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(54) **Title:** DIAGNOSTIC ASSAYS FOR SUPAR- β 3 INTEGRIN DRIVEN KIDNEY DISEASES

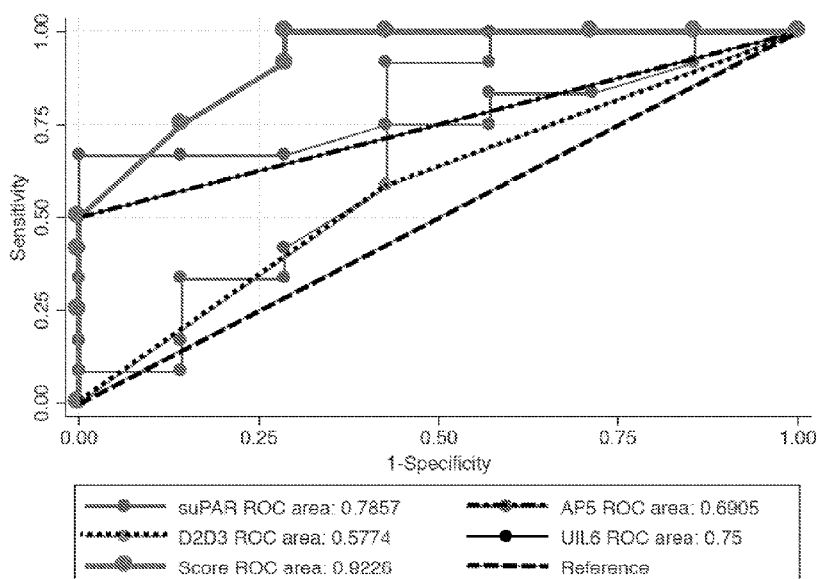


FIG. 10

(57) **Abstract:** Methods for diagnosing suPAR- β 3 integrin driven kidney diseases that can include detection of one, two or more variables, e.g., biomarkers (plasma suPAR levels, urine IL6 levels) and/or bioassays (β 3 integrin activation and presence of distinct suPAR fragments/isoforms in plasma).

Diagnostic Assays
for suPAR- β 3 Integrin Driven Kidney Diseases

CLAIM OF PRIORITY

This application claims the benefit of U.S. Patent Application Serial No. 62/232,606, filed on September 25, 2015. The entire contents of the foregoing are hereby incorporated by reference.

5 **FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT**

This invention was made with Government support under Grant No. 1 R01 DK101350 awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

10 Described herein are methods for diagnosing and treating Chronic kidney diseases (CKDs), e.g., suPAR- β 3 integrin driven kidney diseases. The methods can include detection of one, two or more variables, e.g., biomarkers (plasma suPAR levels, urine IL6 levels) and/or bioassays (β 3 integrin activation and presence of distinct suPAR fragments/isoforms in plasma).

15 **BACKGROUND**

Chronic kidney diseases (CKDs), a progressive loss of renal function over a period of months or years, affects hundreds of millions of people worldwide. The three most common causes of CKD are diabetes mellitus, hypertension, and glomerulonephritis. Historically, kidney disease has been classified according to the
20 part of the renal anatomy involved such as vascular disease, glomerular disease, and tubulointerstitial disease. Glomerular disease comprises a diverse group and is classified into primary glomerular disease such as focal segmental glomerulosclerosis (FSGS) and IgA nephropathy, and secondary glomerular disease such as diabetic nephropathy and lupus nephritis.

25 While major progress has been made in identifying genetic mutations that underlie hereditary forms of FSGS (1-4), kidney biopsy is still widely used for diagnosing glomerular diseases.

SUMMARY

It has been suggested that suPAR drives kidney injury by activating $\beta 3$ integrin on podocytes, thus providing a potential downstream pathogenic pathway that could be used to develop kidney-specific diagnostic tools.

5 Soluble urokinase-type plasminogen activator receptor (suPAR) is the soluble form of the urokinase-type plasminogen activator receptor (uPAR) and is present in plasma and other body fluids. suPAR has different biological forms and is being evaluated as an inflammatory and life style risk biomarker. suPAR was recently identified as a risk factor for both onset as well as progression of CKD regardless of
10 its entomology (5, 6). Elevated suPAR levels in the serum of the patients were originally implicated as a specific risk factor for recurrent FSGS (5), but subsequent studies showed elevated suPAR levels and its association with diabetic nephropathy in patients with type 1 diabetes (see below and 7). Taking into account that suPAR levels could also be elevated due to loss of kidney function (8), and since elevated
15 suPAR levels are associated with diverse pathogenic conditions such as sepsis, cancer (see, e.g., Sier et al., *Thromb Haemost.* 2004 Feb; 91(2):403-11, which found low molecular weight suPAR (e.g., D2D3) in the urine of both healthy and cancer patients, but none in their serum), and coronary artery disease, in addition to diverse chronic kidney diseases such as focal segmental glomerulosclerosis (FSGS) and diabetic
20 nephropathy (DN), additional diagnostics could provide more specificity to diagnose suPAR-driven CKD in patients that already have impaired kidney function.

It has been suggested that suPAR drives kidney injury by activating $\beta 3$ integrin on podocytes, thus providing a potential downstream pathogenic pathway that could be used to develop kidney-specific diagnostic tools. Given the potential of
25 suPAR as a novel therapeutic target in CKD, it was hypothesized that a combination of suPAR levels together with assays that detect suPAR-driven podocyte injury might allow development of the novel molecular diagnostic tools in nephrology that would be able to diagnose suPAR- $\beta 3$ integrin driven pathogenic mechanism in CKD in general, and recurrent FSGS in particular. With novel therapeutics that target $\beta 3$
30 integrin currently being developed and tested in humans (Maile et al., *J Diabetes Res.* 2014; 2014:421827; and Maile et al., *Endocrinology.* 2014 Dec;155(12):4665-75), a diagnostic tool that can specifically detect $\beta 3$ integrin-driven glomerular injury would be helpful in obtaining meaningful results from human trials and for patients with

CKD.

Thus, provided herein are methods comprising two, three, or all four of the following (in any order): determining a level of soluble urokinase-type plasminogen activator receptor (suPAR) protein in a plasma sample from a subject; determining a
5 level of interleukin 6 (IL-6) protein in a urine sample from the same subject; determining a level of low molecular weight suPAR in a plasma sample from the subject; and determining a level of $\beta 3$ integrin activation activity in a plasma sample from the subject.

Also provided herein are methods for detecting the presence of suPAR- $\beta 3$
10 integrin driven kidney disease in a subject. The methods include (a) determining a subject level of two, three, or all four of the following markers (in any order): soluble urokinase-type plasminogen activator receptor (suPAR) protein in a plasma sample from the subject; interleukin 6 (IL-6) protein in a urine sample from the subject; low molecular weight suPAR in a plasma sample from the subject; and $\beta 3$ integrin
15 activation activity in a plasma sample from the subject; (b) comparing the subject level of the marker to a reference level; and (c) detecting the presence of suPAR- $\beta 3$ integrin driven kidney disease in a subject who has at least two markers above the level.

Further provided herein are methods for treating a subject who has chronic
20 kidney disease, comprising (a) determining a subject level of two, three, or all four of the following markers (in any order): soluble urokinase-type plasminogen activator receptor (suPAR) protein in a plasma sample from the subject; interleukin 6 (IL-6) protein in a urine sample from the subject; low molecular weight suPAR in a plasma sample from the subject; and $\beta 3$ integrin activation activity in a plasma sample from
25 the subject; (b) comparing the subject level of the marker to a reference level; and (c) selecting and optionally administering a treatment for suPAR- $\beta 3$ integrin driven kidney disease to a subject who has at least two markers above the level.

In some embodiments, detecting $\beta 3$ integrin activation activity in a plasma sample comprises contacting the plasma sample with cultured human podocytes in
30 vitro and determining a level of $\beta 3$ integrin activation in the sample.

In some embodiments, determining a level of $\beta 3$ integrin activation in the sample comprises contacting the sample with an antibody that binds to beta 3 integrin and an antibody that binds to paxillin and dividing the number of cells expressing beta

3 integrin by the number of cells expressing paxillin.

In some embodiments, the subject has chronic kidney disease.

In some embodiments, the reference value is serum suPAR of ≥ 3 ng/ml (e.g., determined by ELISA assay); $\beta 3$ integrin activation > 1.2 (e.g., determined by AP5/paxillin ratio normalized to healthy serum); presence of low molecular weight suPAR in serum (e.g., determined by a detectable band on Western blot analysis; the detection limit of the assay may depend on the efficacy of the immunoprecipitation procedure); detectable presence of IL6 in the urine (e.g., determined by ELISA assay, e.g., wherein only a positive signal in ELISA (above the background) is considered positive).

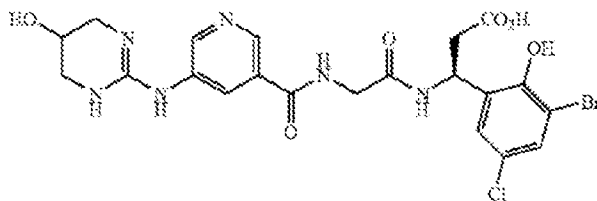
In some embodiments, the methods include determining a score calculated using the following algorithm:

$$\text{score} = \alpha \times (\text{serum suPAR}) + \beta \times (\beta 3 \text{ integrin activation}) + \gamma \times (\text{low molecular weight suPAR}) + \delta \times (\text{urine IL-6}),$$

wherein each of α , β , γ , and δ are empirically determined weights. In some embodiments, the algorithm is:

$$\text{score} = 0.253 \times (\text{serum suPAR}) + 0.282 \times (\beta 3 \text{ integrin activation}) + 0.212 \times (\text{low molecular weight suPAR}) + 0.253 \times (\text{urine IL-6}).$$

In some embodiments, the methods include selecting and/or administering a treatment for suPAR- $\beta 3$ integrin driven kidney disease, e.g., an $\alpha 5 \beta 3$ inhibitor and/or ex vivo removal of suPAR from the subject's circulation. In some embodiments, the $\alpha 5 \beta 3$ inhibitor is a monoclonal antibody that binds specifically to $\alpha 5 \beta 3$; a peptide comprising a RGD binding sequence; or a small molecule $\alpha 5 \beta 3$ inhibitor. In some embodiments, the small molecule $\alpha 5 \beta 3$ inhibitor is a compound of the formula



or a pharmaceutically acceptable salt thereof.

In some embodiments, the monoclonal antibody that binds specifically to $\alpha 5 \beta 3$ is VPI-2960B, CNT095, or anti-CD61.

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

Figures 1A-F. suPAR levels in plasma do not correlate with $\beta 3$ integrin activation.

A) SuPAR levels in serums of patients with different etiologies: healthy individuals, focal segmental glomerulosclerosis (FSGS), diabetic nephropathy (DN), on dialysis due to end stage renal diseases, sepsis.

B-F) Graphs correlating suPAR level in serum with $\beta 3$ integrin activation determined as a ratio between AP5 and paxillin staining. Percent of serums that exhibit $\beta 3$ integrin activation is shown by number and darker color in a Pie Chart.

Figures 2A-C. Immunofluorescence assay that measures $\beta 3$ integrin activation using human plasma.

A) Immunofluorescence (IF) staining of human podocytes incubated with either healthy serum or FSGS serum. Cells were stained for paxillin (marker of focal adhesions) and activated $\beta 3$ integrin using AP5 antibody from Bood Center of Wisconsin.

B) Graph showing $\beta 3$ integrin activation by serum free media (SFM), media with fetal bovine serum (FBS), or serums (#1-4) from healthy individuals. $\beta 3$ integrin activation is determined as a ratio between AP4 and paxillin staining.

C) Representative images of IF analysis using ImageJ program.

Figures 3A-G. D2D3 fragment is potent activator of $\beta 3$ integrin on podocytes and it can be detected in subset of patient serums.

A) Integrin activation of human podocytes treated with healthy serum, FSGS serum (control), FSGS serum incubated with anti-suPAR Ab, FSGS serum from which suPAR was immune precipitated using anti-suPAR Ab (Δ suPAR), or that was incubated with only Protein G beads.

5 B) Western blot analysis of suPAR in human serums before and after they were incubated with Protein G or Protein A beads. Data show that suPAR from the human serum binds Protein G or Protein A beads, even in the absence of specific anti-suPAR antibody.

10 C) Western blot analysis of glycosylated and de-glycosylated suPAR in human serum.

 D) Western blot analysis of D2D3 fragment present in human serum sample #2.

15 E) Integrin activation detected in human podocytes incubated with serum free media (SFM), FSGS serum, serum free media to which 2ng/ml of suPAR or D2D3 was added. Mn²⁺ was used as non-specific control of integrin activation.

 F) Integrin activation detected in human podocytes incubated with healthy serum (HS), FSGS serum and healthy serum with added 2 ng/ml of suPAR or D2D3 fragment.

20 G) D2D3 exhibits cooperative behavior with regard to β 3 integrin activation, in contrast to full length suPAR that activates β 3 integrin at much higher concentrations.

 Figures 4A-G. Integrin activation does not lead to increase in mRNA for integrins.

25 A) Schematic diagram of domains that constitute full length suPAR and D2D3 fragment used in this study.

 B) Silver staining gel of recombinant suPAR from R&D, D2D3 fragment expressed and purified from either bacteria or insect cells, as indicated in the figure.

 C) PLAUR Ab recognizes both suPAR and D2D3 by Western blot analysis.

30 D,E) RT-PCR of mRNA encoding α v, β 3, α 3, β 1 integrins in the presence of suPAR or D2D3 fragment.

 F,G) RT-PCR of mRNA encoding α v, β 3, α 3, β 1 integrins in the presence of different serums. No treatment altered expression levels of above integrins.

Figures 5A-F. D2D3 induces podocyte motility

A-C) High throughput assays determining $\beta 3$ integrin activation at focal adhesions (A), in the cytoplasm (B), or measuring total cell area (C).

5 D) High throughput assay examining podocyte motility in the presence of indicated concentrations of D2D3. LPS treatment was used as a positive control.

E) Bar graph showing podocyte motility under conditions shown in (D).

F) Podocytes viewed by lower magnification, show that at higher D2D3 concentration (25 ng/ml) cells start to detach from the coverslip.

10 Figures 6A-J. D2D3 fragment induces proteinuria and glomerular injury in mice.

A-C) Bar graphs showing proteinuria (kidney injury) or the lack of in animals injected with PBS (A, control), human suPAR (B), or human D2D3 (C). Only
15 injection of D2D3 resulted in transient proteinuria in mice. N=6 animals.

D) Western blot analysis of urine from animals injected with PBS or D2D3. Data show presence of nephrin (podocyte specific protein) in the urine of proteinuric animals, suggesting podocyte injury.

E and H) Schematic diagrams of suPAR transgenes used in this study.

20 F and I) Graphs showing levels of albumin/creatinine (proteinuria) in mice stably expressing mouse D2D3 (F) or Isoform 2 (I) from fat tissue. Animals were fed with high fat diet to induce protein expression.

G and J) Representative image of glomerulus stained with PAS. Image shows that glomerulus exhibits signs of injured glomerulus similar to that observed during
25 diabetic nephropathy.

Figures 7A-B. Alignment of protein sequences encoding uPAR variants as indicated in the Figure.

30 Figures 8A-D. Human isoform 2 induces podocyte detachment.

A) Immunofluorescence (IF) staining of human podocytes incubated with healthy serum (Control), Mn^{2+} or increasing concentrations of human suPAR

variant 2. Cells were stained for paxillin (marker of focal adhesions) and activated $\beta 3$ integrin using AP5 antibody from Blood Center of Wisconsin.

B-D) Bar graphs showing integrin activation (B), number of cells (C) and number of focal adhesions (FA) per cell (D) in cells treated as shown in (A).

5

Figure 9. Table showing representative data sets of 18 FSGS and 5 healthy serums. We originally determined a value for each of the parameters using logistic regression analysis, but that analysis as shown in the Figure determined a similar value for each one of the parameters. Thus, we decided to use a total score (form 0-4) instead of the specific value determined using regression analysis.

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Figure 10. ROC (receiver operating characteristic) curves with 4 different parameters, which separate recurrent from non-recurrent FSGS. Area under the curve was calculated for each one of 4 parameters using ROC analysis in order to separate subjects with recurrent FSGS from non-recurrent FSGS. Area under the curve that is >0.5 is considered statistically significant. Of note, we originally also determined the levels of suPAR in patient's urine, but since the ROC analysis did not show statistically significant area under the curve value (see enclosed Figure, value was 0.327), that parameter was not used in generating the composite score assay. Data shown were generated by using 22 recurrent and 7 non-recurrent FSGS serums.

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Figure 11. Composite score of ≥ 3 efficiently not only separates recurrent from non-recurrent FSGS but is also identifying suPAR- $\beta 3$ integrin pathway in population suffering from DN. Positive value (+) was assigned to: 1. serum suPAR of ≥ 3 ng/ml (Elisa assay); 2. $\beta 3$ integrin activation >1.2 (AP5/paxillin ratio); 3. presence of D2D3 in serum determined by Western blot analysis; 4. detectable presence of IL6 in the urine (ELISA assay). Data were generated using 29 FSGS and 32 DN samples.

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Figures 12 A-D. ROC (receiver operating characteristic) curves with 3 different parameters. Area under the curve was calculated for each one of the 3 indicated parameters (Score 1); combination of any two parameters (Score 3) and finally the combination of three parameters (Score 3). FIG. 12A is a combination of the suPAR, AP5, and IL6 parameters. FIG. 12B is a combination of the suPAR, AP5, and

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low molecular weight suPAR parameters. FIG. 12C is a combination of the suPAR, low molecular weight suPAR, and IL6 parameters. FIG. 12D is a combination of the AP5, low molecular weight suPAR, and IL6 parameters.

DETAILED DESCRIPTION

5 The selectivity of the glomerular filter is maintained by physical, chemical, and signaling interplay among its three core constituents – the glomerular endothelial cells, the glomerular basement membrane (GBM), and podocytes. Injury to or functional impairment of any of these three components of the glomerular filtration barrier can lead to proteinuria (11). Podocytes are injured in many forms of human and experimental glomerular disease, including minimal change disease, focal
10 segmental glomerulosclerosis (FSGS), and diabetes mellitus (12). Podocytes are terminally differentiated visceral epithelial cells of the glomerulus which develop a characteristic architecture specialized for glomerular ultrafiltration. Their structure is traditionally divided into three kinds of subcellular compartment: the cell body,
15 microtubule-driven membrane extensions named primary process, and actin-driven membrane extensions named foot processes (FPs). Adjacent podocytes are interdigitated with each other at their foot processes, which are separated from each other by filtration slits and bridged with a specialized intercellular junction called a slit diaphragm. The foot processes and slit diaphragm serve as an adhesive apparatus
20 to the glomerular basement membrane (GBM), which together with endothelial cells and their glycocalyx forms a filtration barrier.

