.19(-0.79 to 5.18)	NS
Diabetes	-0.73(-2.91 to 1.46)	NS
Hypertension	-1.40(-4.39 to 1.59)	NS

Table 28: Multivariable regression of percentage change in eGFR at 6 months from time of sample collection without proteinuria (n=90)

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-5.18(-10.4 to 0)	0.05
Male sex	2.94(-2.09 to 7.98)	NS
Age	-0.12(-0.31 to 0.08)	NS
Race		
White	Ref	-
African American	-0.011(-6.67 to 6.45)	NS
Hispanic	2.04(-5.64 to 9.72)	NS
Other/Unknown/Missing	3.88(-3.30 to 11.06)	NS
Diabetes	-1.84(-8.11 to 3.42)	NS
Hypertension	-1.17(-8.39 to 6.04)	NS

12 month change in eGFR from time of sample collection:

The mean change at 12 months in participants with detectable levels was higher than those with undetectable levels (-6.21 vs. -0.68 ml/min; $p<0.01$). The mean percent change was also significantly higher in those with detectable levels (-16.4 vs. -0.97; $p<0.01$)

In multivariable logistic regression, detectable ic3b levels were an independent predictor of higher change in eGFR as compared to undetectable levels (Mean change -2.41; $p=0.04$ and *percent* change in eGFR from time of sample collection (Mean percent change -5.99; $p=0.06$) even after adjusting for proteinuria [Tables 29 and 30].

Detectable ic3b was predictive of change in eGFR at 12 months (-2.25; $p=0.06$) as well of percent change in eGFR (-5.42; $p=0.07$) in patients without proteinuria (n=90) [Tables 31 and 32]

In patients with detectable levels, higher tertiles of ic3b were predictive of higher percent changes in eGFR (-10; $p=0.06$ for tertile 3 compared to tertile 1; $p=0.06$) after adjusting for confounders including proteinuria.

Table 29: Multivariable regression of change in eGFR at 12 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-2.41(-4.80 to -0.02)	0.04
Proteinuria > 0.5	-5.56(-8.01 to -3.11)	<0.01
Male sex	-0.32(-2.39 to 1.75)	NS
Age	0.08(0.01 to 0.16)	0.02
Race		
White	Ref	-
African American	1.50(-1.34 to 4.34)	NS
Hispanic	0.20(-3.08 to 3.48)	NS
Other/Unknown/Missing	2.02(-0.87 to 4.91)	NS
Diabetes	-2.42(-4.6 to -0.27)	0.03
Hypertension	-1.56(-4.85 to 1.74)	NS

Table 30: Multivariable regression of percentage change in eGFR at 6 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-5.99(-12.2 to 0.25)	0.06
Proteinuria > 0.5	-17.1(-23.6 to -10.7)	<0.01
Male sex	0.36(-5.04 to 5.78)	NS
Age	0.23(0.04 to 0.41)	0.02
Race		
White	Ref	-
African American	2.26(-5.18 to 9.70)	NS
Hispanic	-2.19(-10.8 to 6.4)	NS
Other/Unknown/Missing	5.19(-2.36 to 12.7)	NS
Diabetes	-4.41(-10 to 1.21)	NS
Hypertension	-5.91(-14.5 to 2.69)	NS

Table 31: Multivariable regression of change in eGFR at 12 months from time of sample collection in patients without proteinuria (n=90)

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-2.25(-4.78 to 0.27)	0.06
Male sex	0.77(-1.68 to 3.21)	NS
Age	0.03(-0.07 to 0.12)	NS
Race		
White	Ref	-
African American	1.99(-1.19 to 5.18)	NS
Hispanic	1.12(-2.61 to 4.84)	NS
Other/Unknown/Missing	2.64(-0.85 to 6.13)	NS
Diabetes	-1.78(-4.34 to 0.78)	NS
Hypertension	-1.12(-4.63 to 2.38)	NS

Table 32: Multivariable regression of percentage change in eGFR at 12 months from time of sample collection without proteinuria (n=90)

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-5.42(-11.8 to 0.5)	0.07
Male sex	2.94(-2.09 to 7.98)	NS
Age	-0.12(-0.31 to 0.08)	NS
Race		
White	Ref	-
African American	-0.011(-6.67 to 6.45)	NS
Hispanic	2.04(-5.64 to 9.72)	NS
Other/Unknown/Missing	3.88(-3.30 to 11.06)	NS
Diabetes	-1.84(-8.11 to 3.42)	NS
Hypertension	-1.17(-8.39 to 6.04)	NS

18 month change in eGFR from time of sample collection:

The mean change at 18 months in participants with detectable levels was higher than those with undetectable levels (-8.79 vs. -0.40 ml/min; $p<0.01$).

In multivariable logistic regression, detectable ic3b levels were an independent predictor of higher change in eGFR (Mean change -4.63; $p<0.01$) and a higher percent change in eGFR (Mean percent change -10.3; $p<0.01$) as compared to undetectable levels including proteinuria. [Tables 33 and 34]

Detectable ic3b was predictive of change in eGFR at 18 months (-4.87; $p<0.01$) as well of percent change in eGFR (-10.3; $p<0.01$) in patients without proteinuria (n=90) [Tables 35 and 36]

In patients with detectable levels, higher tertiles of ic3b were predictive of higher percent changes in eGFR at 18 months (-15.4; $p=0.03$ for tertile 3 compared to tertile 1) after adjusting for confounders but lost significance after adjusting for proteinuria.

Table 32: Multivariable regression of change in eGFR at 18 months from time of sample collection

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-4.63(-7.65 to -1.61)	0.01
Proteinuria > 0.5	-5.66(-8.76 to -2.56)	<0.01
Male sex	-1.14(-3.76 to 1.48)	NS
Age	0.11(0.02 to 0.3)	0.01
Race		
White	Ref	-
African American	-0.70(-4.30 to 2.91)	NS
Hispanic	-1.08(-5.24 to 3.07)	NS
Other/Unknown/Missing	0.75(-2.91 to 4.40)	NS
Diabetes	-3.81(-6.51 to -1.09)	0.01
Hypertension	-2.63(-6.79 to 1.53)	NS

Table 34: Multivariable regression of percentage change in eGFR at 18 months from time of sample collection

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-10.3(-17.9 to -2.65)	0.01
Proteinuria > 0.5	-18.9(26.7 to -11.1)	<0.01
Male sex	-1.85(-8.45 to 4.76)	NS
Age	0.29(0.06 to 0.52)	0.01
Race		
White	Ref	-
African American	-3.26(-12.3 to 5.82)	NS
Hispanic	-2.19(-10.8 to 6.4)	NS
Other/Unknown/Missing	1.09(-8.12 to 103)	NS
Diabetes	-7.51(-14.4 to 0.67)	0.03
Hypertension	-8.24(-24.2 to 12.9)	NS

Table 35: Multivariable regression of change in eGFR at 18 months from time of sample collection in patients without proteinuria (n=90)

	Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-4.87(-7.96 to -1.77)	<0.01
Male sex	0.99(-0.11 to 0.12)	NS
Age	0.01(-0.11 to 0.12)	NS
Race		
White	Ref	-
African American	-0.23(-4.14 to 3.68)	NS
Hispanic	-0.77(-5.35 to 3.81)	NS
Other/Unknown/Missing	0.69(-3.59 to 4.96)	NS
Diabetes	-3.21(-6.34 to -0.01)	0.04
Hypertension	-1.24(-5.53 to 3.06)	NS

Table 36: Multivariable regression of percentage change in eGFR at 18 months from time of sample collection without proteinuria (n=90)

	Percent Change in eGFR at 6 months Coefficient (95% Confidence Interval)	P
Detectable ic3b levels	-10.3(-17.4 to -3.19)	0.07
Male sex	1.86(-5.02 to 8.74)	NS
Age	-0.04(-0.31 to 0.22)	NS
Race		
White	Ref	-
African American	-2.99(-11.9 to 5.96)	NS
Hispanic	-0.71(-11.2 to 9.77)	NS
Other/Unknown/Missing	0.55(-9.25 to 10.4)	NS
Diabetes	-7.53(-14.7 to -0.34)	0.04
Hypertension	-1.09(-10.9 to 8.75)	NS

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for assessing the progression of chronic kidney disease (CKD) in a subject, comprising

- (a) obtaining a first urine sample from a human subject;
- (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample;
- (c) obtaining a second urine sample from the human subject, wherein the second sample is obtained following a predetermined time interval;
- (d) measuring the level of the one or more complement C3 cleavage fragments in the second urine sample;
- (e) comparing the measured levels in the first urine sample with the measured levels in the second urine sample; and
- (f) identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of the subject having a high or increased likelihood of CKD progression.

2. A method for monitoring the progression of kidney dysfunction, chronic kidney disease or diabetic kidney dysfunction in a subject, comprising

- (a) obtaining a first urine sample from a human subject at a first time point;
- (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample;
- (c) obtaining a second urine sample from the human subject, wherein the second sample is obtained at a second time point;
- (d) measuring the level of the one or more complement C3 cleavage fragments in the second urine sample;
- (e) comparing the measured levels in the first urine sample with the measured levels in the second urine sample; and
- (f) identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of a high or increased likelihood of CKD progression.

3. A method of monitoring the progression of kidney dysfunction, chronic kidney disease or diabetic kidney dysfunction in a subject, comprising
- (a) obtaining a first urine sample from a human subject;
 - (b) measuring the level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b in the first urine sample;
 - (c) comparing the measured levels to one or more predetermined, statistically significant reference levels; and
 - (d) identifying an increase in the measured levels of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a high or increased likelihood of kidney dysfunction, chronic kidney disease or diabetic kidney dysfunction progression.
4. The method of any one of claims 1 or 2, wherein a 2- to 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold increase in the level of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample is indicative of a likelihood of CKD progression.
5. The method of any one of claims 1 or 2, wherein a 2-fold to 10-fold increase in the level of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample is indicative of a likelihood of CKD progression.
6. The method of claim 1, wherein the predetermined time interval is about 1 month to about 12 months, about 2 to about 10 months, about 3 to about 8 months, about 3 to about 7 months, about 4 to about 6 months, or about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, or about 12 months.
7. The method of claim 6, wherein the predetermined time interval is about 3 months.

8. The method of claim 6, wherein the predetermined time interval is about 6 month

9. The method of claim 1, wherein the predetermined time interval is about 1 week to about 12 weeks, about 2 to about 10 weeks, about 3 to about 8 weeks, about 3 to about 7 weeks, about 4 to about 6 weeks, or about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, or about 12 weeks.

10. The method of any one of claims 1 to 9, wherein the subject has diabetes.

11. The method of any one of claims 1 to 10, wherein the subject has hypertension.

12. The method of any one of claims 1 to 11, wherein the levels are measured by ELISA, immunoassays, enzymatic assays, mass spectrophotometry, colorimetry, or fluorometry.

13. The method of claim 2 or 10-12, wherein obtaining the second urine sample from the human subject at a second time point is performed at about 1 month to about 12 months, about 2 to about 10 months, about 3 to about 8 months, about 3 to about 7 months, about 4 to about 6 months, or about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, or about 12 months after obtaining the first urine sample from the human subject at the first time point.

14. The method of claim 2 or 10-12, wherein obtaining the second urine sample from the human subject at a second time point is performed at about 3 months after obtaining the first urine sample from the human subject at the first time point.

15. The method of any one of claims 2 or 10-12, wherein obtaining the second urine sample from the human subject at a second time point is performed at about 6 months after obtaining the first urine sample from the human subject at the first time point.