 Regardless of the underlying cause of glomerular disease, the early pathogenic events are characterized by molecular alterations in the slit diaphragm without visible morphological changes or, more obviously, by a reorganization of the FPs structure
25 with fusion of filtration slits termed “FP effacement” (12-14). Although it is possible to have proteinuria without significant FP effacement, for over 50 years FPs effacement has been a cardinal feature of proteinuria. While the mechanistic significance of FPs effacement with regard to proteinuria has long been a mystery, over the last decade numerous studies demonstrated that FPs effacement represents a
30 change in the organization of the actin cytoskeleton (12, 15).

 As noted above, one of the pathogenic pathways implicated in CKD is suPAR- β 3 integrin pathway (Wei et al., Nature medicine. 2011;17(8):952-60; Wei et al., Nat Med. 2008 Jan;14(1):55-63). By determining the ability of the low molecular weight

suPAR as well as splice variant 2 to induce proteinuria and glomerular injury in mice via activating $\beta 3$ integrin on podocytes, the data presented herein establish methods including a composite scoring system that efficiently separate non-recurrent from recurrent FSGS. The same scoring system also identified a subset of patients with DN as positive subjects. Given current and future attempts to develop novel therapeutic targets for CKD, and given that CKD encompasses a highly diverse group of patients, it is become more and more important to identify patients that are predicted to respond to target-specific therapy. The methods described herein can be used to identify subjects that have suPAR- $\beta 3$ integrin driven kidney diseases, and thus are expected to respond (i.e., have a stabilized or improved condition) to either suPAR and/or $\beta 3$ blocking therapies.

Described herein are methods that use a combination of biomarkers (plasma suPAR levels, urine IL6 levels) and/or bioassays ($\beta 3$ integrin activation and presence of low molecular weight suPAR, e.g., fragments/isoforms, in plasma) together to generate a diagnostic score that predicts $\beta 3$ -integrin driven kidney injury. Unexpectedly, as shown herein, a positive score was associated with a majority of patients with recurrent FSGS and with a subset of patients with DN.

Subjects

In the present methods a subject who may be evaluated using the present methods can be a human or other mammal, typically one who is at risk of developing or has a disorder characterized by proteinuria, is at risk for or is undergoing kidney failure, has received a kidney graft, or any combination thereof. A disorder characterized by proteinuria includes, for example, kidney or glomerular diseases (e.g., FSGS), membranous glomerulonephritis, focal segmental glomerulonephritis, minimal change disease, nephrotic syndromes, pre-eclampsia, eclampsia, kidney lesions, collagen vascular diseases, stress, strenuous exercise, benign orthostatic (postural) proteinuria, focal segmental glomerulosclerosis, IgA nephropathy, IgM nephropathy, membranoproliferative glomerulonephritis, membranous nephropathy, end-stage kidney disease, sarcoidosis, Alport's syndrome, diabetes mellitus (e.g., diabetic nephropathy), kidney damage due to drugs, Fabry's disease, infections, aminoaciduria, Fanconi syndrome, hypertensive nephrosclerosis, interstitial nephritis, sickle cell disease, hemoglobinuria, multiple myeloma, myoglobinuria, Wegener's granulomatosis, and glycogen storage disease type 1. In some embodiments, the

subject may be affected by one or more of the foregoing disorders, may be a heterozygote for the polymorphism Leu33Pro in the human integrin $\beta 3$ gene, may be a homozygote for the polymorphism Leu33Pro in the human integrin $\beta 3$ gene, may have at least about 3 ng suPAR per ml blood in the circulation, or any combination thereof.

In some embodiments, subjects who may be evaluated using the present methods include those who have chronic kidney disease (CKD) or are at risk of developing CKD, e.g., who have acute kidney injury (AKI) or another condition noted above, e.g., a disorder characterized by proteinuria.

The stages of CKD are classified as follows:

Stage 1: Kidney damage with normal or increased GFR (>90 mL/min/1.73 m²);

Stage 2: Mild reduction in GFR (60-89 mL/min/1.73 m²);

Stage 3a: Moderate reduction in GFR (45-59 mL/min/1.73 m²);

Stage 3b: Moderate reduction in GFR (30-44 mL/min/1.73 m²);

Stage 4: Severe reduction in GFR (15-29 mL/min/1.73 m²); and

Stage 5: Kidney failure (GFR <15 mL/min/1.73 m² or dialysis).

In stage 1, given the relatively normal GFR, a diagnosis may be confirmed by the presence of one or more of the following:

Albuminuria (albumin excretion >30 mg/24 hr or albumin:creatinine ratio >30 mg/g [>3 mg/mmol]);

Urine sediment abnormalities;

Electrolyte and other abnormalities due to tubular disorders;

Histologic abnormalities;

Structural abnormalities detected by imaging; or

History of kidney transplantation.

See, e.g., Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney Int Suppl. 2013. 3:1-150. Standard laboratory and clinical methods can be used to establish a diagnosis.

Focal segmental glomerulosclerosis (FSGS) is a significant cause of end-stage kidney disease. It affects both native kidneys and transplanted kidney grafts. It starts in kidney glomeruli. In the early stage of FSGS, it mainly targets the visceral

epithelium (also called podocytes) that comprise cells with foot processes to regulate functioning of the renal filtration barrier. Effacement of podocyte foot processes can mark the first or one of the first ultrastructural step(s) that is/are associated with loss of plasma proteins into the urine. While gene defects in podocytes have been
5 identified for hereditary FSGS, there are also cases that occur in the absence of gene defects or with post-transplant recurrence in about 30% of patients receiving a kidney graft. These observations led to the suggestion that development of FSGS can be associated with a "FSGS permeability factor" in the patient's circulation (see Savin et al., Translational Res. 151 :288-292, 2008). Without wishing to be bound by theory,
10 suPAR is likely to be that factor; see WO 2010/054189 and WO 2012/154218.

Methods of Diagnosis and Monitoring

Included herein are methods for diagnosing suPAR- β 3 integrin driven kidney diseases. The methods include detection of one, two or more variables, e.g., biomarkers (plasma suPAR levels, urine IL6 levels) and/or bioassays (β 3 integrin
15 activation and presence of distinct lower molecular weight suPAR fragments/isoforms in plasma). The methods can include obtaining or providing a plasma and/or urine sample from a subject, and determining one, two, three, or all four of the following variables: (1) the presence and/or level of total suPAR in the plasma; (2) the presence and/or level of low molecular weight suPAR (e.g., suPAR fragments/isoforms that are
20 not full length) in the plasma; (3) presence and/or levels of IL6 in the urine; and/or (4) presence and/or levels of β 3 integrin activation (e.g., in an *in vitro* assay).

The low molecular weight suPAR detected in the present methods include those that are not full length suPAR, and thus have a lower molecular weight than full length suPAR. Full length suPAR is approximately 50 kD when fully glycosylated
25 and approximately 32.5 kD when deglycosylated. The low molecular weight suPAR fragments/isoforms can include those that lack D1 (e.g., as a result of proteolysis, e.g., D2D3 fragment) or are splice variants, e.g., suPAR2. The low molecular weight suPAR fragments/isoforms have a molecular weight of less than 50 kD, e.g., about 25-45 kD, e.g., about 30 kD, when fully glycosylated and less than 32.5 kD, e.g.,
30 about 20-30 kD, e.g., about 25 kD when deglycosylated. In this context, "about" means $\pm 10\%$.

As used herein the term "sample", when referring to the material to be tested for the presence of a biological marker using the method of the invention, unless

otherwise specified can include *inter alia* tissue, whole blood, plasma, serum, urine, sweat, saliva, breath, exosome or exosome-like microvesicles (U.S. Patent No. 8.901.284), lymph, feces, cerebrospinal fluid, ascites, bronchoalveolar lavage fluid, pleural effusion, seminal fluid, sputum, nipple aspirate, post-operative seroma or wound drainage fluid. The type of sample used may vary depending upon the identity of the biological marker to be tested and the clinical situation in which the method is used. Various methods are well known within the art for the identification and/or isolation and/or purification of a biological marker from a sample. An “isolated” or “purified” biological marker is substantially free of cellular material or other contaminants from the cell or tissue source from which the biological marker is derived, i.e., partially or completely altered or removed from the natural state through human intervention. For example, nucleic acids contained in the sample are first isolated according to standard methods, for example using lytic enzymes, chemical solutions, or isolated by nucleic acid-binding resins following the manufacturer’s instructions.

The presence and/or level of a protein (e.g., of IL-6, suPAR total, and/or low molecular weight suPAR, e.g., suPAR fragments and isoforms) can be evaluated using methods known in the art, e.g., using standard electrophoretic and quantitative immunoassay methods for proteins, including but not limited to, Western blot, e.g., with immunoprecipitation of specific proteins; enzyme linked immunosorbent assay (ELISA); biotin/avidin type assays; protein array detection; radio-immunoassay; immunohistochemistry (IHC); immune-precipitation assay; FACS (fluorescent activated cell sorting); mass spectrometry (Kim (2010) *Am J Clin Pathol* 134:157-162; Yasun (2012) *Anal Chem* 84(14):6008-6015; Brody (2010) *Expert Rev Mol Diagn* 10(8):1013-1022; Philips (2014) *PLOS One* 9(3):e90226; Pfaffe (2011) *Clin Chem* 57(5): 675-687). The methods typically include revealing labels such as fluorescent, chemiluminescent, radioactive, and enzymatic or dye molecules that provide a signal either directly or indirectly. As used herein, the term “label” refers to the coupling (i.e. physically linkage) of a detectable substance, such as a radioactive agent or fluorophore (e.g. phycoerythrin (PE) or indocyanine (Cy5), to an antibody or probe, as well as indirect labeling of the probe or antibody (e.g. horseradish peroxidase, HRP) by reactivity with a detectable substance. Antibodies to suPAR and IL-6 are known in the art and commercially available. Antibodies that bind

specifically to low molecular weight suPAR can be generated using known methods (see, e.g., Sier et al., *Thromb Haemost.* 2004 Feb; 91(2):403-11). See also WO2012154218 for additional information on measuring levels of suPAR in serum.

In some embodiments, an ELISA method may be used, wherein the wells of a
5 microtiter plate are coated with an antibody against which the protein is to be tested. The sample containing or suspected of containing the biological marker is then applied to the wells. After a sufficient amount of time, during which antibody-antigen complexes would have formed, the plate is washed to remove any unbound moieties, and a detectably labelled molecule is added. Again, after a sufficient period of
10 incubation, the plate is washed to remove any excess, unbound molecules, and the presence of the labeled molecule is determined using methods known in the art. Variations of the ELISA method, such as the competitive ELISA or competition assay, and sandwich ELISA, may also be used, as these are well-known to those skilled in the art.

15 In some embodiments, an IHC method may be used. IHC provides a method of detecting a biological marker *in situ*. The presence and exact cellular location of the biological marker can be detected. Typically, a sample is fixed with formalin or paraformaldehyde, embedded in paraffin, and cut into sections for staining and subsequent inspection by confocal microscopy. Current methods of IHC use either
20 direct or indirect labelling. The sample may also be inspected by fluorescent microscopy when immunofluorescence (IF) is performed, as a variation to IHC.

Mass spectrometry, and particularly matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS), is useful for the detection of biomarkers of this
25 invention. (See U.S. Patent No. 5,118,937; 5,045,694; 5,719,060; 6,225,047). Such methods may be particularly useful for determining presence and/or levels of low molecular weight suPAR fragments and isoforms.

Integrin activation can be determined by an assay such as the one described herein. In this exemplary assay, $\beta 3$ integrin activation on podocytes is measured *in*
30 *vitro*. The podocytes are cultured in the presence of serum from the subject for a time sufficient to allow activation of integrin and/or development of focal adhesions (e.g., approximately 0.1-10%, approximately 5-10%, or approximately 10% human serum in media; for example, 1 ml media will have 0.1 ml of human serum; time can be

approximately 12 to 24 hours, or approximately 24 hours) and the ratio of activated integrin levels over levels of total amount of focal adhesions is determined. Activated integrin levels can be determined, e.g., using an antibody that specifically detects the active form of $\beta 3$ integrin, e.g., AP5 (see, e.g., Honda et al., 270(20):11947-54 (1995); Faccio et al., Journal of Cell Science 115, 2919-2929 (2002); and Wei et al., Nature Medicine 17:952-960 (2011). AP5 antibodies are available commercially from Kerafast (Boston, MA). The number of focal adhesions can be determined using known methods, e.g., by staining with paxillin and determining the number or level of focal adhesions within a selected field.

In the present methods, two, three, or more of the variables can be determined, e.g., as follows:

Levels of total suPAR in plasma	Levels of IL-6 in urine	Levels of low molecular weight suPAR in plasma	Integrin activation
X	X		
X		X	
X			X
	X	X	
	X		X
		X	X
X	X	X	
	X	X	X
X		X	X
X	X		X
X	X	X	X

The methods can include comparing the presence and/or level of each variable with one or more references, e.g., a control reference that represents a normal level, e.g., a level in an unaffected subject, and/or a disease reference that represents a level associated with suPAR- $\beta 3$ integrin driven kidney diseases, e.g., a level in a subject having FSGS, e.g., a subject having an increased risk of reoccurrence of FSGS after kidney transplant. Methods of determining threshold or reference levels are known in the art, and exemplary methods are described herein. In some embodiments, the threshold levels are: suPAR in the serum above 3 ng/ml; $\beta 3$ integrin activation above 1.2; presence of any detectable low molecular weight suPAR if determined by fragment IP followed by the western blot (e.g., a level above the lowest level of detection for a standard assay); presence of any detectable levels of IL6 in the urine (e.g., a level above the lowest level of detection for a standard assay). In some

embodiments, the methods also include detecting suPAR in the urine (with an exemplary threshold of 3 ng/ml or above).

The methods can also include calculating a score based on the variables that can be compared to a reference score, wherein a score that is above the reference score indicates that the subject has suPAR- β 3 integrin driven kidney disease and/or a high risk of, or is likely to have, recurrence of kidney disease after transplant, or is predicted to have a positive response to therapy targeting suPAR- β 3 integrin pathway; a score below the reference score indicates that the subject has a low risk of recurrence of disease after transplant, or is predicted to have no or a poor response to therapy targeting suPAR- β 3 integrin pathway. a “high” risk as used herein indicates that the subject has a statistically increased (e.g., at least greater than 50%) chance of recurrence or response as compared to someone with a score associate with a “low” risk.

In some embodiments, the levels of each of the evaluated variables can be assigned a value (e.g., a value that represents the level of the biomarker or activation level, e.g., normalized as described herein). For example, value of 0 or 1 can be assigned to each of the evaluated parameters. That value (optionally weighted to increase or decrease its effect on the final score) can be summed or otherwise mathematically manipulated to produce a final score. One of skill in the art could optimize such a method to determine an optimal algorithm for determining a score; one exemplary method is described herein.

For example, a weighted average formula can be used to generate a composite score assay. In some embodiments, a composite score can be calculated based on all four of the variables, using the following algorithm:

$$\text{score} = \alpha \times (\text{serum suPAR}) + \beta \times (\beta 3 \text{ integrin activation}) + \gamma \times (\text{low molecular weight suPAR}) + \delta \times (\text{urine IL-6})$$

Wherein each of α , β , γ , and δ are empirically determined weights. An exemplary formula with weights included can be:

$$\text{score} = 0.253 \times (\text{serum suPAR}) + 0.282 \times (\beta 3 \text{ integrin activation}) + 0.212 \times (\text{low molecular weight suPAR}) + 0.253 \times (\text{urine IL-6})$$

Threshold levels can be determined empirically. One of skill in the art will appreciate that references can be determined using known epidemiological and statistical methods, e.g., by determining a score, or protein or activation levels, in an appropriately stratified cohort of subjects, e.g., subjects who have or do not have a recurrence of disease after transplant.

In exemplary embodiments, the thresholds can be as follows: suPAR in the serum above 3 ng/ml; $\beta 3$ integrin activation above 1.2; presence of any detectable low molecular weight suPAR if determined by fragment IP followed by the western blot (e.g., a level above the lowest level of detection for a standard assay); presence of any detectable levels of IL6 in the urine (e.g., a level above the lowest level of detection for a standard assay). A score above a certain level, e.g., a score of > 0.5 or > 0.7 , can be considered positive for suPAR- $\beta 3$ integrin driven podocyte injury.

The threshold level for each variable or for the overall score can be determined using known epidemiological and statistical methods; in some embodiments the level can be, e.g., a median or mean, or a level that defines the boundaries of an upper or lower quartile, tertile, or other segment of a clinical trial population that is determined to be statistically different from the other segments. 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 association with risk of developing disease or presence of disease 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 or presence of disease 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-quartiles (i.e., n regularly spaced intervals) the lowest of the n-quartiles being subjects with the lowest risk and the highest of the n-quartiles being subjects with the highest risk.

As noted above, any of the two variables evaluated herein can be used, rather than all four, with reasonably good sensitivity; using additional variables increases the specificity. In some embodiments, all 4 variables are evaluated, and a positive score on any two, or any 3 in combination (score of 0.5 or 0.7) will be very specific for suPAR- $\beta 3$ pathway. However, depending on the specificity desired, a subset can also

be used. When two are used, the presence of two positive results indicates can be considered positive for suPAR- β 3 integrin driven podocyte injury. When three are used, the presence of two or three positive results indicates can be considered positive for suPAR- β 3 integrin driven podocyte injury.

5 Subjects associated with predetermined values are typically referred to as reference subjects. For example, in some embodiments, a control reference subject does not have a disorder described herein (e.g., does not have suPAR- β 3 integrin driven kidney disease or does not have a recurrence of disease after kidney transplant). In some cases, it may be desirable that the control subject has kidney
10 disease (e.g., FSGS), and in other cases it may be desirable that a control subject has no kidney disease. In some cases, it may be desirable that the control subject has a recurrence of disease after transplant and in other cases it may be desirable that a control subject does not have a recurrence after transplant.

 Thus, in some cases the variable in a subject being greater than or equal to a
15 reference variable is indicative of a clinical status (e.g., indicative of a disorder as described herein, e.g., suPAR- β 3 integrin driven kidney disease or high risk of recurrence after transplant). In other cases, the level of the variable in a subject being less than or equal to the reference variable is indicative of the absence of suPAR- β 3 integrin driven kidney disease or normal or low risk of recurrence. In some
20 embodiments, the amount by which the level in the subject is the less than the reference level is sufficient to distinguish a subject from a control subject, and optionally is a statistically significantly less than the level in a control subject. In cases where the variable in a subject being equal to the reference variable, the “being equal” refers to being approximately equal (e.g., not statistically different).

25 The predetermined value can depend upon the particular population of subjects (e.g., human subjects) selected. For example, an apparently healthy population will have a different ‘normal’ range of levels of the measured variables than will a population of subjects which have, are likely to have, or are at greater risk to have, a disorder described herein. Accordingly, the predetermined values selected
30 may take into account the category (e.g., sex, age, health, risk, presence of other diseases) 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 characterizing likelihood, or risk, numerous predetermined values can be established.