16. The method of any one of claims 2 or 10-12, wherein obtaining the second urine sample from the human subject at a second time point is performed at about 1 week to about 12 weeks, about 2 to about 10 weeks, about 3 to about 8 weeks, about 3 to about 7 weeks, about 4 to about 6 weeks, or about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, or about 12 weeks after obtaining the first urine sample from the human subject at the first time point.

17. The method of any one of claims 1 to 16, wherein the levels of the one or more complement C3 cleavage fragments is measured using antibodies, wherein at least one antibody is capable of specifically binding to each of complement C3 cleavage fragments.

18. The method of any one of claims 1 to 17, further comprising measuring the level of total protein in the first urine sample, second urine sample, or both.

19. The method of any one of claims 1 to 17, further comprising measuring the level of total creatinine in the first urine sample, second urine sample, or both.

20. The method of any one of claims 1 to 17, further comprising determining the total protein to creatinine ratio (UPCR) in the first urine sample, second urine sample, or both.

21. The method of any one of claims 1 to 20, further comprising administering to a subject having measured levels indicative of a high or increased likelihood of CKD progression a compound which inhibits the renin-angiotensin system selected from the group consisting of angiotensin-receptor-blockers and angiotensin converting enzyme (ACE) inhibitors.

22. The method of claim 21, wherein the angiotensin-receptor-blocker is selected from the group consisting of candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan.

23. The method of claim 21, wherein the angiotensin converting enzyme (ACE) inhibitor is selected from the group consisting of tamipril, enalapril, lisinopril, and perindopril.

24. The method of any one of claims 1 to 20, further comprising referring a subject having measured levels indicative of a high or increased likelihood of CKD progression to a renal specialist.

25. The method of any one of claims 1 to 20, further comprising modifying the clinical record of a subject having measured levels indicative of a high or increased likelihood of CKD progression to identify the subject having high or increased likelihood of CKD progression.

26. The method of any one of claims 1 to 20, further comprising instructing the insurance provider for a subject having measured levels indicative of a high or increased likelihood of CKD progression to approve payment for further treatment, specialist diagnostics or specialty care medical practices.

27. The method of any one of claims 3, 10-13 or 17-26, wherein the reference levels take into account one or more of the subject's age, sex, body mass index, fasting glucose level, hypertension, fasting insulin, blood pressure, glomerular filtration rate, urinary albumin to creatinine ratio (UACR), or urinary total protein to creatinine ratio (UPCR).

28. The method of any one of claims 3, 10-12 or 17-26, wherein a 2-fold to 10-fold increase in the level of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a likelihood of CKD progression.

29. The method of any one of claims 1-28, wherein the complement C3 cleavage fragment is C3a.

30. The method of any one of claims 1-28, wherein the complement C3 cleavage fragment is C5b-9.

31. The method of any one of claims 1-28, wherein the complement C3 cleavage fragment is iC3b.

32. A method, comprising intervening in a human subject's health care by:

- i) administering a renin-angiotensin compound to a human subject or instructing the human subject to self-administer a renin-angiotensin compound, or
- ii) referring a human subject to a renal specialist;

provided that the human subject is selected for intervention if a urine sample from the subject tested according to any one of claims 1 to 3 is indicative of the subject having a likelihood of CKD progression.

33. A kit for diagnosing the risk of chronic kidney disease (CKD) progression, comprising one or more antibodies that bind to one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b; and a reagent useful for the detection of a binding between said antibody and said complement C3 cleavage fragment.

34. A kit, comprising:

- (a) one or more reagents suitable for performing an ELISA or mass spectrometry analysis on a urine sample from a human subject to measure a level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b; and
- (b) instructions to measure the levels of the one or more complement C3 cleavage fragments from a first urine sample collected at a first time point and from a second urine sample collected at a second time point.
- (c) optionally, one or more control samples;
- (c) instructions or software for comparing the measured levels at the first time point to the measured levels at the second time point; and
- (d) instructions or software for identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the second urine sample compared to the measured levels in the first urine sample as indicative of a likelihood of CKD progression.

35. A kit, comprising:

- (a) one or more reagents suitable for performing an ELISA or mass spectrometry analysis on a urine sample from a human subject to measure a level of one or more complement C3 cleavage fragments selected from the group consisting of C3a, C5b-9 and iC3b; and
- (b) one or more reference samples predetermined reference levels of the one or more complement C3 cleavage fragments;
- (c) instructions or software for comparing the measured levels to one or more predetermined reference levels for the same complement C3 cleavage fragments; and
- (d) instructions or software for identifying an increase in measured levels of the one or more complement C3 cleavage fragments in the first urine sample compared to the reference levels as indicative of a likelihood of CKD progression.

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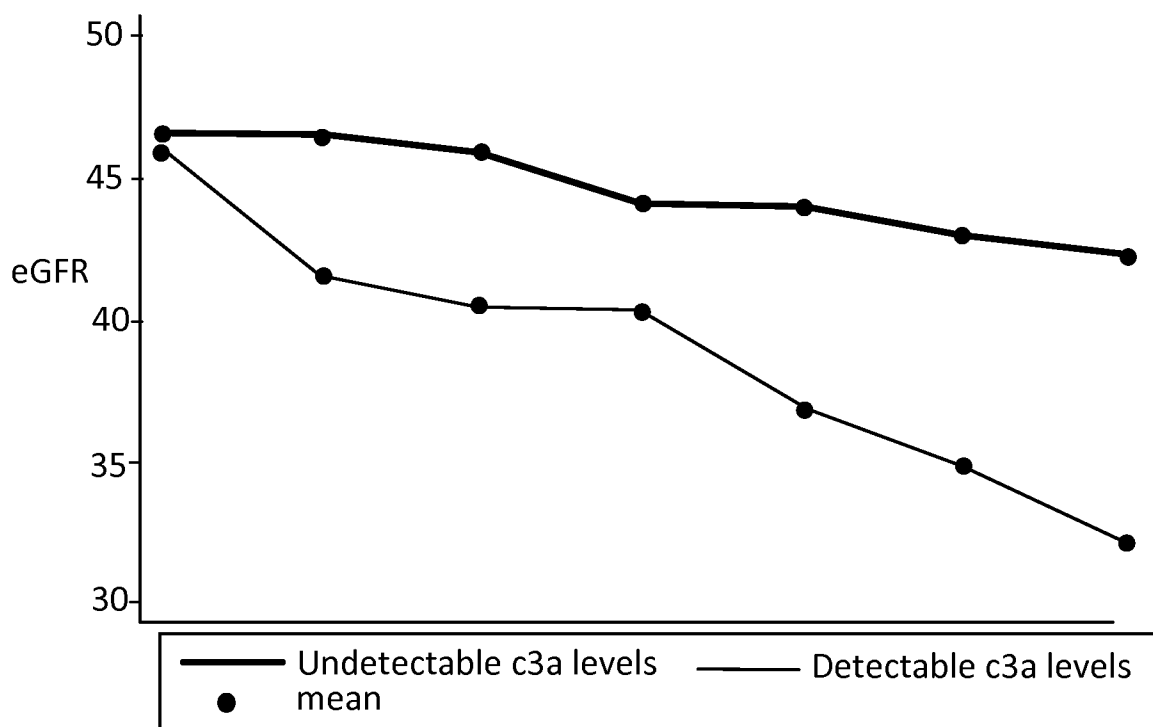