The present methods can also be performed multiple times on the same subject, e.g., before, after, and during treatment, to monitor the effectiveness of the treatment, or without any treatment, e.g., to monitor the subject's condition (e.g., the severity of their disease). An increase in levels of the measured markers and/or activation over time indicates that the subject's condition is worsening (for example, progressing from acute kidney injury (AKI) to CKD); no change in levels means that the subject is stable (in a subject with progressive disease, this may indicate that the treatment has stabilized the disease); and a decrease indicates that the subject's condition is improving (e.g., the treatment is effective). The methods can be used to determine if or when to begin treatment, for example, when a subject has progressed to severe enough disease to warrant further intervention. The methods can be used to select a treatment; for example, the methods can be used to select subjects who would be most likely to benefit from a treatment directed at affecting suPAR- β 3 integrin driven pathogenesis; those with higher levels (e.g., levels above a threshold) of the measured markers and/or activation would be more likely to benefit. Furthermore, the methods can be used to identify those who are most likely to have a relapse of FSGS after transplant (i.e., those with higher levels (e.g., levels above a threshold) of the measured markers and/or activation), and are thus better candidates for a cadaver organ rather than from a living donor.

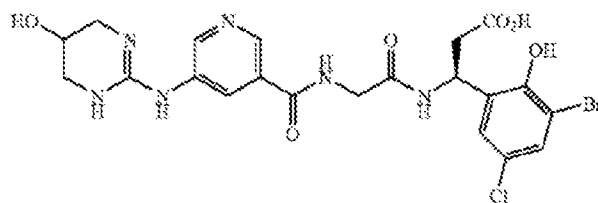
Methods of Treatment

The methods described herein can include selecting and/or administering a treatment for kidney disease to a subject determined to have a score above a reference score, or a level of one or more of the variables evaluated herein above a reference level.

A number of treatments for kidney disease are known in the art. For example, standard treatments can include one or more of administration of medications to control blood pressure; e.g., angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs), with a target blood pressure of less than 130/80 mm Hg; vitamin D supplementation, e.g., with synthetic analogs such as paricalcitol; treatment of hyperlipidemia, e.g., with statins; treatment of any hypothyroidism, e.g., with thyroid hormone replacement therapy (THRT) with L-

thyroxine; controlling blood glucose levels (target hemoglobin A1c [HbA1C] < 7%),
 e.g., with antidiabetic drugs or insulin; administration of renin-angiotensin system
 (RAS) blockers in subjects with diabetic kidney disease (DKD) and proteinuria; and
 administration of angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin-
 5 receptor blockers (ARBs) in patients with proteinuria. In more advanced stages, other
 treatments can be added as needed, e.g., for anemia (erythropoiesis-stimulating
 agents, for example epoetin alfa or darbepoetin alfa); hyperphosphatemia (dietary
 phosphate binders and dietary phosphate restriction); hypocalcemia (Ca^{2+}
 supplements with or without calcitriol); volume overload (loop diuretics or
 10 ultrafiltration); metabolic acidosis (oral alkali supplementation); hyperparathyroidism
 (calcitriol, vitamin D analogues, or calcimimetics); and/or uremic manifestations
 (long-term renal replacement therapy (hemodialysis, peritoneal dialysis, or renal
 transplantation).

Alternatively or in addition, the methods can include selecting and/or
 15 administering a treatment directed at affecting suPAR- β 3 integrin driven
 pathogenesis, e.g., an agent which inhibits soluble urokinase receptor (suPAR)
 activity and/or function and/or modulates expression soluble and/or membrane bound
 urokinase receptor (uPAR) and/or pathways associated with urokinase receptor, e.g.,
 an antibody, aptamer, antisense oligonucleotide, a natural agent, or synthetic agent
 20 (see, e.g., WO 2010/054189), e.g., an α 5 β 3 inhibitor, e.g., a monoclonal antibody that
 binds specifically to α 5 β 3 and/or α 5 β 5, e.g., VPI-2960B (Vascular Pharma, Research
 Triangle Park, NC) or CNTO95 as described in PCTUS2011/49563, or anti-CD61; a
 peptide comprising a RGD binding sequence, e.g., cyclo-[Arg-Gly-Asp-D-Phe-Val],
 or a small molecule α 5 β 3 inhibitor, e.g., a compound of the formula



or a pharmaceutically acceptable salt thereof, or another compound as described in
 US2010/0297139; and/or ex vivo removal of suPAR from the subject's circulation,
 e.g., as described in WO2012/154218. Combinations of any of the above can also be
 administered.

EXAMPLES

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

Example 1. Composite diagnostic assays predict suPAR- β 3 integrin driven kidney diseases

Reagents:

Level of human suPAR in serum was determined using commercially available ELISA assays from R&D. Level of suPAR in the urine was determined using identical ELISA.

Level of human IL6 in urine was determined using commercially available ELISA assay from Thermo Fisher Scientific (Life Technologies). AP5 antibody that recognizes active form of β 3 integrin was from Blood Center of Wisconsin.

Methods:

AP5 Staining: AP5 AB: Blood Center of Wisconsin; Paxillin: Abcam (Cat. #: a β 32084). Human podocytes were proliferated at 33°C and differentiated at 37C for 10 days on coverslips in a 6-well plate. On day 10, the podocytes were serum starved overnight (RPMI 1640+anti-anti). Cells were then incubated with 10% healthy serum or patient sample for 24h at 37C. Healthy serum served as the negative control, and healthy serum supplemented with MnCl₂ at a final concentration of 625 μ M served as the positive control. After treatment, samples were fixed with 4% paraformaldehyde for 20 minutes, washed and permeabilized with 0.3% Triton-X for 3 minutes, and blocked (5% donkey serum, 5% goat serum, in 1x PBS) for 45 minutes. The cells were incubated with the first primary antibody (anti-Paxillin, 1:300 Rabbit) for 1 hour. The secondary antibody goat-anti-rabbit 568 (1:2000, Invitrogen) was used. The cells were incubated with the second primary antibody (AP5, 1:50 Mouse) for 1 hour. The secondary antibody goat-anti-mouse 488 (1:1000, Invitrogen) was used. The coverslips were mounted with Flouoroshield with DAPI (abcam, ab104139). Images were acquired using Zeiss LSM 5 Pascal. The detector gain, amplifier offset, and laser power settings were kept consistent for the collection of all images. The images were analyzed using ImageJ.

Additional Information for image analysis: The files were opened in ImageJ and the channels were separated and images inverted. The threshold was set for each channel and kept consistent for the analysis of all images. For beta 3 integrin images,

the nuclear staining was excised from each cell. Using the same freehand selection function, the cell was circled and the integrity density was measured for each channel. The data were exported into Microsoft Excel and the integrity density value of beta 3 integrin (AP5) staining of each cell was divided by the integrity density value of focal adhesion (paxillin) staining of each cell, and normalized to the negative control.

Detection of low molecular weight proteins using IP and Western blot

analysis: Serum samples were diluted 1:1 with RIPA buffer (Pierce RIPA Buffer, Product number 89901) containing protease inhibitor cocktail tablet (Roche, Product number 11836170001). Streptavidin Mag Sepharose beads (GE Healthcare, Product code: 28-9857-99) were rinsed twice with RIPA buffer containing protease inhibitor and added to the serum samples (beads:serum samples =1:20) and incubated for 1 hour at room temperature on a tube rotator RotoFlex (Argos, Catalog number R2000). uPAR (R4)-BSA Free (Novusbio Product number NBP2-41379-0.2mg) or ATN 615 antibody was biotinylated using EZ-link Micro Sulfo-NHS-Biotinylation Kit (ThermoFisher Scientific, Catalog number: 21925) and stored at 4 °C until use. To the precleared serum samples biotinylated uPAR (R4)-BSA Free or ATN615 antibody was added (antibody:dilute precleared serum=1:530) and incubated on a tube rotator for 1 h at room temperature. Washed Streptavidin Mag Sepharose beads (beads:serum samples =1:20) were then added to the samples and incubated for another hour on the tube rotator at room temperature. The unbound protein fractions were removed and the beads were rinsed once and then washed for 10 min with RIPA buffer on the tube rotator. To the bound fraction 25.5 µL N-Glycanase reaction buffer (working solution) and 2.5 µL of denaturation solution (PROzyme, Code: WS0012) were added and the samples were heated at 70 °C for 3 min. The samples were then cooled on ice for 1 min. Detergent solution (2.5 µL) (PROzyme, Code: WS0013) and 2-4 µL N-Glycanase (working solution) were then added to the samples and incubated overnight at 37 °C. Working solution of N-Glycanase reaction buffer was prepared by diluting the buffer stock (PROzyme, Code: WS0010) 5x with DI water. Working stock of N-Glycanase was prepared by diluting the enzyme stock (PROzyme, Code:GKE-5006A) 10x with the working solution of N-Glycanase reaction buffer.

The proteins were eluted from the beads by boiling the samples in Laemmli Sample Buffer (Biorad, catalog number 161-0747) supplemented with additional SDS solution and 2-mercaptoethanol. The samples were then subjected to SDS-PAGE

analysis using 4-20% Mini-PROTEAN TGX gels (Biorad, Catalog number 456-1094). The proteins were then transferred to a PVDF membrane and subjected to Western blot analysis using Rabbit anti-UPAR (Bethyl, Product number A304-462A) (1:1250) or Anti-PLAUR antibody produced in rabbit (Sigma-Aldrich, Product
 5 number HPA050843-100uL) (1:1000), followed by goat anti-rabbit-HRP (1:3333).

Example 1.1: suPAR levels in plasma do not correlate with β 3 integrin activation

It has been suggested that suPAR drives podocyte injury by activating β 3 integrin on podocytes (5). Thus, we examined correlation between suPAR
 10 concentrations and β 3 integrin activation (Figure 1). As seen before, suPAR levels in the plasma were elevated upon multiple pathogenic conditions, such as focal segmental glomerulosclerosis (FSGS), diabetic nephropathy (DN), in patients on dialysis, or in sepsis (Figure 1A).

Since the original published assay that detected β 3 integrin activation in
 15 human cultured podocytes lacked a quantifiable component, and in order to compare β 3 integrin activation induced by different serums in a most relevant and quantifiable manner, we modified the original assay (Figure 2) so that it measured the ratio of activated integrin level (represented by AP5 staining in Figure 2A) over total amount of focal adhesions (FAs, determined by Paxillin staining in green in Figure 2A). No
 20 significant difference in the level of β 3 integrin activation between different healthy serums (HS), or that compared to the addition of 10% fetal bovine serum (FBS) standardly used to grow podocytes in culture was detected (Figure 2B). In order to compare data sets performed on different days, the levels of β 3 integrin activation in the presence of different sera were compared to that of a healthy serum, which was
 25 used as a standard in each experiment. The signal was quantified using ImageJ (see also Methods), and representative data set are show in Figure 2C. Ratio between AP5 staining and paxillin for healthy serum was adjusted to 1, and all other ratios are calculated with respect to the healthy serum in each experiment. Addition of Mn^{2+} is used to non-selectively activate all integrins on the surface of podocytes including β 3
 30 integrin, thus determining the maximal level of β 3 integrin activation in the assay (Figure 2C). Ratios between AP5 signal and paxillin that were >1.2 were considered a positive signal (presence of β 3 integrin activation).

As shown in Figure 1, only ~12% of serum samples from healthy individuals exhibited $\beta 3$ integrin activation (Figure 1B), and that number was increased to ~25% for serums from patients on dialysis or in sepsis (Figure 1C and 1D). Importantly, $\beta 3$ integrin activation did not correlate with suPAR concentrations. Indeed, extremely high levels of suPAR detected in serums of patients in sepsis (10-30 ng/ml) did not necessarily lead to $\beta 3$ integrin activation (Figure 1D). Interestingly, the majority of serum samples from patients suffering from FSGS (~76%) exhibited $\beta 3$ integrin activation. Even more surprising was the fact that high percent of serums (~67%) from patients with DN also exhibited $\beta 3$ integrin activation, suggesting that in some instances DN pathology might also be driven by suPAR- $\beta 3$ pathway.

Example 1.2: $\beta 3$ integrin activation by human serums is in part suPAR dependent

Despite the fact that $\beta 3$ integrin activation was originally linked to elevated levels of suPAR in FSGS subjects (5), lack of correlation between suPAR levels and $\beta 3$ integrin activation suggested three distinct possibilities. First, $\beta 3$ integrin activation by human serums was not suPAR-dependent. Second, $\beta 3$ integrin activation was in part suPAR-dependent but it required a yet unidentified modifiable factor. Third, suPAR per se might be distinctly modified.

We first attempted to test whether observed $\beta 3$ integrin activation was suPAR-dependent. To this end, FSGS serum was incubated with anti-suPAR antibody to test whether this procedure could block integrin activation. As shown in Figure 3A, addition of anti-suPAR antibody significantly diminished ability of FSGS serum to activate $\beta 3$ integrin. In addition, removal of suPAR using anti-suPAR antibody bound to Protein G beads (immunoprecipitation procedure) also significantly lowered the ability of FSGS serum to activate $\beta 3$ integrin (Figure 3A, Δ suPAR column). Ability of Protein G beads that are not conjugated to anti-suPAR antibody to also significantly lower ability of FSGS serum to activate $\beta 3$ integrin (Figure 3A, Protein G column) was due to propensity of suPAR to non-specifically bind Protein-G and Protein-A beads (Figure 3B). Together, those data demonstrated that detected $\beta 3$ integrin activation was indeed suPAR-dependent.

We next examined whether there was a difference in the form(s) of suPAR present in FSGS serums that activate $\beta 3$ integrin versus those present in healthy serums. While uPAR is encoded by one gene, at least 3 different splice variants have

been identified so far (Figure 7). In addition, uPAR consists of three domains (D1, D2, D3) and can be cleaved between D1 and D2 domain to generate two fragments: D1 and D2D3 fragment (9). D2D3 fragments have been shown to promote cell motility (10) and to bind $\beta 3$ integrin. In addition, it has been show that mouse variant 2 (Figure7) when expressed in mouse causes proteinuria and glomerular injury similar to FSGS (5). Thus, we next attempted to examine suPAR status in serum. Since suPAR is present at very low concentrations (healthy levels are <3 ng/ml), in order to detect suPAR in serum we immunoprecipitated (IP) suPAR using anti-suPAR antibody, and examined the precipitated proteins using Western blot analysis (Figure 3C). To confirm specificity of suPAR using this procedure, recombinant human suPAR (Figure 3C, lane 1) was added to human serum of suPAR (Figure 3C, lane 2). Given the fact that suPAR is also contains 5 glycosylation sites, samples were de-glycosylated using N-glycanase. As shown in Figure 3, this procedure efficiently and specifically IP-ed suPAR from the human serum (Figure 3C, lane 4), but only in the presence of anti-suPAR antibody (Figure 3C, lanes 5, 6). When multiple serums were tested by this procedure, lower molecular weight proteins were detected (Figure 3D, lane S2 and red arrows). Those lower molecular weight proteins migrated with the speed of D2D3 fragment generated by cleaving recombinant human suPAR using chymotrypsin, suggesting presence of D2D3 fragment in serums on some of the patients.

In order to test whether D2D3 fragment had ability to activate $\beta 3$ integrin by itself, we expressed and purified human D2D3 fragment (schematic diagram shown in Figure 4A) in insect cells and bacteria. The purity of the proteins is shown in Figure 4B. Both proteins, full length suPAR and D2D3 fragment were recognized by PLAUR antibody, though D2D3 fragment to a lesser extent, suggesting that levels of fragment detected in human serum might be underestimated. Importantly, addition of D2D3 fragment potently activated $\beta 3$ integrin on human podocytes (Figure 3E). Since activation was present using serum free media, this data demonstrate that D2D3 fragment does not require an additional serum modifier to potently activate $\beta 3$ integrin. In addition, at exact same physiological concentration (2 ng/ml) full-length suPAR did not induce significant activation (Figure 3E). Addition of D2D3 fragment to healthy serum transformed the serum from non-activating (HS bar graph in Figure 3F) to activating. Of note, addition of suPAR or D2D3 fragment did not alter

expression levels of $\alpha V\beta 3$ and $\alpha 3\beta 1$ (Figure 4D,E), further suggesting that observed $\beta 3$ integrin activation was indeed due to conformational switch within $\beta 3$ integrin and not due to overall increase in $\beta 3$ integrin levels in the cell. Consistent with these experiments, comparison between activating (recurrent FSGS serum), non-activating serum and healthy serums did not detect significant alterations in expression levels of $\alpha V\beta 3$ and $\alpha 3\beta 1$ integrins in human podocytes (Figures 4F,G) further demonstrating specific effects of activating serums on the conformational switch within $\beta 3$ integrin, and not its levels.

Concentration dependence of $\beta 3$ integrin activation with regard to D2D3 exhibited cooperative behavior (small changes in the concentration had significant consequences on $\beta 3$ integrin activation) (Figure 3G). The peak of activation was observed at ~ 2 ng/ml, with higher concentrations leading to lower activation, most likely due to so called “hyper activation” that can result in integrin internalization (ref). Identical activating profile was observed using high throughput assays (Figure 5A-C). In addition, D2D3-induced integrin activation increased podocyte motility (Figure 5 D, E). Importantly, the concentration of D2D3 fragment (2 ng/ml) that was associated with the highest level of $\beta 3$ integrin activation also resulted in the greatest motility. Increase in D2D3 concentrations (5-25 ng/ml) was associated with lower cell motility and indeed cell detachment (Figure 5F), most likely due to internalization of $\beta 3$ integrin due to hyper-activation. In summary, our data suggest that presence of D2D3 fragment in the serum might underlie ability of that serum to induce $\beta 3$ integrin activation.

Example 1.3: D2D3 fragment induces podocyte damage and proteinuria in mice

Ability of D2D3 fragment to induce potent $\beta 3$ integrin activation and motility suggested that D2D3 might cause podocyte injury leading to proteinuria when present in circulation. Thus, we next injected recombinant proteins into the tail vein of mice. As shown in Figures 6A and 6B, animals injected with PBS (vehicle control) or suPAR did not exhibit proteinuria (determined based on Albumin/creatinine ratio in Figure 6). In contrast, injection of D2D3 resulted in transient proteinuria and led to detectable presence of nephrin in the urine of proteinuric animals. Since nephrin is a transmembrane protein specifically present in podocytes, and since it has been shown that podocyte injury often leads to release of nephrin from the podocytes together, those data show that D2D3 in circulation can induce podocyte injury.

To further examine the ability of D2D3 fragment to induce podocyte injury we generated D2D3-transgenic mice expressing D2D3 from adipocytes (Figure 6E). The protein expression was induced by putting the animals onto the fat diet at 2 months of age (Figures 6F). While a number of animals exhibited microalbuminuria, approximately 15% of animals developed significant proteinuria and their glomerulus showed signs of injury such as moderate mesangial expansion (Figure 6G). Together, these data show that D2D3 can cause podocyte injury when present in the circulation.