FIG. 1

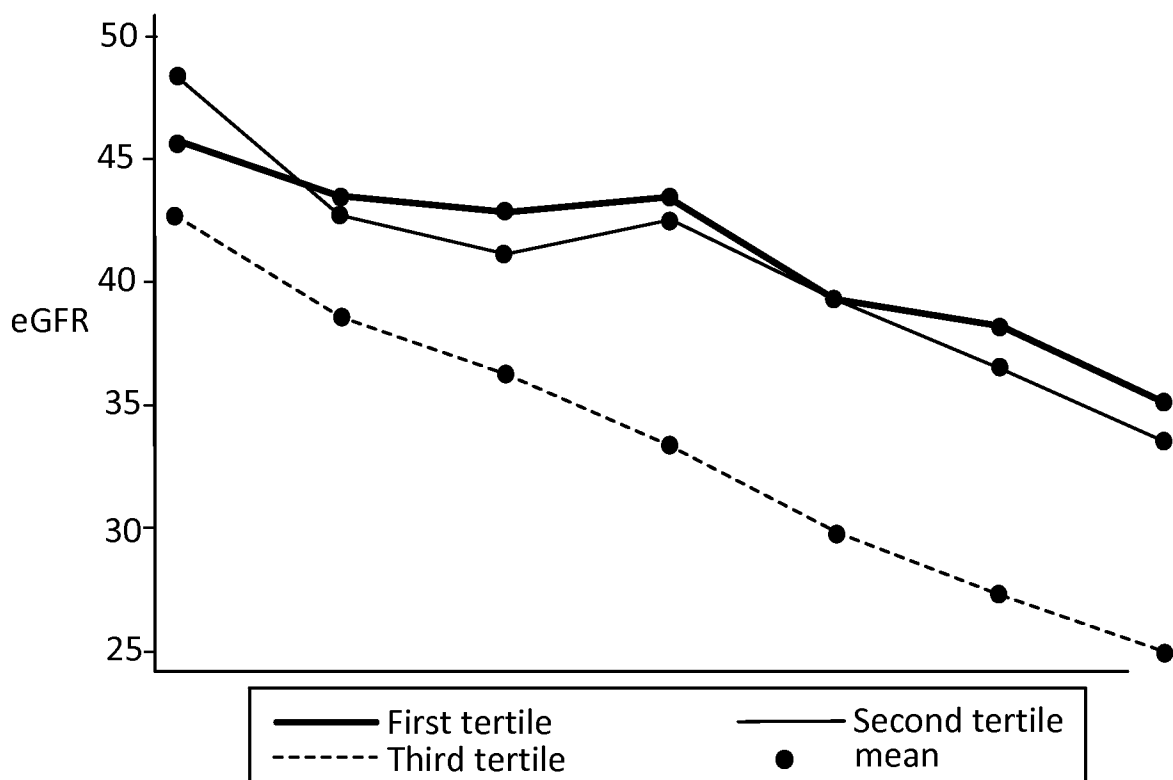


FIG. 2

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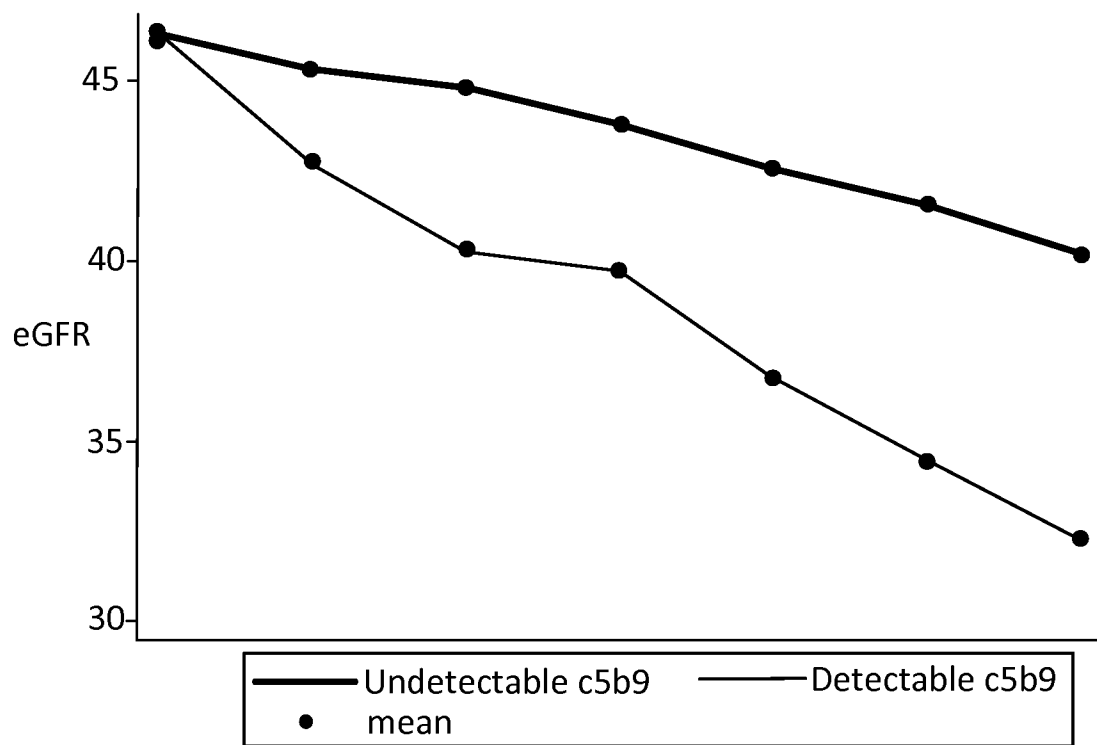


FIG. 3

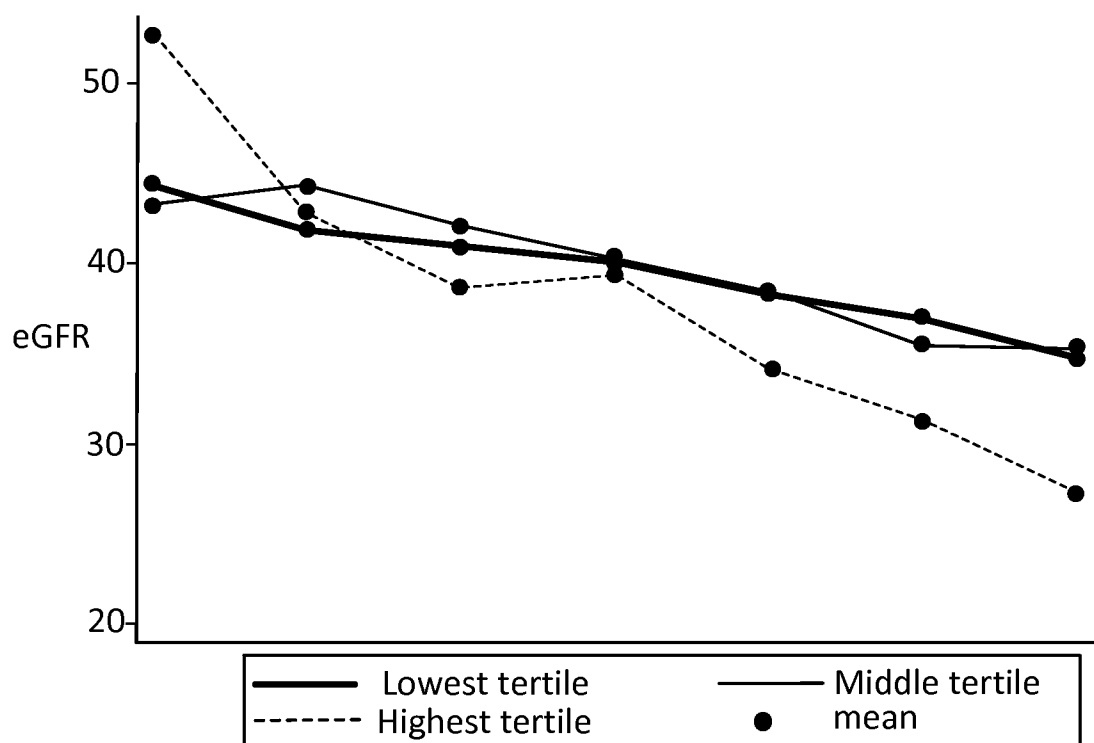


FIG. 4

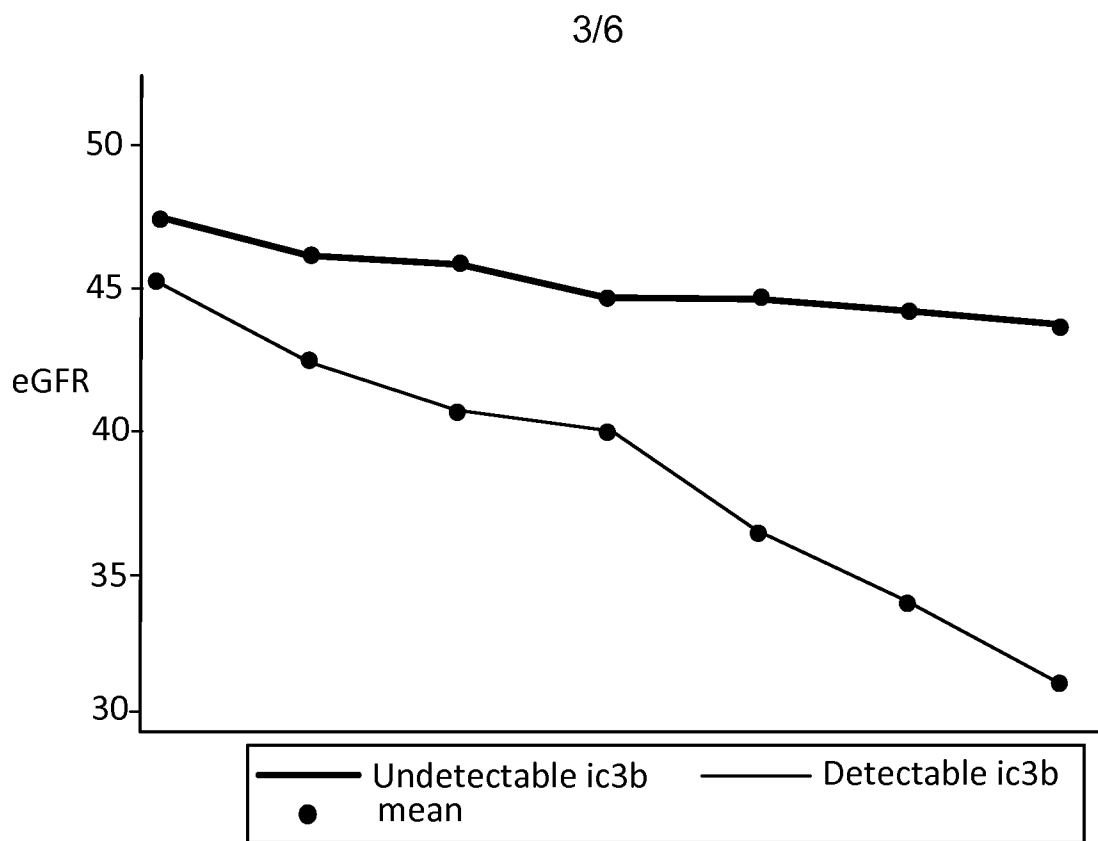


FIG. 5