Example 1.4: suPAR isoform 2 causes proteinuria and glomerular injury

Originally, it was shown that expression of mouse splice variant 2 causes FSGS type of glomerular injury in mice(5) and Figure 7. Those experiments were performed by electroporating DNA encoding mouse isoform 2 protein in mice (5). Consistent with those original observations, constitutive expression of isoform 2 in mouse form adipocytes (Figure 6H) resulted in ~27% of animals exhibiting signs of proteinuria (Figure 6I) and glomerular injury (Figure 6J). Together those data suggested that while presence of D2D3 in circulation can induce podocyte injury, expression of isoform 2 can do the same. Indeed, while addition of human suPAR isoform 2 induced moderate $\beta 3$ integrin activation at sub physiological concentration (Figures 8A and 8B, 0.5 ng/ml), isoform 2 induced potent cell detachment (Figure 8C) due to loss of focal adhesions (FA in Figure 8D). It is worth nothing that isoform 2 lacks part of the domain 3 as well as GPI-anchor sequence (Figure 7) thus it is expected to be directly secreted into the circulation and to exhibit lower molecular weight then the full length protein. The last observation is important given the presence of lower molecular weight proteins in human serums by Western blot analysis. Together, our data suggest that suPAR in circulation have multiple ways of activating $\beta 3$ integrin, and thus inducing podocyte injury: via formation of D2D3 fragment, and/or expression of distinct splice variant such as isoform 2.

Example 1.5: Establishment of composite score that identifies suPAR- $\beta 3$ integrin in podocyte injury

Based on our studies, it seemed reasonable to suggest that glomerular injury in subset of patients suffering from FSGS might be driven by suPAR- $\beta 3$ integrin pathway. In addition, given ability of some of DN serums to activate $\beta 3$ integrin (Figure 1F), data suggested that this pathway might also underlie some other types of

CKD such as DN. To explore this idea further we established a composite score assay that can efficiently identify suPAR- β 3 integrin pathogenic pathway in a non-invasive way. Since this pathway has been implicated specifically in recurrent FSGS we decided to test ability of several assays/biomarkers to efficiently distinguish recurrent from non-recurrent FSGS. In addition, recurrent vs non-recurrent FSGS represented highly uniform patient population since all patients were diagnosed using kidney biopsy, they all went through ESRD (end stage renal disease) and dialysis, they all got new kidney and are on similar immunosuppression therapies. What distinguished them is that in certain instances diseases recurred and in some it did not. Thus, we measured suPAR levels in their serums and urine, we determined the ability of serums to activate β 3 integrin, we determined whether their serums contain lower molecular weight proteins using Western blot analysis and we also measured level of IL6 in their urine.

Samples from patients with recurrent FSGS (19 samples) were compared to samples from patients with non-recurrent FSGS (7 samples) using a weighted average formula to generate a composite score assay.

The composite score was calculated based on the following algorithm:

$$\text{score} = 0.253 \times (\text{serum suPAR}) + 0.282 \times (\beta 3 \text{ integrin activation}) + 0.212 \times (\text{D2D3 fragment}) + 0.253 \times (\text{urine IL-6})$$

A value of 0 or 1 was assigned to each of the four parameters as described above. In the present cohort, a score of > 0.5 was considered positive for suPAR- β 3 integrin driven podocyte injury.

Representative scoring is shown in Figure 9. As shown in Figure 10, ROC (receiver operating characteristic) analysis showed that while each parameter exhibited significant ability to separate non-recurrent from recurrent FSGS, the joined score of 0.922 was impressive when all four parameters were determined. Presence of 3 positive parameters was detected in 60% of subject with recurrent FSGS and not in the single subject with non-recurrent FSGS. Interestingly, $\sim 23\%$ DN subjects exhibited $\geq 3+$ further suggesting that the suPAR- β 3 integrin pathway might underlie renal pathology in a subset of patients suffering from DN (Figure 11). Together, these data establish the composite score assay as a viable tool to identify suPAR- β 3 integrin pathway that underlies podocyte injury in CKD.

Figures 12A-D show ROC (receiver operating characteristic) curves with single or combinations of 2 or 3 different parameters. Area under the curve was calculated for each one of the 3 indicated parameters (Score 1); combination of any two parameters (Score 3) and finally the combination of three parameters (Score 3).

Figure 12A is suPAR, AP5, and IL6 parameters. Figure 12B is suPAR, AP5, and low molecular weight suPAR parameters. Figure 12C is suPAR, low molecular weight suPAR, and IL6 parameters. Figure 12D is AP5, low molecular weight suPAR, and IL6 parameters. Data show that any given parameter exhibited statistically significant value (area under the curve of ROC was between 0.6 and 0.76 in all combinations).

While the combination of any two given parameters increased statistical significance (Score 2 was between 0.92 to 0.669) by increasing sensitivity, it also resulted in drop of specificity. This means that while any 2 given values could separate healthy from patients that got a kidney transplant (FSGS subjects), this would not be sufficient to predict that those FSGS subject exhibit suPAR-b3 integrin pathogenic pathway.

Indeed, inclusion of the third parameter in all combinations decreased ROC values (0.65-0.69) due to drop in both sensitivity and specificity. Only when all 4 parameters were included, as in Figure 10, did ROC value become 0.922.

References:

1. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science*. 1999;286(5438):312-5.
2. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, et al. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nature genetics*. 2000;24(3):251-6.
3. Santin S, Garcia-Maset R, Ruiz P, Gimenez I, Zamora I, Pena A, et al. Neph rin mutations cause childhood- and adult-onset focal segmental glomerulosclerosis. *Kidney international*. 2009;76(12):1268-76.
4. Brown EJ, Schlondorff JS, Becker DJ, Tsukaguchi H, Tonna SJ, Uscinski AL, et al. Mutations in the formin gene INF2 cause focal segmental glomerulosclerosis. *Nature genetics*. 2010;42(1):72-6.

5. Wei C, El Hindi S, Li J, Fornoni A, Goes N, Sageshima J, et al. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nature medicine*. 2011;17(8):952-60.
6. Hayek SS, Sever S, Ko YA, Trachtman H, Awad M, Wadhvani S, et al. Soluble Urokinase Receptor and Chronic Kidney Disease. *The New England journal of medicine*. 2015;373(20):1916-25.
7. Theilade S, Lyngbaek S, Hansen TW, Eugen-Olsen J, Fenger M, Rossing P, et al. Soluble urokinase plasminogen activator receptor levels are elevated and associated with complications in patients with type 1 diabetes. *Journal of internal medicine*. 2015;277(3):362-71. Epub 2014 Jun 23.)
8. Huang J, Liu G, Zhang YM, Cui Z, Wang F, Liu XJ, et al. Plasma soluble urokinase receptor levels are increased but do not distinguish primary from secondary focal segmental glomerulosclerosis. *Kidney international*. 2013;84(2):366-72.
9. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. *Nature reviews Molecular cell biology*. 2010;11(1):23-36.
10. Kjoller L. The urokinase plasminogen activator receptor in the regulation of the actin cytoskeleton and cell motility. *Biological chemistry*. 2002;383(1):5-19.
11. Haraldsson B, Nystrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. *Physiological reviews*. 2008;88(2):451-87.
12. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *The Journal of clinical investigation*. 2001;108(11):1583-7.
13. Reiser J, von Gersdorff G, Simons M, Schwarz K, Faul C, Giardino L, et al. Novel concepts in understanding and management of glomerular proteinuria. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2002;17(6):951-5.
14. Reiser J, Sever S. Podocyte biology and pathogenesis of kidney disease. *Annual review of medicine*. 2013;64:357-66.
15. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends in cell biology*. 2007;17(9):428-37.

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
5 the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

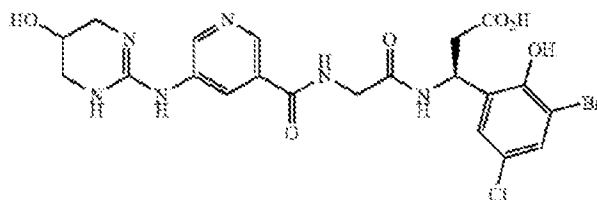
WHAT IS CLAIMED IS:

1. A method, comprising two, three, or all four of the following in any order:
determining a level of soluble urokinase-type plasminogen activator receptor (suPAR) protein in a plasma sample from a subject;
determining a level of interleukin 6 (IL-6) protein in a urine sample from the same subject;
determining a level of low molecular weight suPAR in a plasma sample from the subject; and
determining a level of $\beta 3$ integrin activation activity in a plasma sample from the subject.
2. A method of detecting the presence of suPAR- $\beta 3$ integrin driven kidney disease in a subject, the method comprising
 - (a) determining a subject level of two, three, or all four of the following markers:
soluble urokinase-type plasminogen activator receptor (suPAR) protein in a plasma sample from the subject;
interleukin 6 (IL-6) protein in a urine sample from the subject;
low molecular weight suPAR in a plasma sample from the subject; and
 $\beta 3$ integrin activation activity in a plasma sample from the subject;
 - (b) comparing the subject level of the marker to a reference level; and
 - (c) detecting the presence of suPAR- $\beta 3$ integrin driven kidney disease in a subject who has at least two markers above the level.
3. A method of treating a subject who has chronic kidney disease, the method comprising
 - (a) determining a subject level of two, three, or all four of the following markers:
soluble urokinase-type plasminogen activator receptor (suPAR) protein in a plasma sample from the subject;
interleukin 6 (IL-6) protein in a urine sample from the subject;
low molecular weight suPAR in a plasma sample from the subject; and
 $\beta 3$ integrin activation activity in a plasma sample from the subject;
 - (b) comparing the subject level of the marker to a reference level; and
 - (c) selecting and optionally administering a treatment for suPAR- $\beta 3$ integrin driven kidney disease to a subject who has at least two markers above the level.

4. The method of claims 1 to 3, wherein detecting $\beta 3$ integrin activation activity in a plasma sample comprises contacting the plasma sample with cultured human podocytes in vitro and determining a level of $\beta 3$ integrin activation in the sample.
5. The method of claim 4, wherein determining a level of $\beta 3$ integrin activation in the sample comprises contacting the sample with an antibody that binds to beta 3 integrin and an antibody that binds to paxillin and dividing the number of cells expressing beta 3 integrin by the number of cells expressing paxillin.
6. The method of claims 1 or 2, wherein the subject has chronic kidney disease.
7. The method of claims 2 or 3, wherein the reference value is serum suPAR of ≥ 3 ng/ml (by ELISA assay); $\beta 3$ integrin activation > 1.2 (AP5/paxillin ratio normalized to healthy serum); presence of low molecular weight suPAR in serum; detectable presence of IL6 in the urine.
8. The method of claims 1-3, further comprising determining a score calculated using the following algorithm:

$$\text{score} = \alpha \times (\text{serum suPAR}) + \beta \times (\beta 3 \text{ integrin activation}) + \gamma \times (\text{low molecular weight suPAR}) + \delta \times (\text{urine IL-6}),$$
 wherein each of α , β , γ , and δ are empirically determined weights.
9. The method of claim 8, wherein the algorithm is:

$$\text{score} = 0.253 \times (\text{serum suPAR}) + 0.282 \times (\beta 3 \text{ integrin activation}) + 0.212 \times (\text{low molecular weight suPAR}) + 0.253 \times (\text{urine IL-6}).$$
10. The method of claim 3, wherein the treatment for suPAR- $\beta 3$ integrin driven kidney disease is an $\alpha 5\beta 3$ inhibitor and/or ex vivo removal of suPAR from the subject's circulation.
11. The method of claim 9, wherein the $\alpha 5\beta 3$ inhibitor is a monoclonal antibody that binds specifically to $\alpha 5\beta 3$; a peptide comprising a RGD binding sequence; or a small molecule $\alpha 5\beta 3$ inhibitor.
12. The method of claim 10, wherein the small molecule $\alpha 5\beta 3$ inhibitor is a compound of the formula



or a pharmaceutically acceptable salt thereof.

13. The method of claim 9, wherein the monoclonal antibody that binds specifically to $\alpha 5 \beta 3$ is VPI-2960B, CNTO95, or anti-CD61.