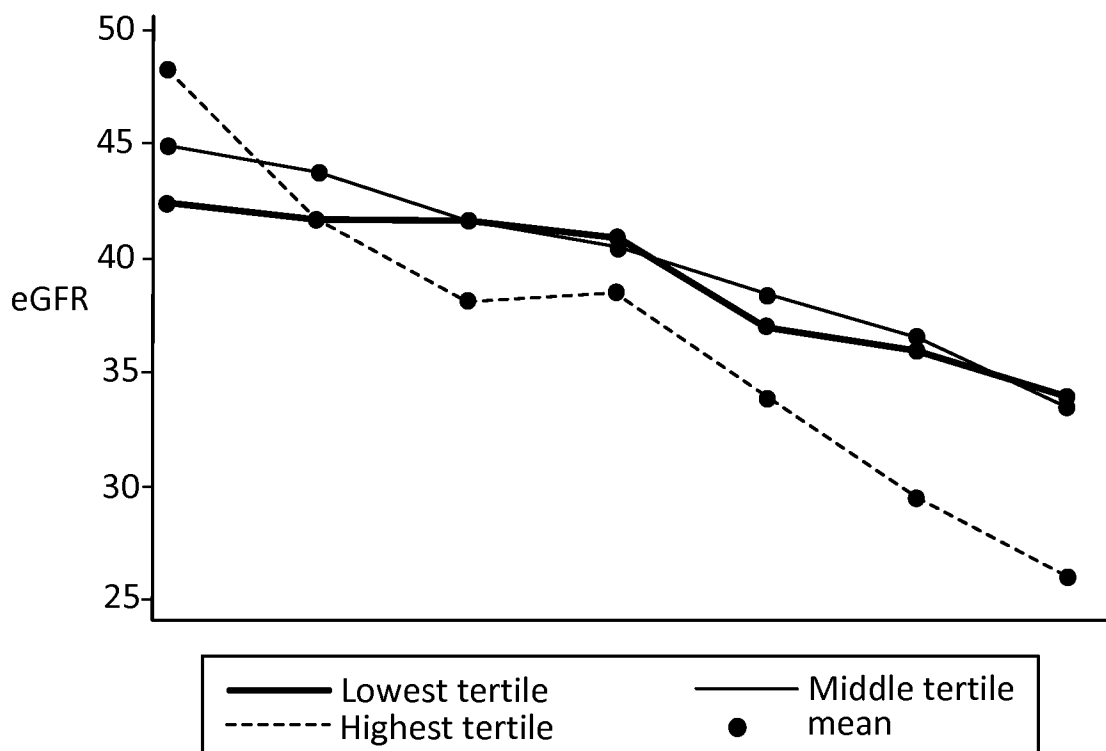


FIG. 6

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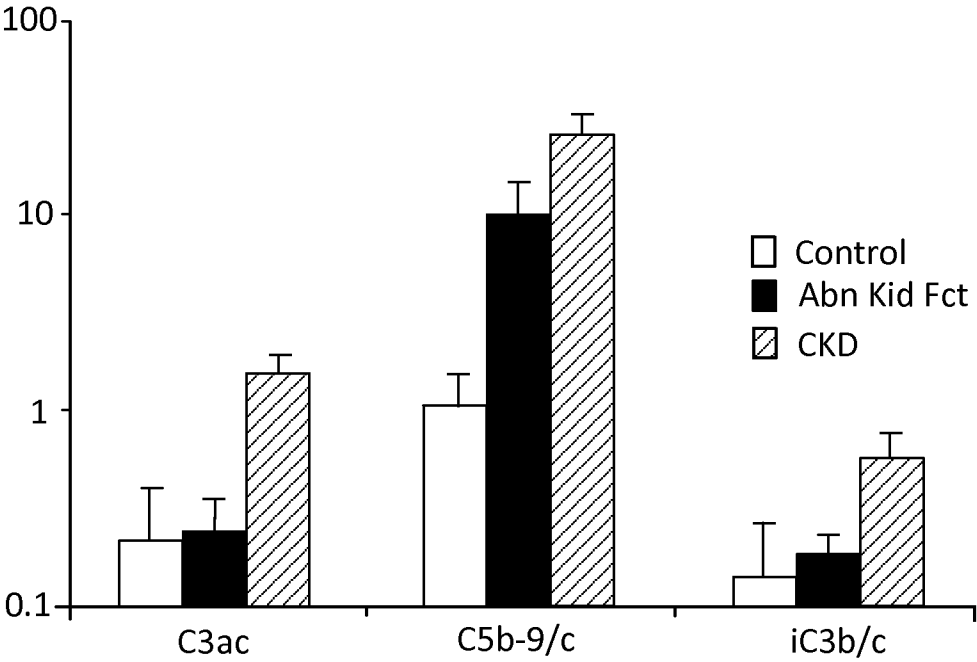


FIG. 7

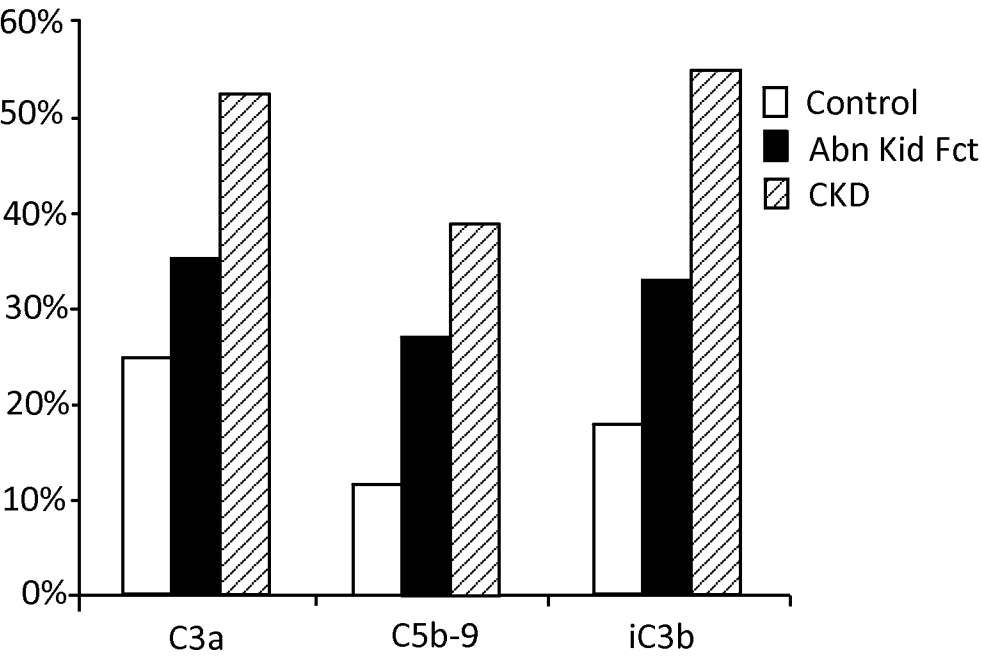


FIG. 8

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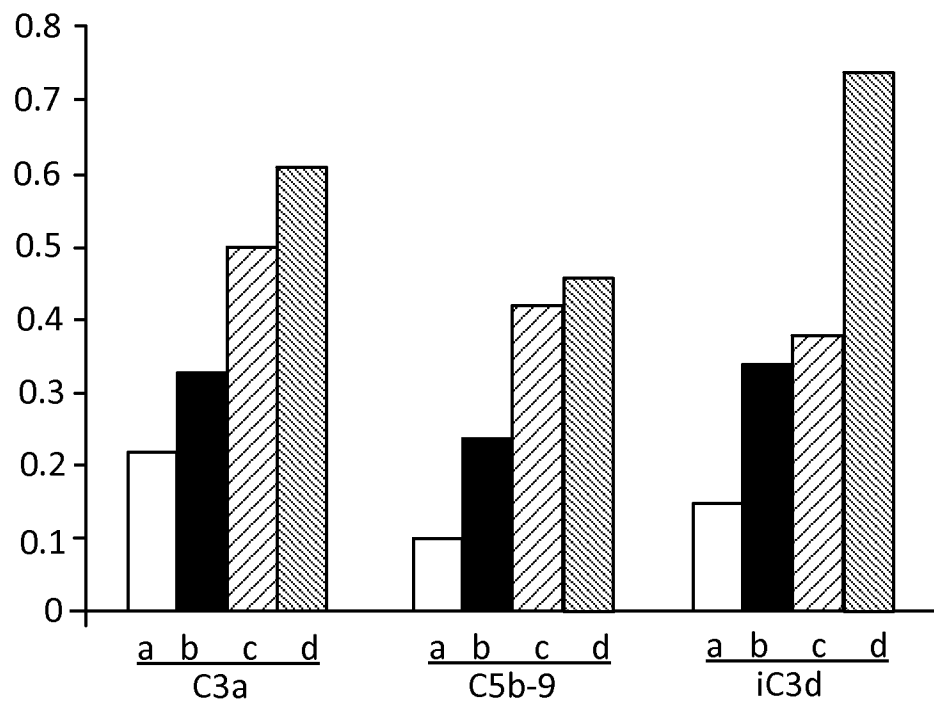