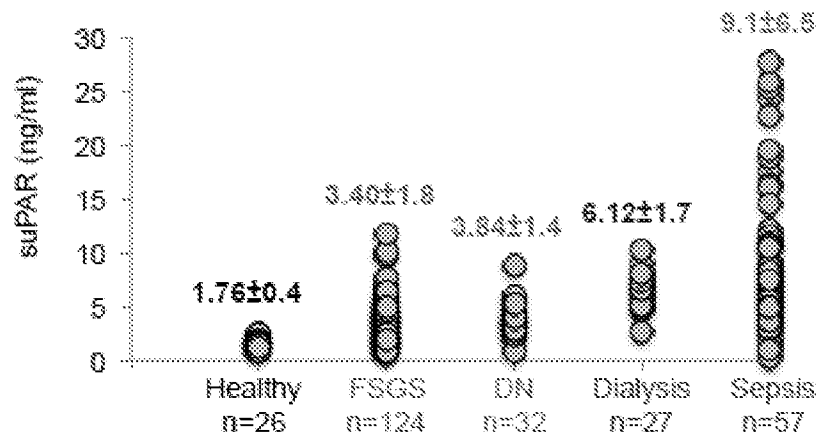


FIG. 1A

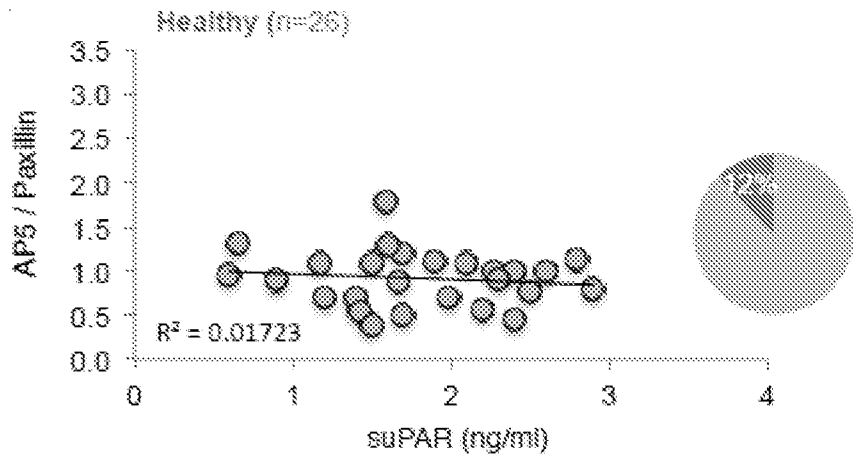


FIG. 1B

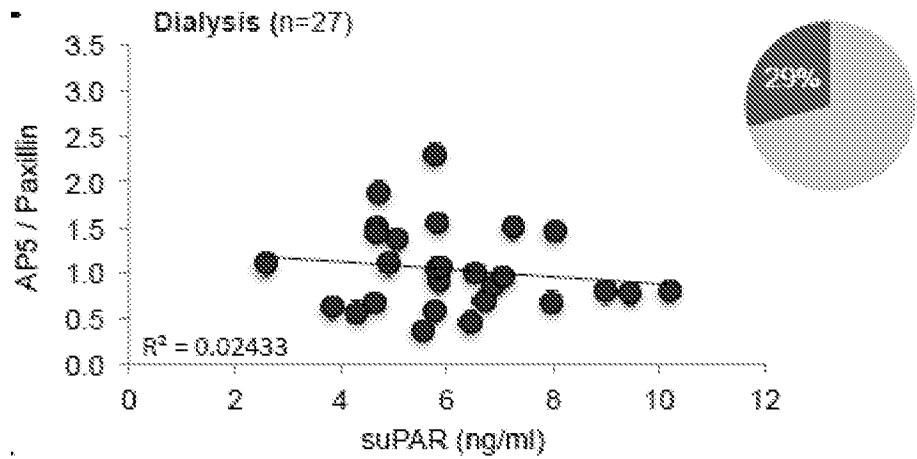


FIG. 1C

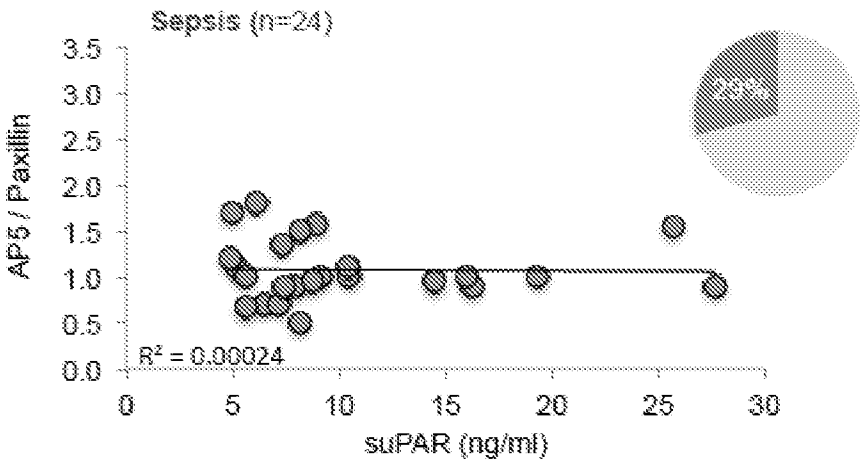


FIG. 1D

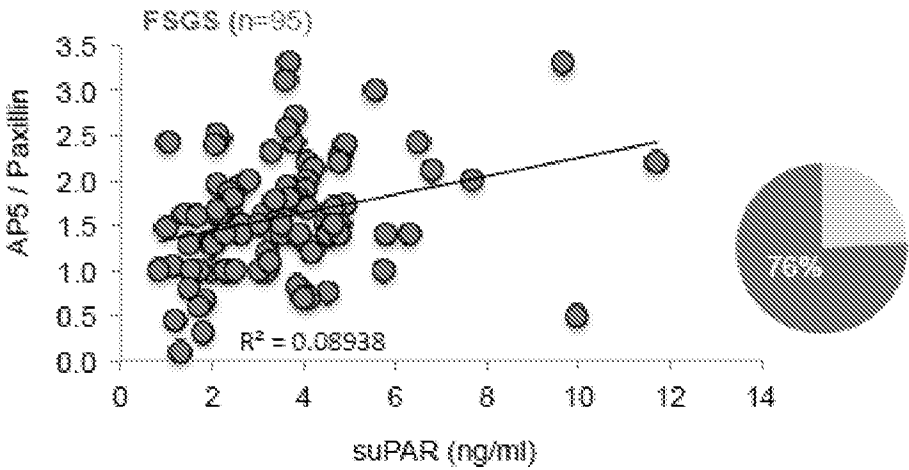


FIG. 1E

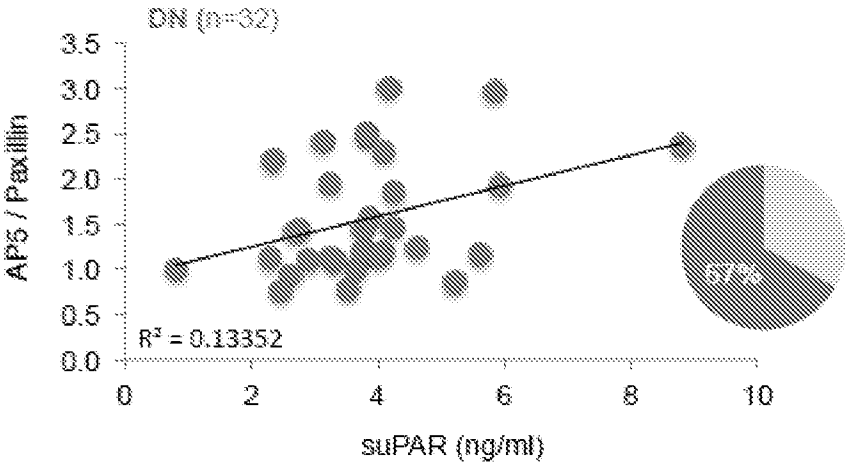


FIG. 1F

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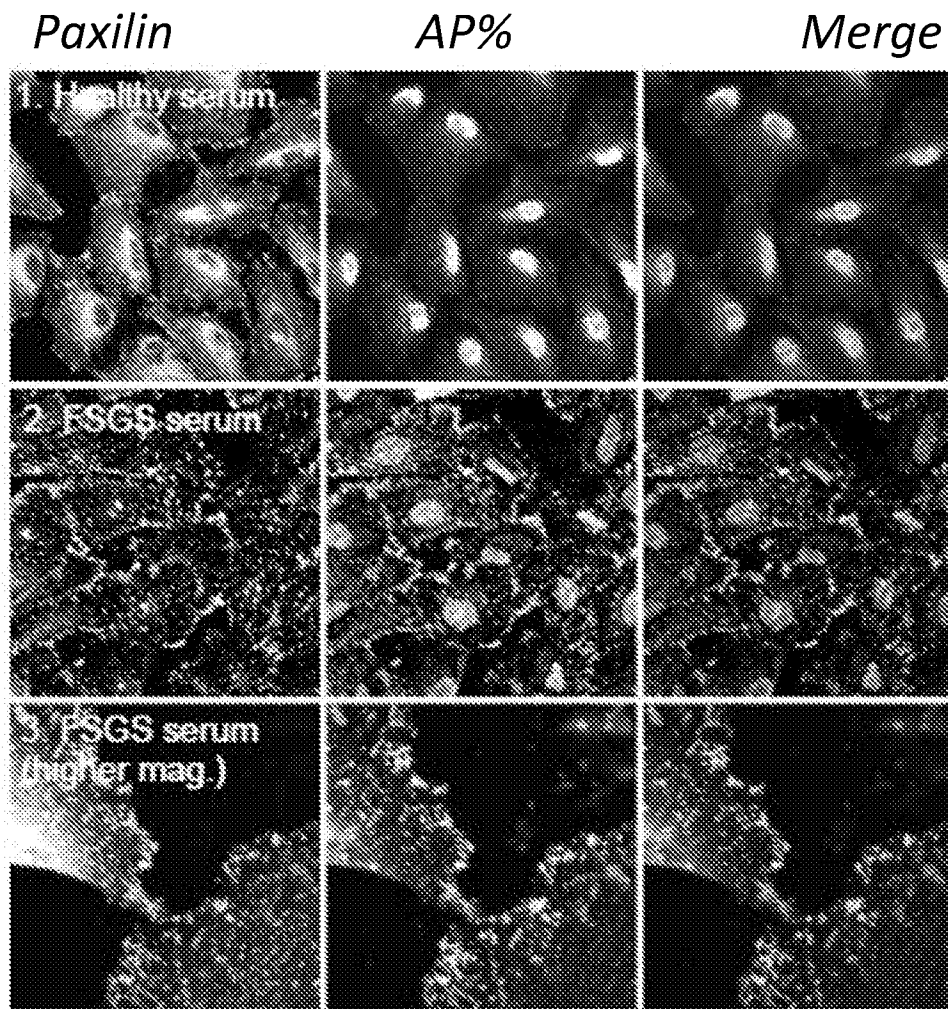


FIG. 2A

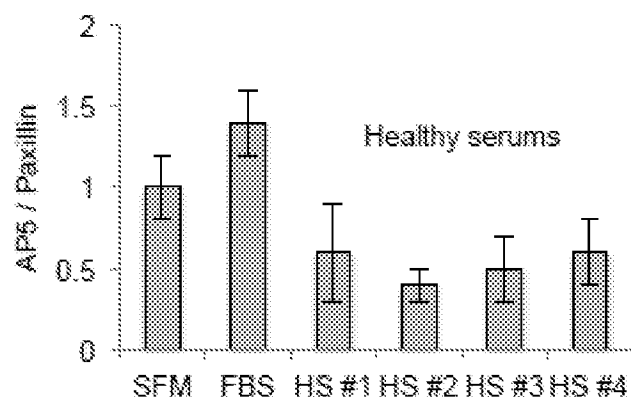


FIG. 2B

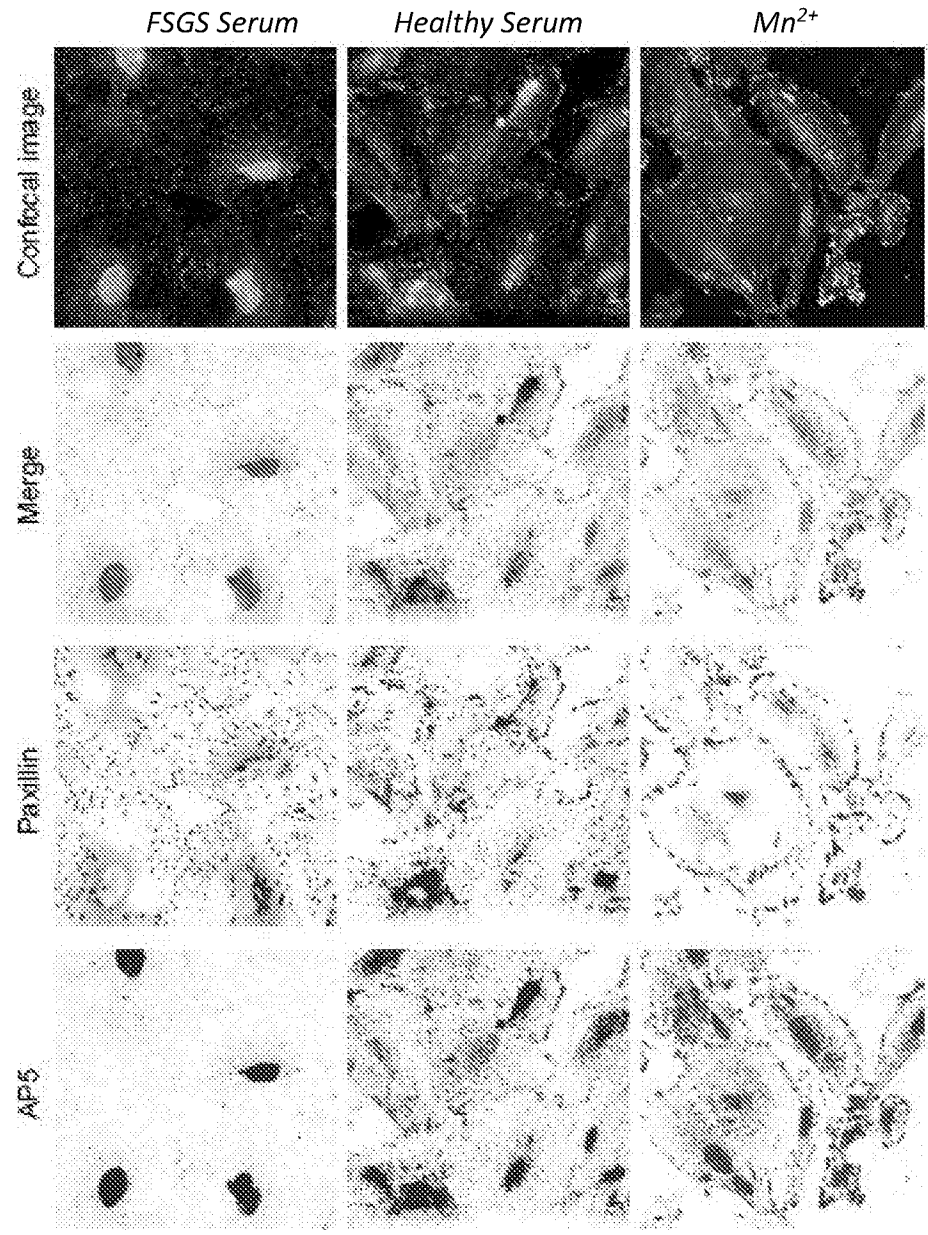


FIG. 2C

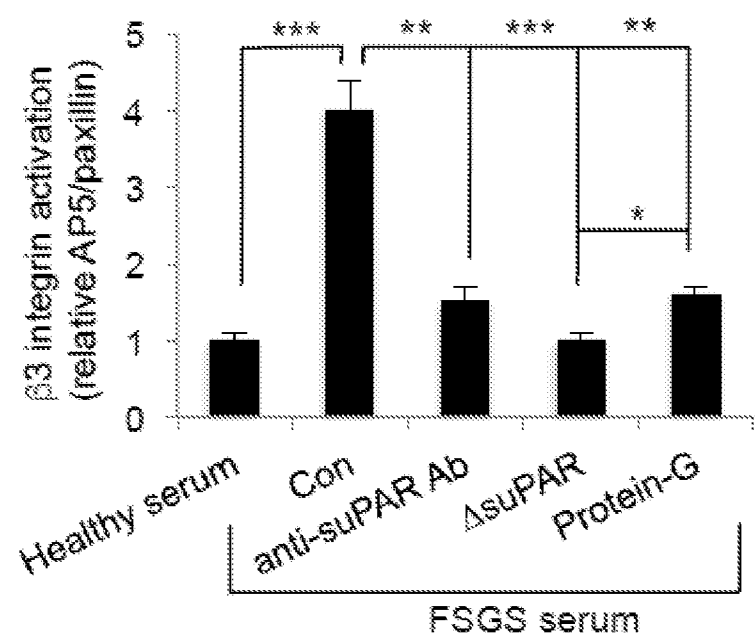


FIG. 3A

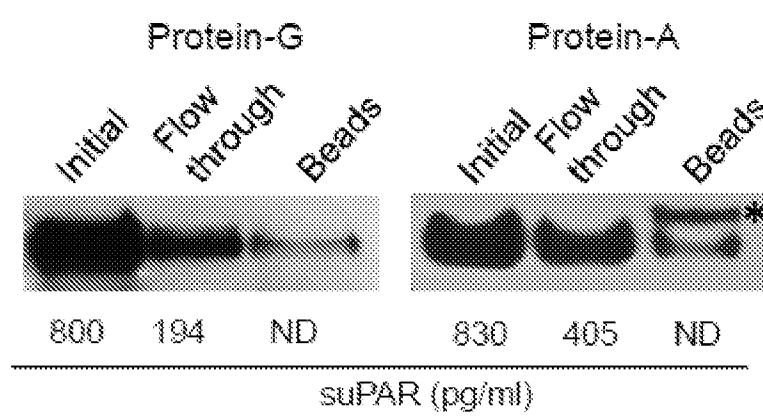


FIG. 3B

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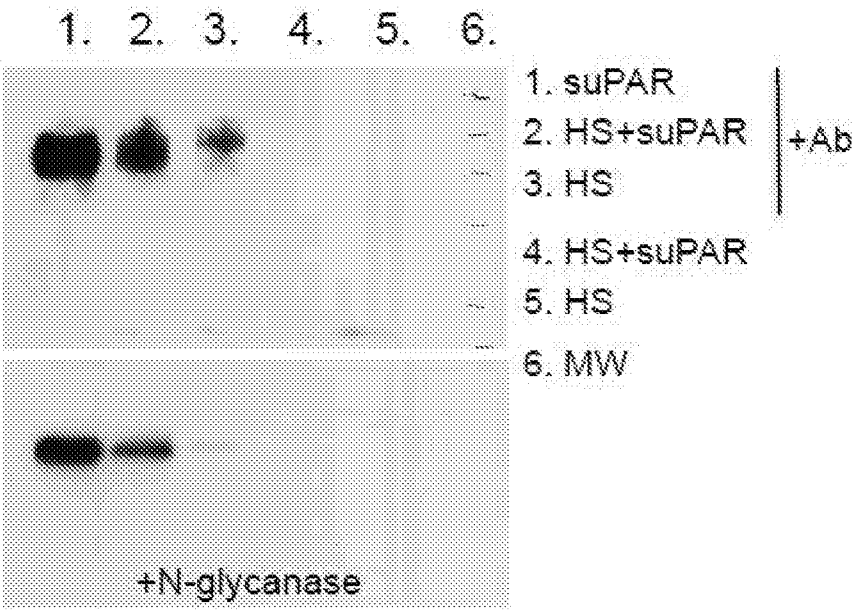


FIG. 3C

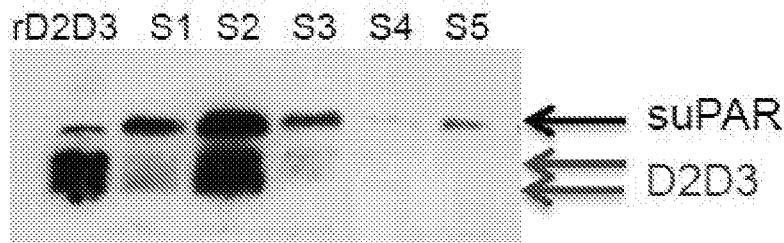


FIG. 3D

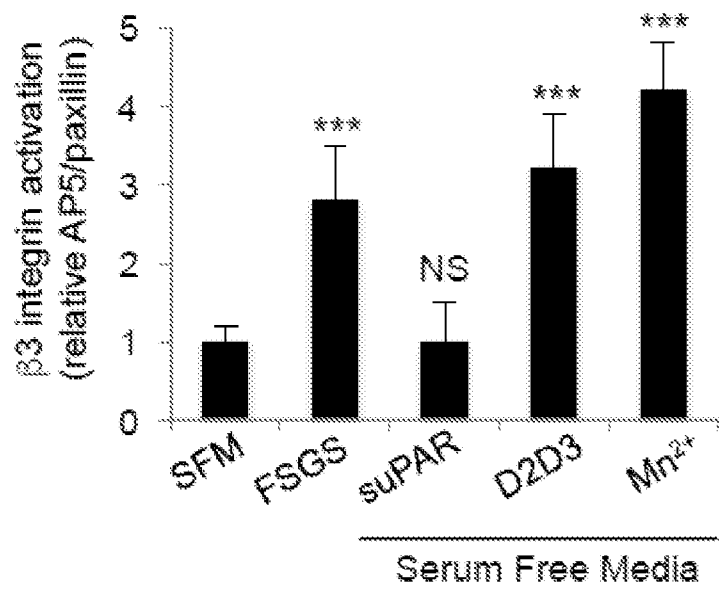


FIG. 3E

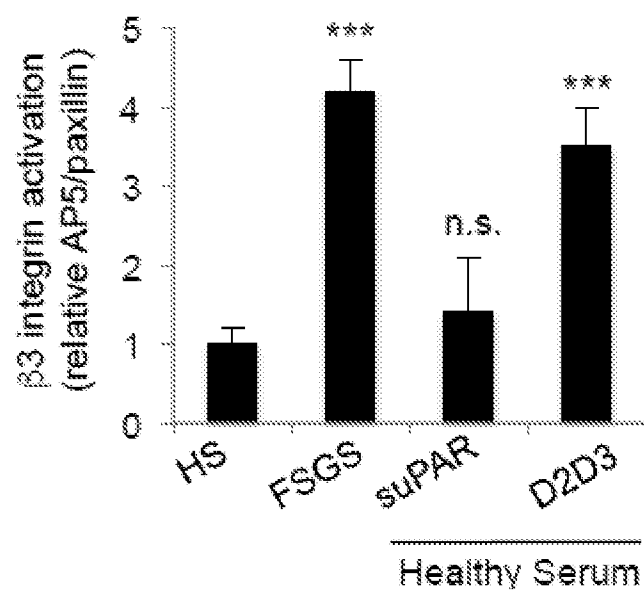


FIG. 3F

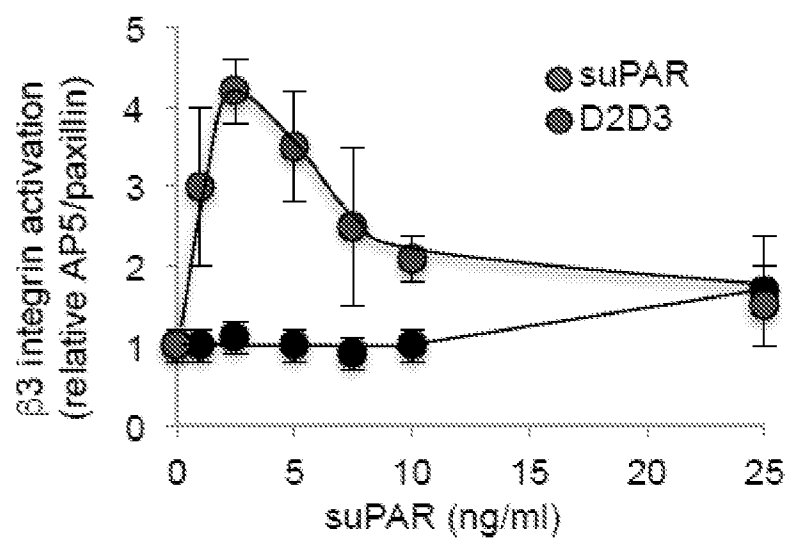


FIG. 3G

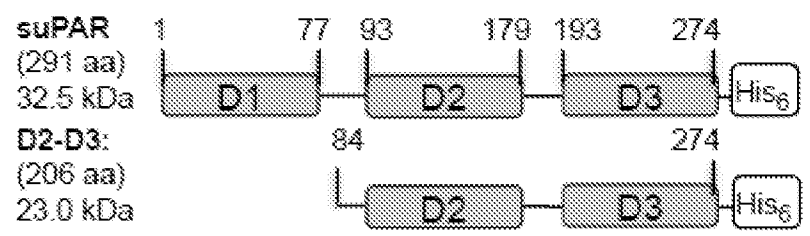


FIG. 4A

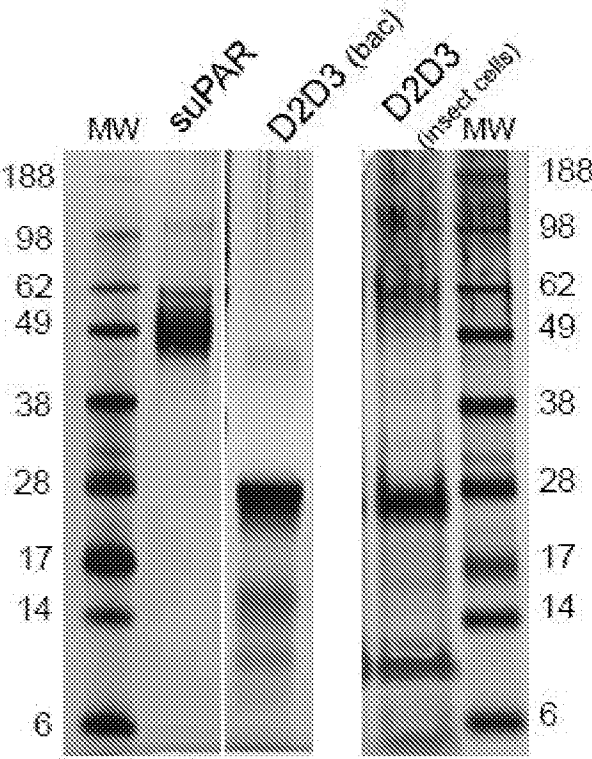


FIG. 4B

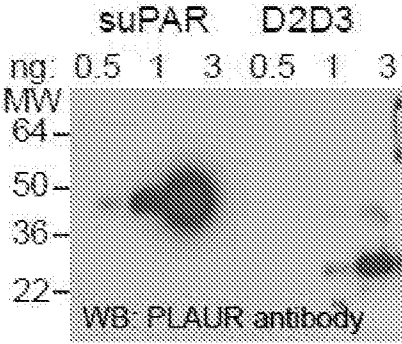


FIG. 4C

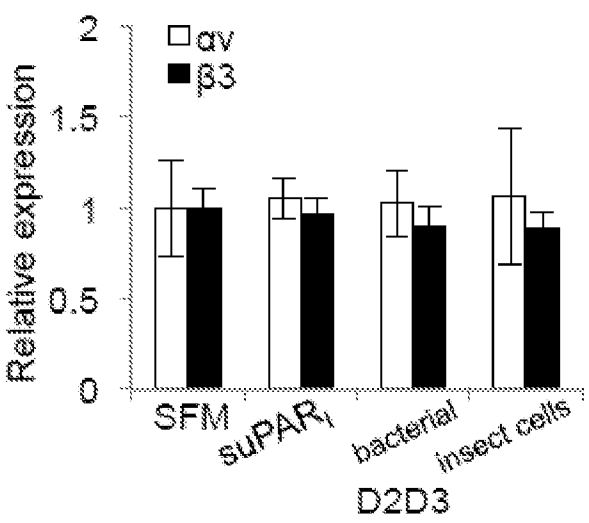


FIG. 4D

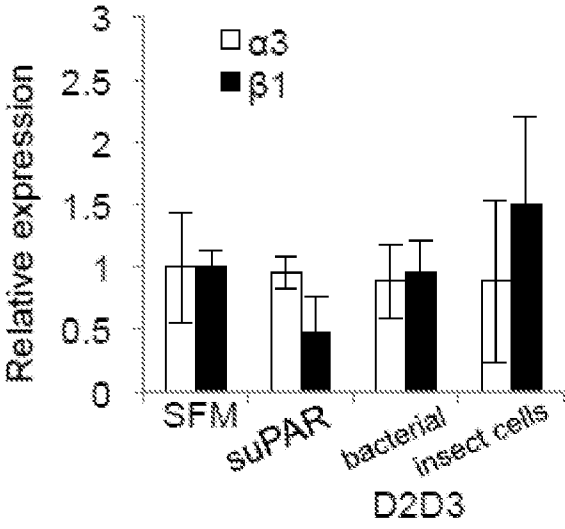


FIG. 4E

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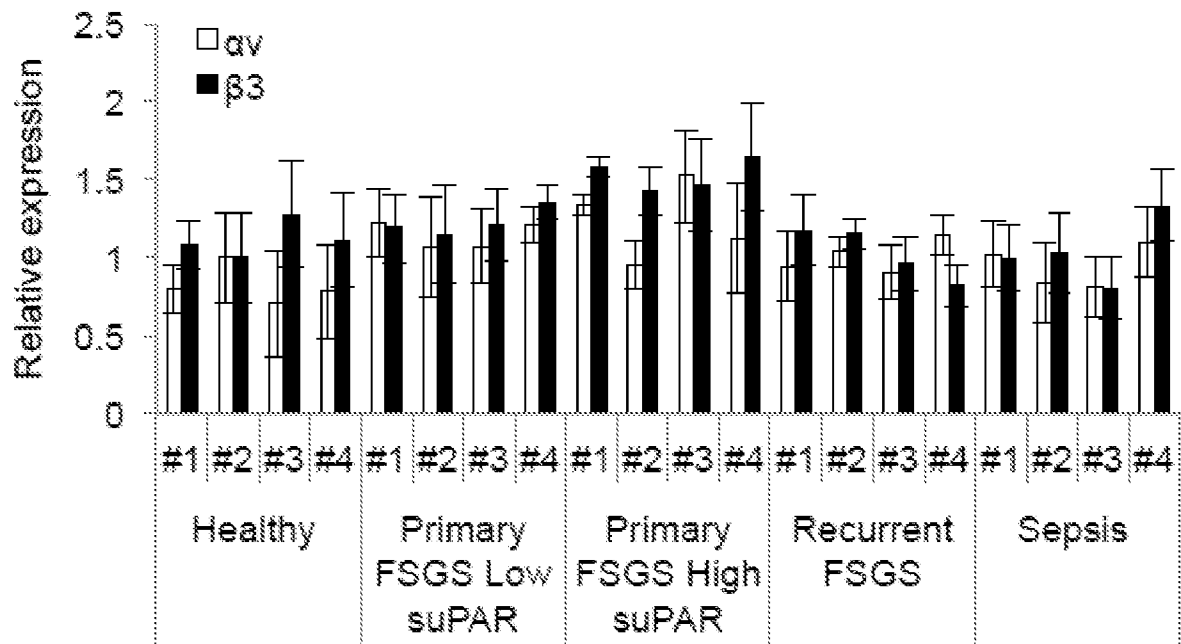


FIG. 4F

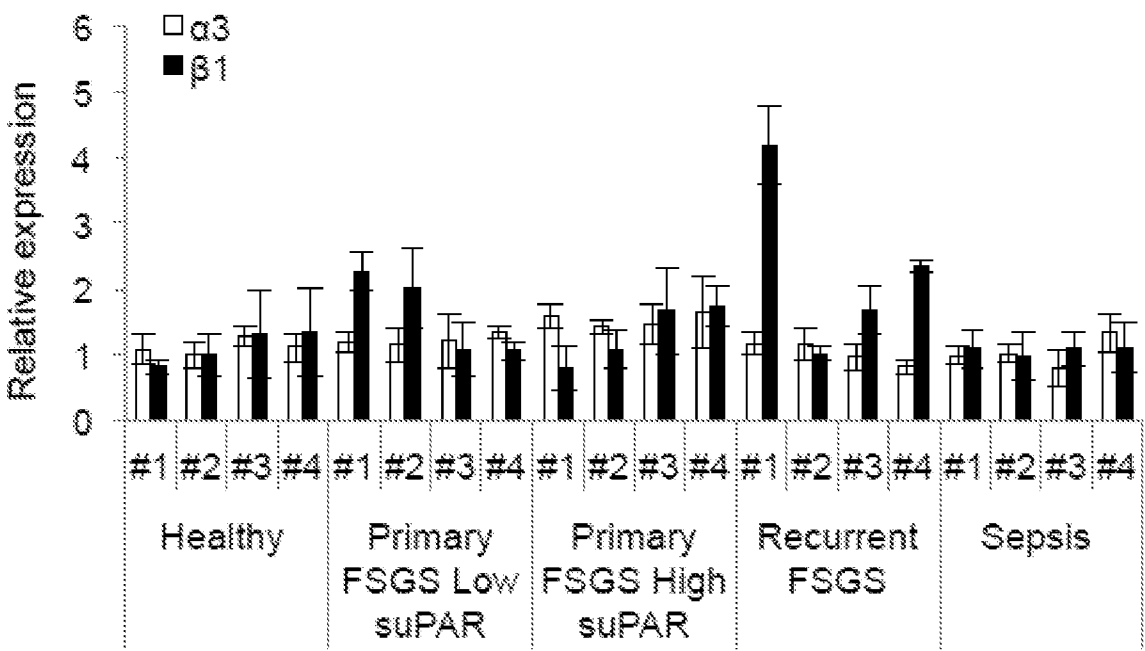


FIG. 4G

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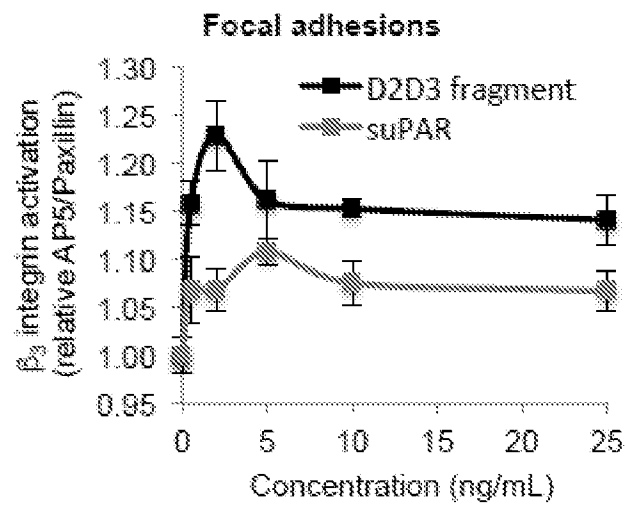


FIG. 5A

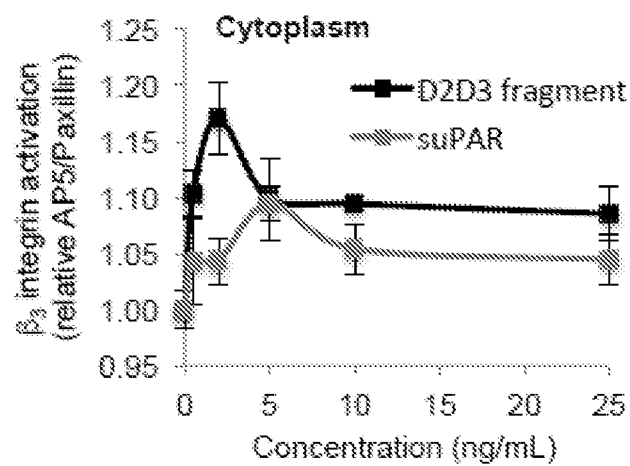


FIG. 5B

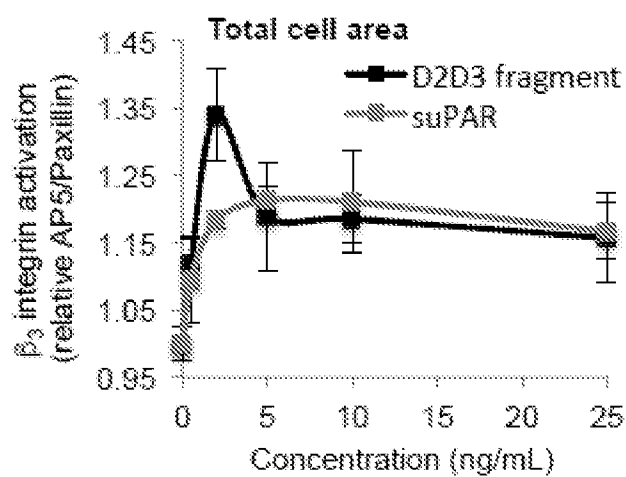


FIG. 5C

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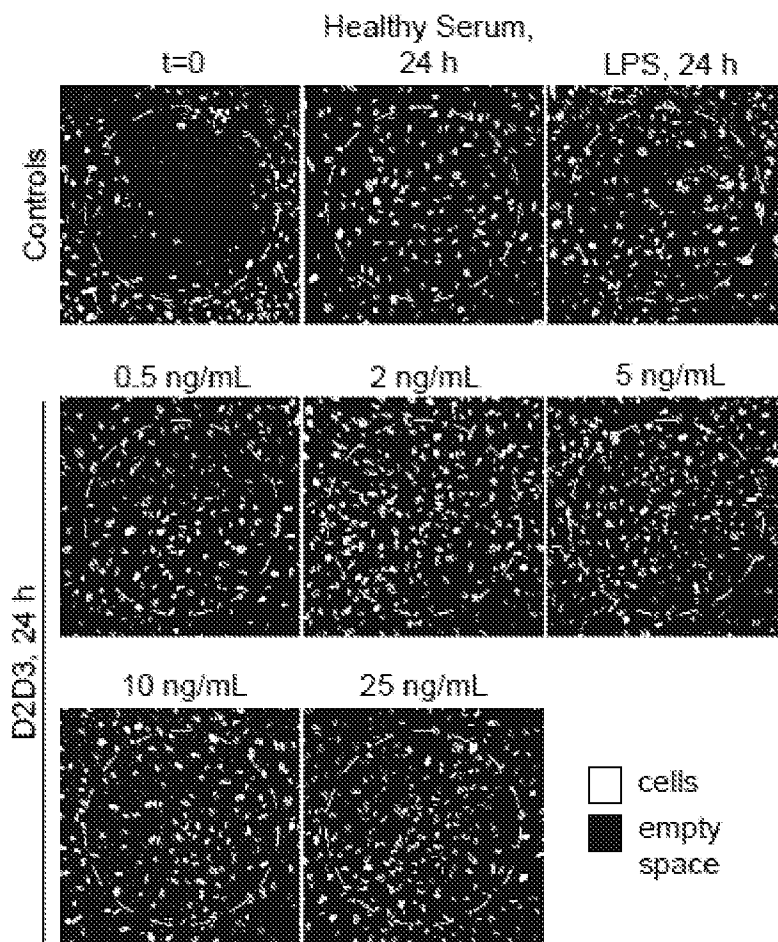


FIG. 5D

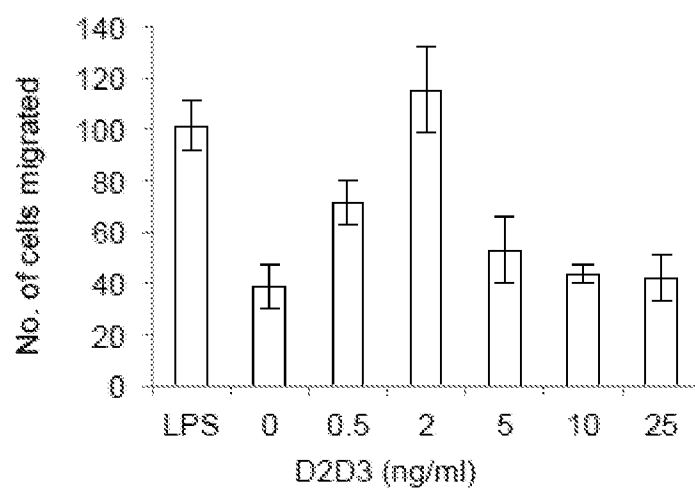


FIG. 5E

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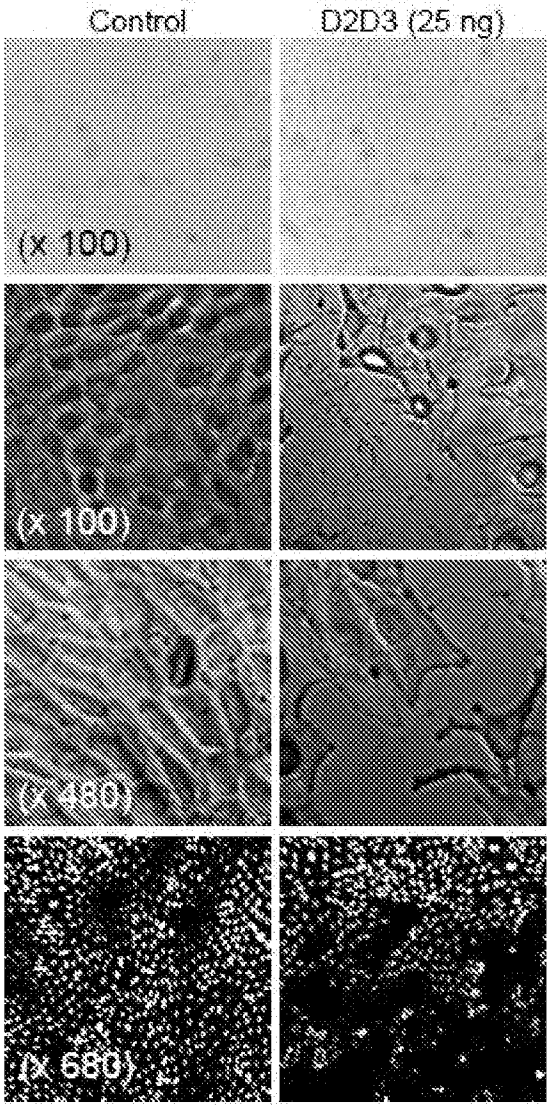


FIG. 5F

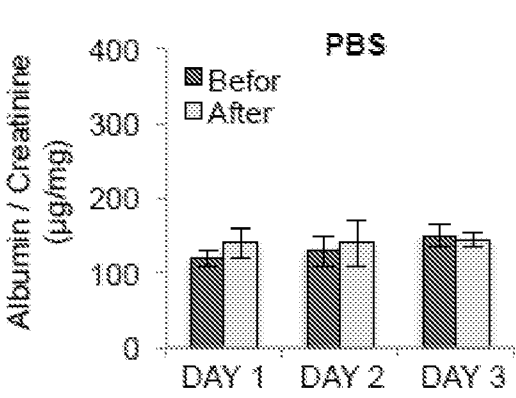


FIG. 6A

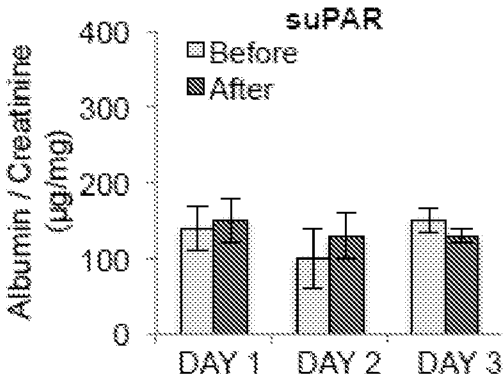


FIG. 6B

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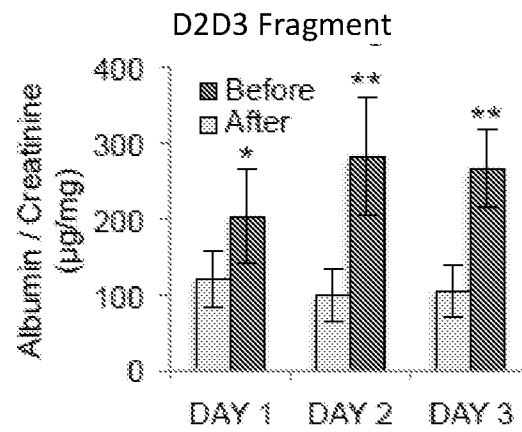


FIG. 6C

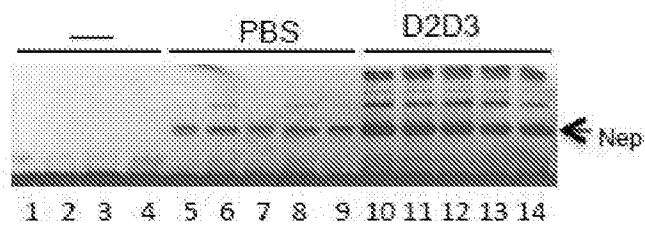
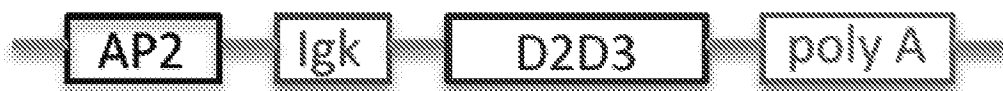