FIG. 9

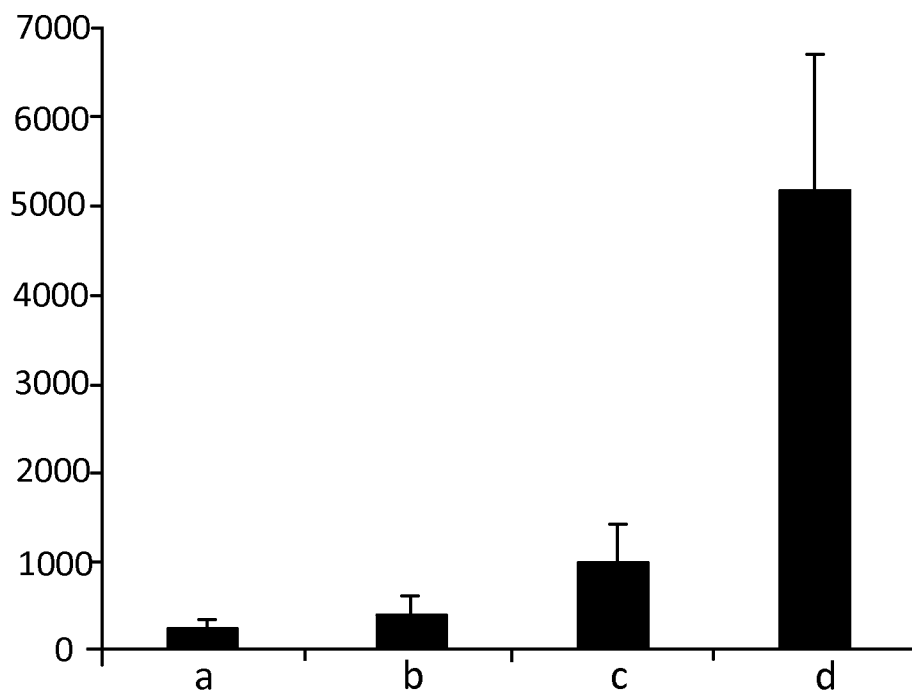


FIG. 10

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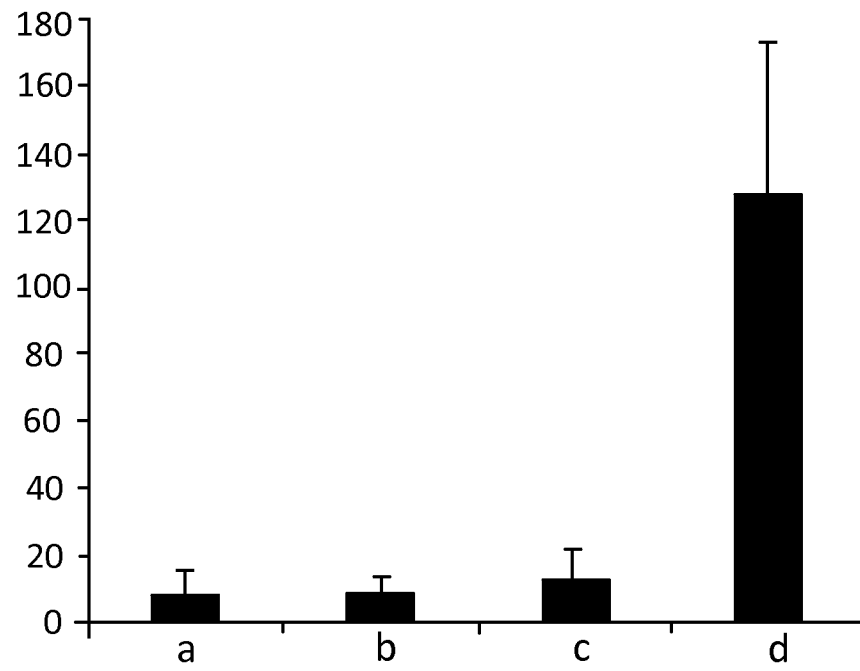


FIG. 11

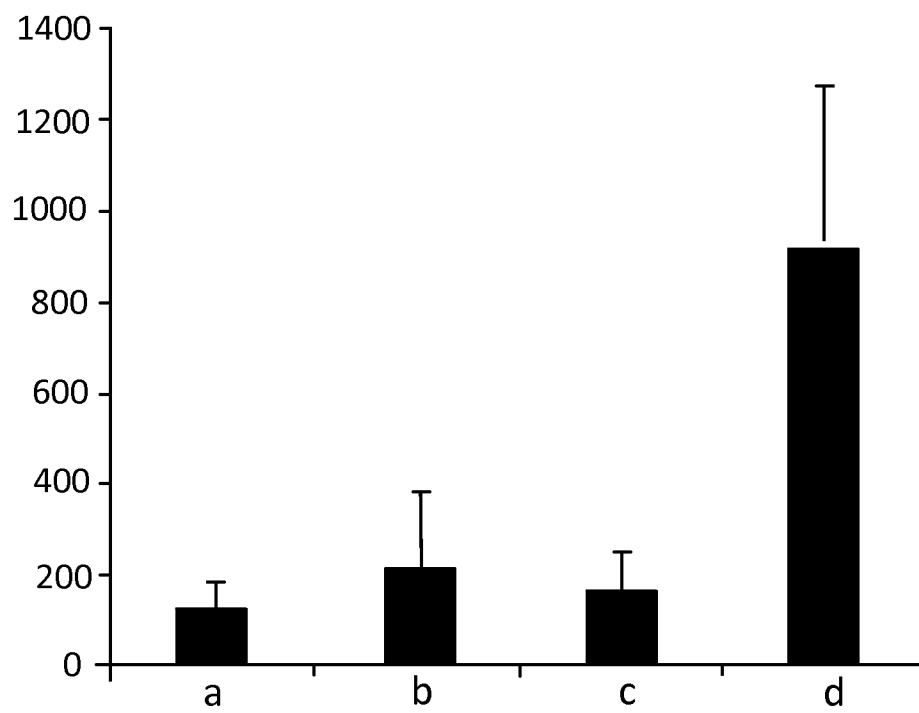


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/064592

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/68 (2014.01)

CPC - G01N 33/68 (2014.12)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - G01N 33/00, 33/48, 33/50, 33/53, 33/68 (2014.01)

USPC - 435/4, 7.1, 7.4, 7.9, 7.92

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - G01N 33/00, 33/48, 33/50, 33/53, 33/68 (2014.12)

(keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, PubMed

Search terms used: method assess monitor chronic kidney disease CKD second urine sample human levels c3 cleavage fragment time interval kit antibody renin angiotensin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0258095 A1 (DEMOPULOS et al) 11 October 2012 (11.10.2012) entire document	1-9, 34, 35
Y		32
X	CA 2 714 714 A1 (CENTRE FOR EYE RESEARCH AUSTRALIA) 15 January 2012 (15.01.2012) entire document	33
Y	WO 2006/042192 A2 (THE BRIGHAM AND WOMENS'S HOSPITAL, INC) 20 April 2006 (20.04.2006) entire document	32
A	US 2010/0303832 A1 (HAGEMAN) 02 December 2010 (02.12.2010) entire document	1-9, 32-35
A	US 2012/0195900 A1 (GHAYUR et al) 02 August 2012 (02.08.2012) entire document	1-9, 32-35

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

05 January 2015

Date of mailing of the international search report

23 FEB 2015

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Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/064592

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 10-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.