FIG. 6D



D2D3 suPAR transgene



FIG. 6E

FIG. 6F

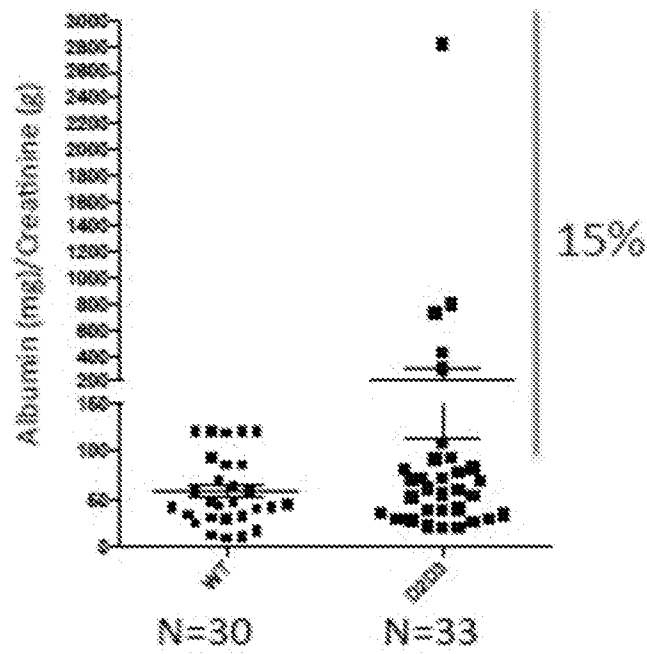


FIG. 6G

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Isoform 2 suPAR transgene



FIG. 6H

FIG. 6I

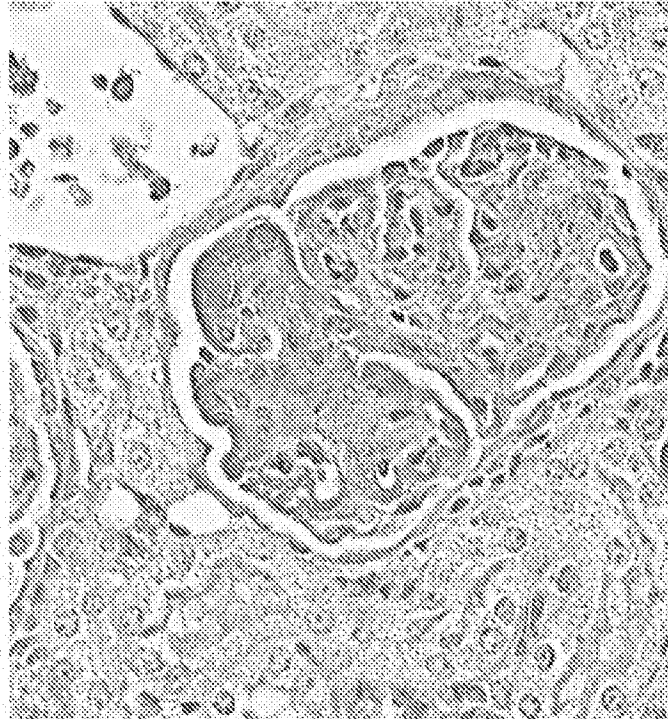
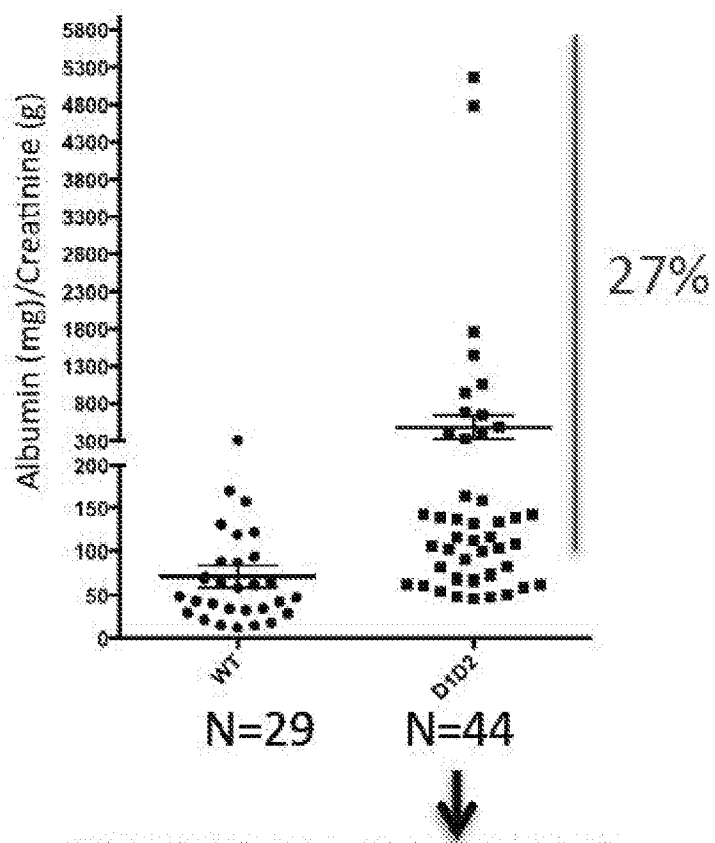


FIG. 6J

FIG. 7A

uPAR2 Homo sapiens	NGFHNNDTFFFLKCCNTTKCNEGP ILELENLPQNGRQCYSCKGNSTHGCSSEETFLIDCR
uPAR3 Homo sapiens	-----VLELENLPQNGRQCYSCKGNSTHGCSSEETFLIDCR
uPAR1 Homo sapiens	NGFHNNDTFFFLKCCNTTKCNEGP ILELENLPQNGRQCYSCKGNSTHGCSSEETFLIDCR
3BT1_u	NGFHNNDTFFFLKCCNTTKCNEGP ILELENLPQNGRQCYSCKGNSTHGCSSEETFLIDCR
uPAR Mus musculus	AGFHSNQTFHFLKCCNYTHCNGGPFVLDLQSFPPNGFQCYSCGNNTLGCSSEEEASLINCR
uPAR2 Mus musculus	EIFKSWEQSA-SKRQLNPHTVTGPTFSVTGS-----SGSLDQLGSDQEPSPSYLVMSR
	:.: . . . * . . . * : . *
uPAR2 Homo sapiens	GPMNQCLVATGTHERSLWGSWLPCKST-----T-ALRPPCCEEAQATHV-
uPAR3 Homo sapiens	GPMNQCLVATGTHEPKNQSYMVRGCATASMCQHAHLGDAFSMN-HIDVSCCTKSGCNHPD
uPAR1 Homo sapiens	GPMNQCLVATGTHEPKNQSYMVRGCATASMCQHAHLGDAFSMN-HIDVSCCTKSGCNHPD
3BT1_u	GPMNQCLVATGTHEPKNQSYMVRGCATASMCQHAHLGDAFSMN-HIDVSCCTKSGCNHPD
uPAR Mus musculus	GPMNQCLVATGLDVLGNRSYTVRGCATASWCQGSHVADSFPTHNLNVSVSCCHGSGCNSP-
uPAR2 Mus musculus	ILL-----SF-----
	:
uPAR2 Homo sapiens	-----SEQ ID NO: 1
uPAR3 Homo sapiens	LDVQYRSGAAPQGPAPHLSLTITLLMTARLWGGTLLWTSEQ ID NO: 2
uPAR1 Homo sapiens	LDVQYRSGAAPQGPAPHLSLTITLLMTARLWGGTLLWTSEQ ID NO: 3
3BT1_u	LDVQYR-----SEQ ID NO: 4
uPAR Mus musculus	-----TGGAPRPGPAQLSLIASLLLTGLWGV-LLWTSEQ ID NO: 5
uPAR2 Mus musculus	-----SEQ ID NO: 6

FIG. 7B

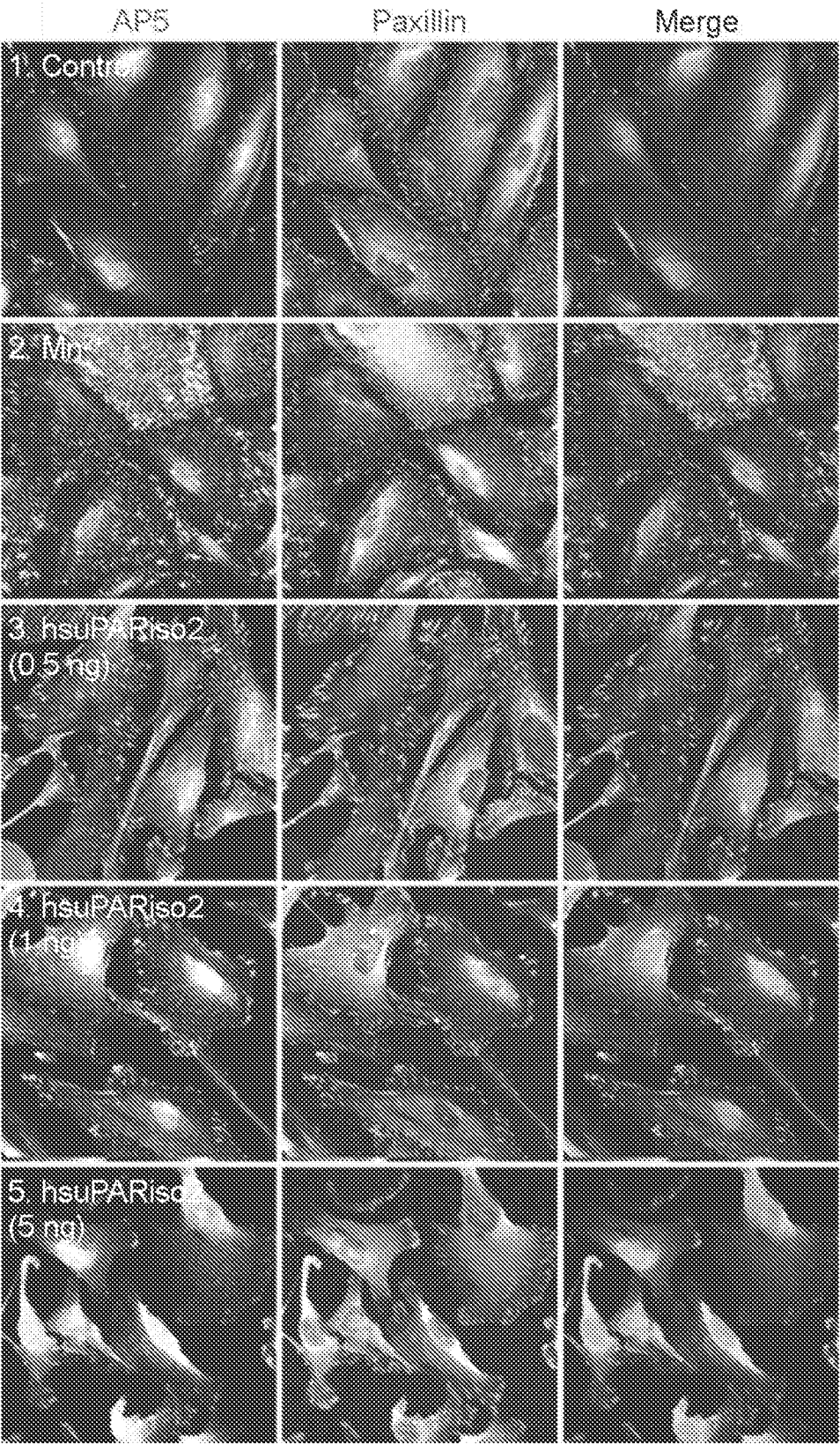


FIG. 8A

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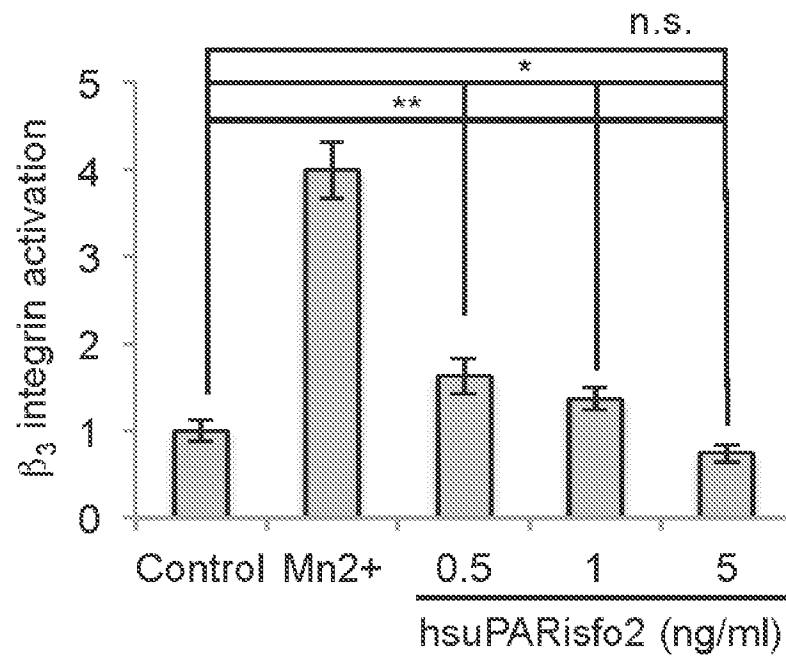


FIG. 8B

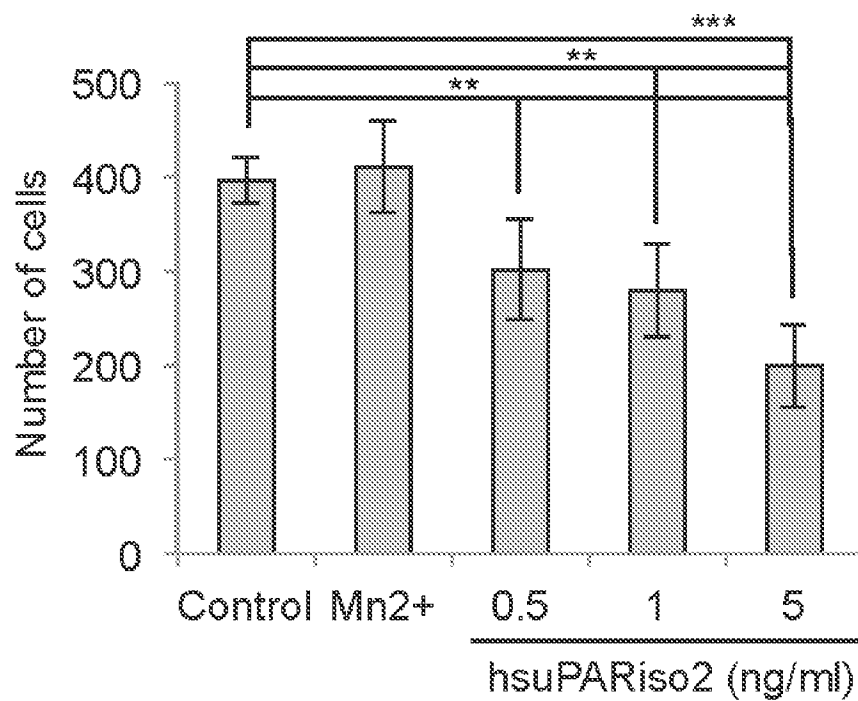


FIG. 8C

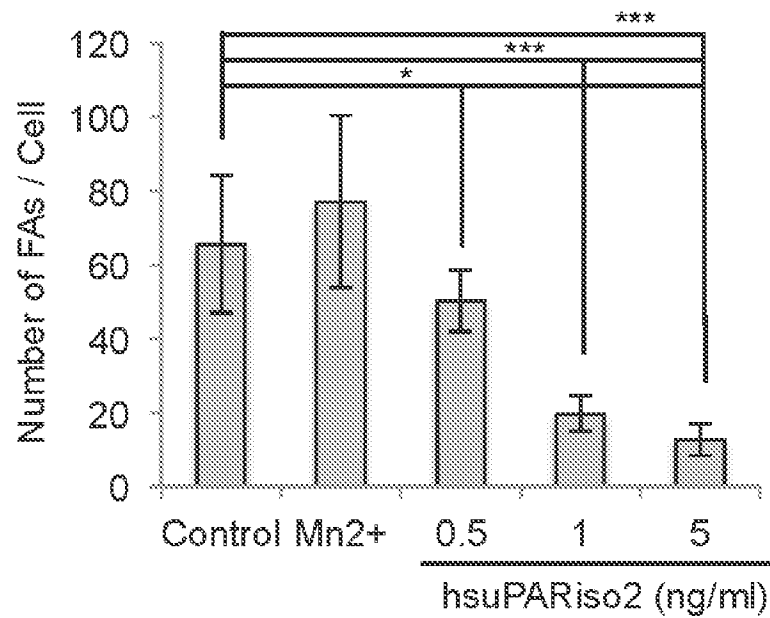


FIG. 8D

		cut off 3		1		
		Elisa	IF	IP Western blot	Elisa	
	Samples	1. serum suPAR	2. AP5 activation	3. D2-D3 fragment	4. urine IL-6	TOTAL
	Healthy	0	0	0	0	0
	D2-D3	1	1	1	1	4
	Serum					
1	Sample 1	3.31 (1)	2.4 (1)	no (0)	10.9	3
2	Sample 2	2.61 (0)	1.5 (1)	no (0)	0	1
3	Sample 3	2.27 (0)	1.4 (1)	no (0)	0	1
4	Sample 4	2.45 (0)	1.8 (1)	yes (1)	241	3
5	Sample 5	4.63 (1)	1.5 (1)	yes (1)	5.4	4
6	Sample 6	5.58 (1)	3.2 (1)	yes (1)	89	4
7	Sample 7	2.17 (0)	1.6 (1)	yes (1)	0	2
8	Sample 8	4.78 (1)	1.4 (1)	no (0)	17.8	3
9	Sample 9	3.21 (1)	1 (0)	yes (1)	0	2
10	Sample 10	4.14 (1)	1.5 (1)	yes (1)	0	3
11	Sample 11	2.76 (0)	0.8 (0)	no (0)	1.5	1
12	Sample 12	5.7 (1)	2.7 (1)	no (0)	2.1	3
13	Sample 13	2.5 (0)	1.06 (0)	no (0)	0	0
14	Sample 14	1.5 (0)	1.35 (1)	no (0)	0	1
15	Sample 15	4.9	2.39	no (0)	13.2	3
16	Sample 16	6.1 (1)	1.76 (1)	yes (1)	37.4	4
17	Sample 17	16.2 (1)	0.98 (0)	no (0)	1238	2
18	Sample 18	10.5 (1)	1.02 (0)	no (0)	1226.2	2
19	Healthy-1	1.79 (0)	0.7 (0)	no	0	0
20	Healthy-2	2.33 (0)	0.5 (0)	no	0	0
21	Healthy-3	1.59 (0)	1.2 (0)	no	0	0
22	Healthy-4	1.98 (0)	0.9 (0)	no	0	0
23	Healthy-5	2.91 (0)	1 (0)	no	0	0

FIG. 9

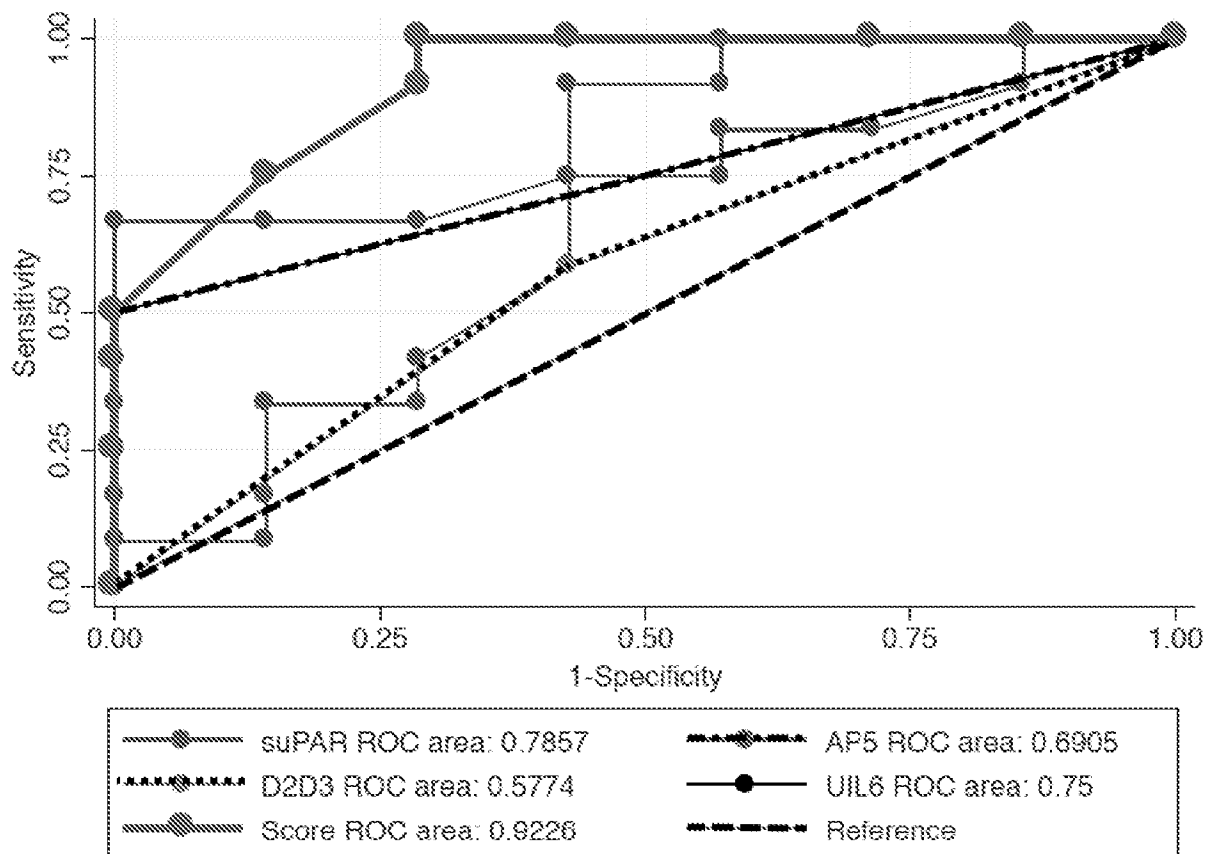
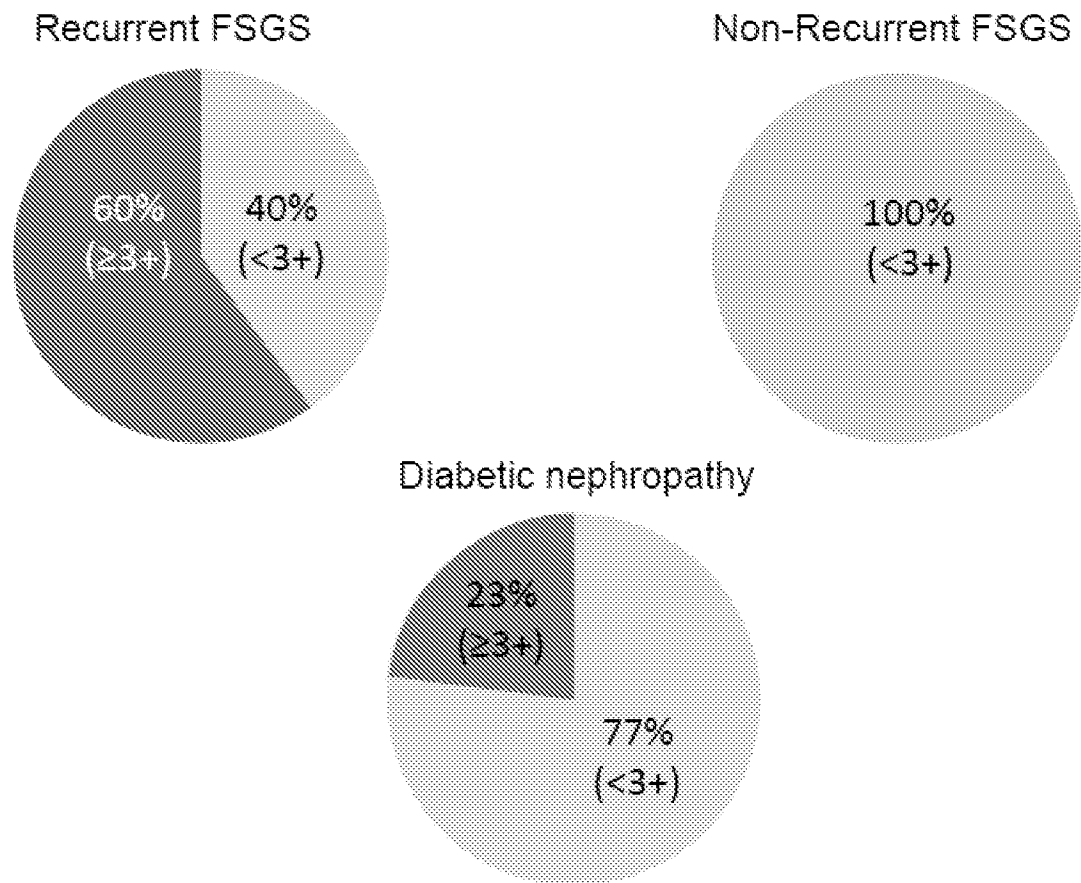


FIG. 10

*FIG. 11*

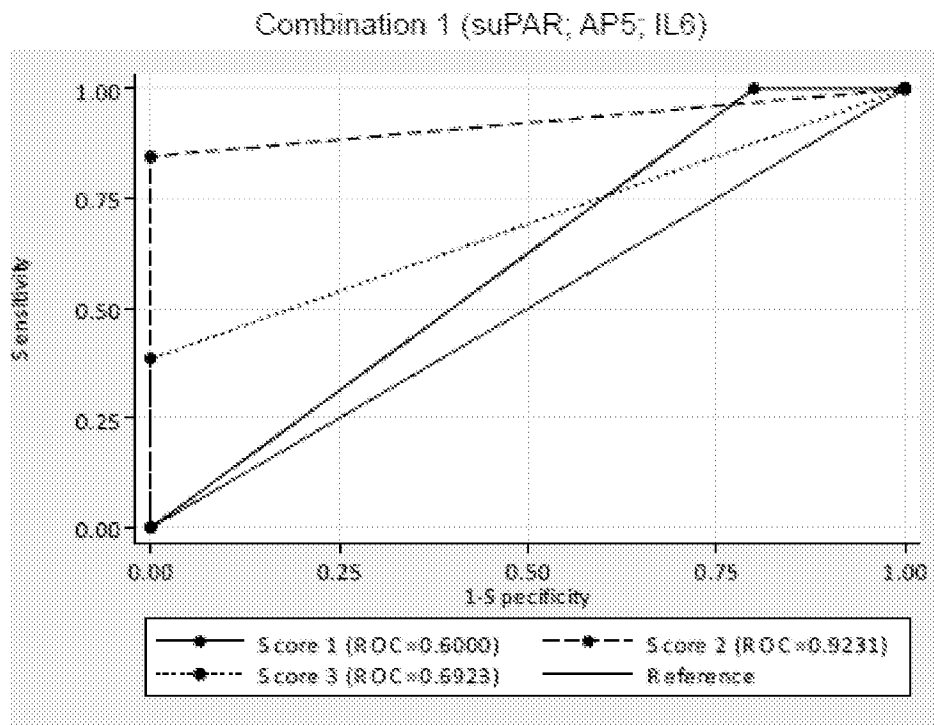


FIG. 12A

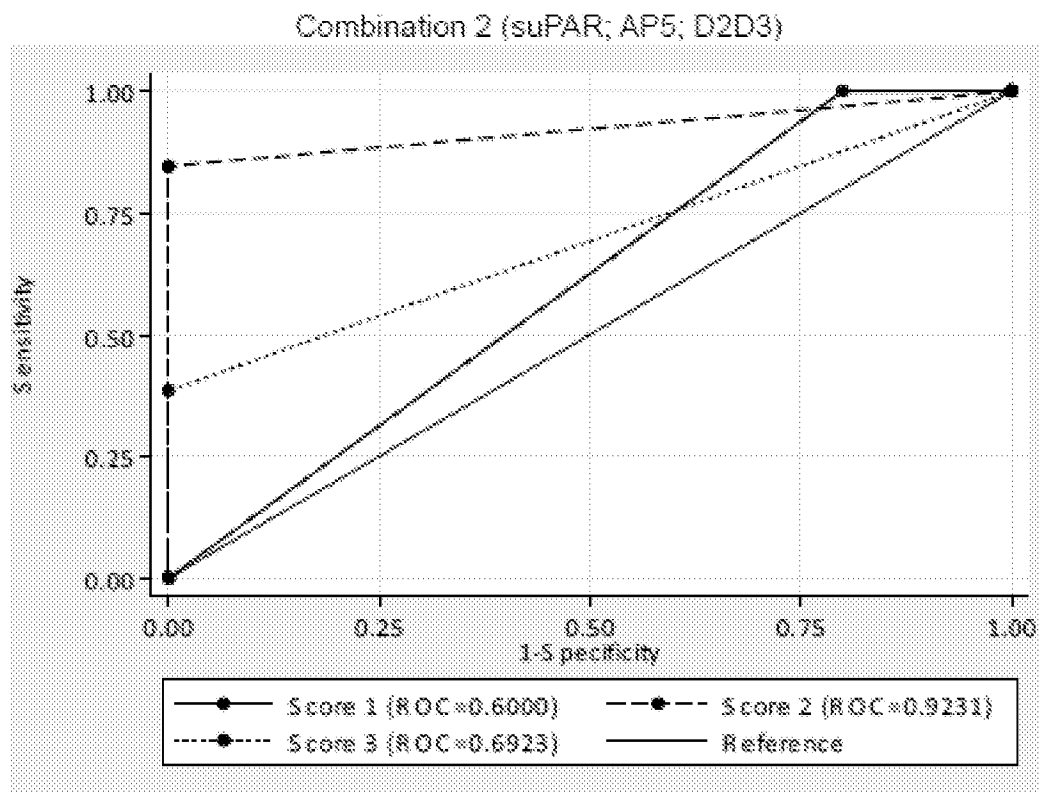


FIG. 12B

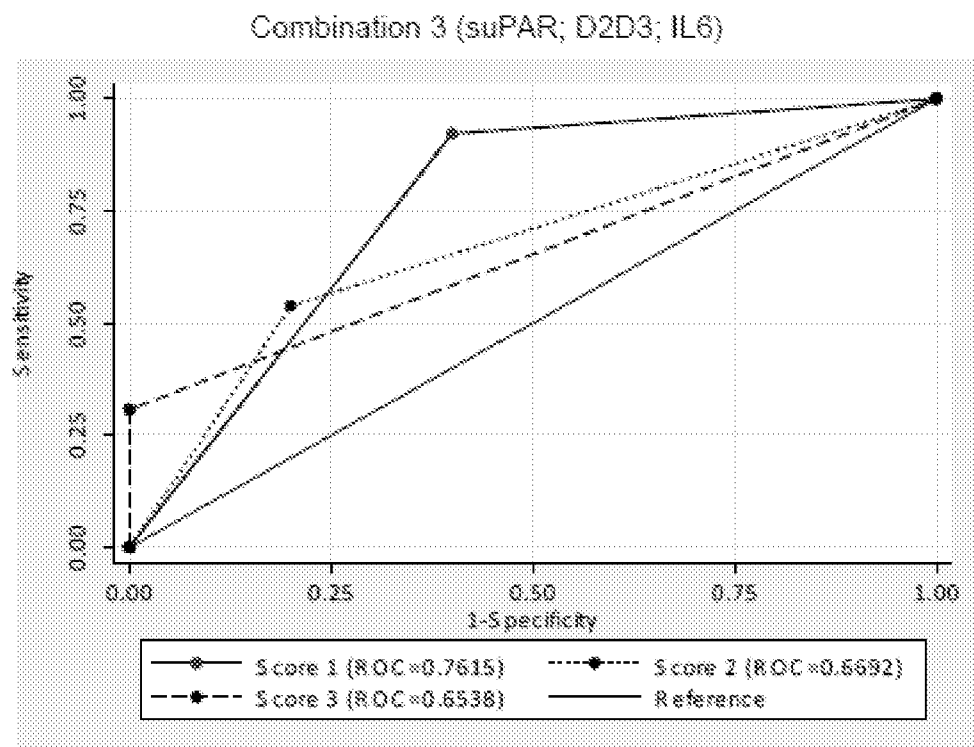


FIG. 12C

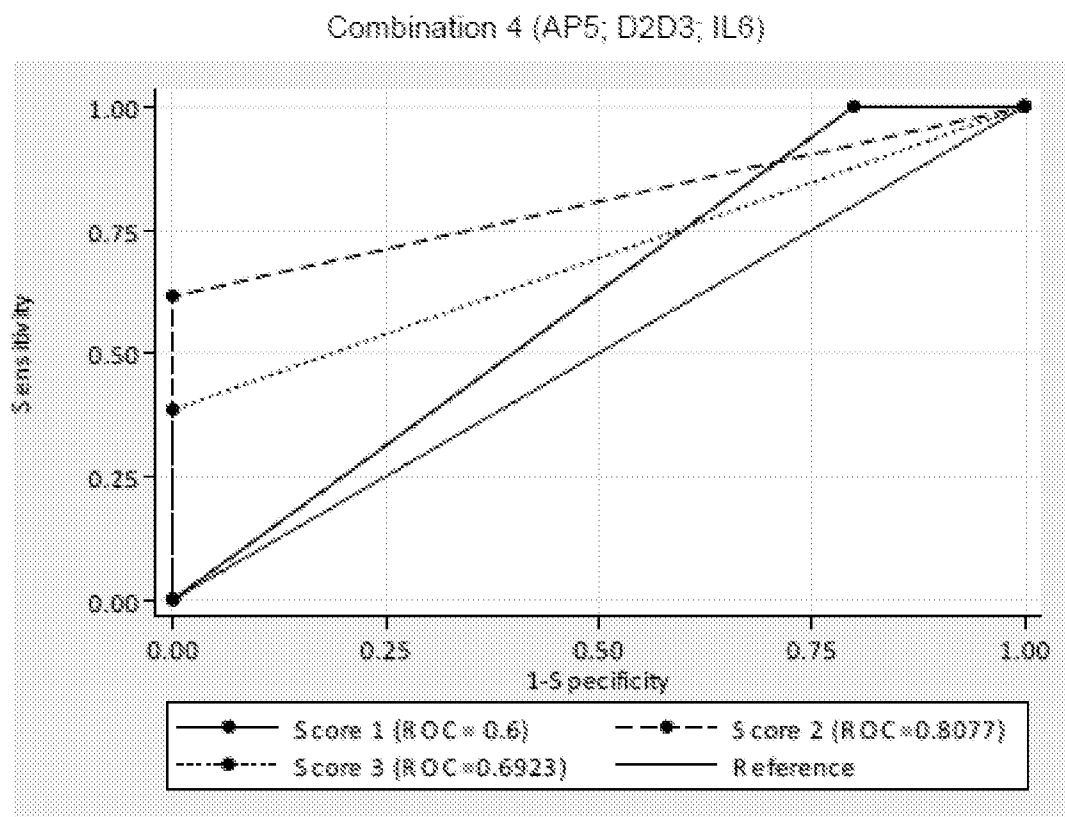


FIG. 12D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/53788

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/53, C07K 14/705 (2016.01)

CPC - G01N 33/6893, G01N 2333/9723, G01N 2800/042, C07K 14/705, G01N 33/53, G01N 2333/70596

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 33/53, C07K 14/705 (2016.01)

CPC - G01N 33/6893, G01N 2333/9723, G01N 2800/042, C07K 14/705, G01N 33/53, G01N 2333/70596

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

CPC - G01N 2800/50, G01N 33/6827, G01N 33/6893, G01N 2800/347

USPC- 424/172.1, 435/7.1, 435/7.9, 435/7.92, 436/501

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

PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: SEVER, Sanja; REISER, Jochen; Soluble urokinase-type plasminogen activator receptor; suPAR; VPI-2960B, CNT095, CNT0 95, anti-CD61 antibody, RGD-peptidomimetic, S247, estimated glomerular filtration rate, eGFR, single chain urokinase type plasminogen activator, scuPA, DI, DII, DIIDIII, D2D3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Outinen, et al. Urine soluble urokinase-type plasminogen activator receptor levels correlate with proteinuria in Puumala hantavirus infection. J Intern Med. 2014, 276(4): 387-395; pg 3 [according to the posted document]	1
X -- Y	WO 2015/017386 A1 (GENERAL HOSPITAL CORP., et al.) 05 February 2015 (05.02.2015) claims 1, 5, 12; pg 2, ln 10-15; pg 3, ln 9-11; pg 7, ln 20-28; pg 27, ln 10-15; pg 36, ln 20-25; pg 39, ln 31-32	1, 2, (4,5)/(1,2), 6 ----- 5/3, 7-9, 11, 13
X -- Y	US 2014/0083945 A1 (Reiser) 27 March 2014 (27.03.2014) Abstract; claims 10, 20, 22, 25; para [0004], [0008], [0018], [0058], [0060], [0061], [0065], [0067], [0093].	1-4, 6, 10 ----- 5/3, 12, 7-9, 11, 13
Y	US 2010/0297139 A1 (Reiser) 25 November 2010 (25.11.2010) para [0220]	12
Y	Pecoits-Filho, et al. Update on interleukin-6 and its role in chronic renal failure. Nephrol. Dial. Transplant. 2003, 18(6):1042-1045; pg 1042, col 1; pg 1043, Fig 1 and its legend	7-9, 11, 13
Y	US 2012/0213775 A1 (Reiser) 23 August 2012 (23.08.2012) claims 1, 4, 13; para [0012]	11, 13

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

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

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"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

04 November 2016

Date of mailing of the international search report

27 DEC 2016

Name and mailing address of the ISA/US

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