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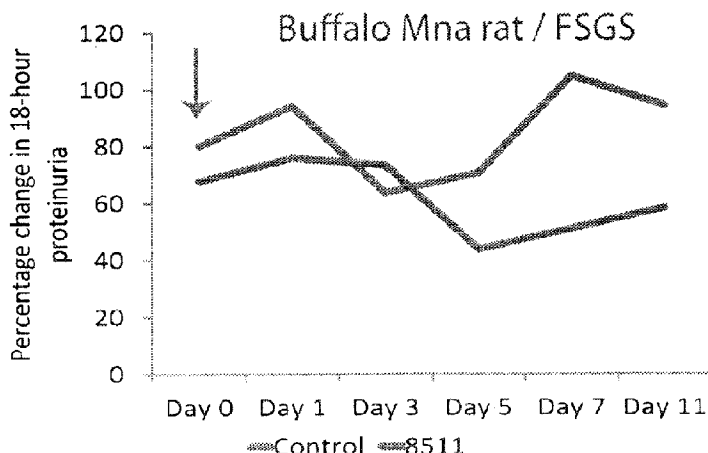
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(54) Title: METHODS FOR TREATMENT OF NEPHROTIC SYNDROME AND RELATED CONDITIONS



(57) Abrégé/Abstract:

The present disclosure provides a polypeptide and method for treating and/or preventing nephrotic syndrome, such as but not limited to those associated with minimal change disease and membranous nephropathy, and conditions related to nephrotic syndrome, such as but not limited to, proteinuria and edema, as well as diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The present disclosure further provides methods for reducing proteinuria and other disease states as discussed herein. Such methods comprise the therapeutic delivery of an Angptl4 polypeptide or Angptl4 polypeptide derivative to a subject.

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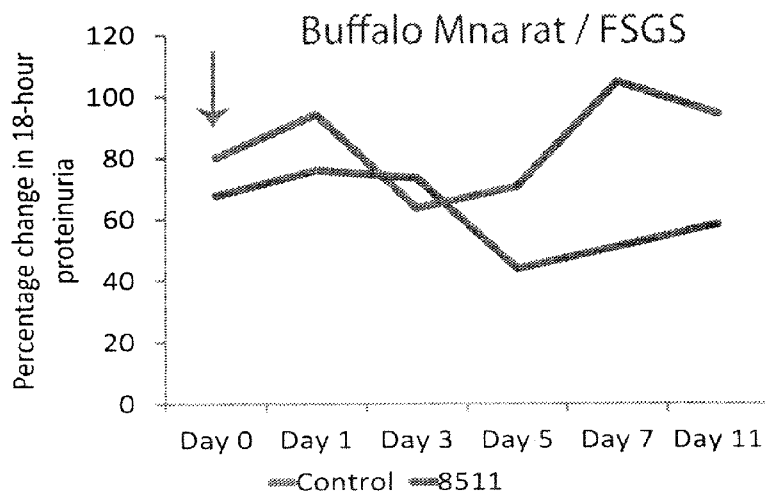
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(54) Title: METHODS FOR TREATMENT OF NEPHROTIC SYNDROME AND RELATED CONDITIONS

FIG. 19



(57) Abstract: The present disclosure provides a polypeptide and method for treating and/or preventing nephrotic syndrome, such as but not limited to those associated with minimal change disease and membranous nephropathy, and conditions related to nephrotic syndrome, such as but not limited to, proteinuria and edema, as well as diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The present disclosure further provides methods for reducing proteinuria and other disease states as discussed herein. Such methods comprise the therapeutic delivery of an Angptl4 polypeptide or Angptl4 polypeptide derivative to a subject.

METHODS FOR TREATMENT OF NEPHROTIC SYNDROME AND RELATED CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Application 13/841,240, filed March 15, 2013
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15 FIELD OF THE DISCLOSURE

The present disclosure is directed to methods for the treatment and prevention of nephrotic
syndrome and conditions related thereto, such as, but not limited to, proteinuria and edema.

BACKGROUND

Nephrotic syndrome (NS) is a general term that refers to the loss of protein in the urine
20 (proteinuria), hyperlipidemia (hypercholesterolemia and hypertriglyceridemia), and edema.
Nephrotic syndrome involves changes in the pathology of cells in the kidney, such as podocytes.
Proteinuria is defined as the presence of an excess of serum proteins in the urine. Albuminuria, a
specific type of proteinuria, is a pathological condition wherein albumin is present in the urine.

Podocytes (or visceral epithelial cells) are cells in the outer layer of the glomerular capillary
25 loop in the kidneys. The glomerulus filters blood, holding back large molecules such as proteins, and
passing through small molecules such as water, salts, and sugar, as the first step in forming urine.
The long projections, or "foot processes," of the podocytes wrap around the capillaries, and come to
rest on the glomerular basement membrane. The foot processes are connected by a porous
structure called the slit diaphragm. The innermost layer of the glomerular capillary loop is made of
30 fenestrated endothelial cells. Kidneys affected by nephrotic syndrome have abnormalities in the
glomerular capillary loop that cause leakage of blood proteins, resulting in proteinuria.

When protein is lost in the urine, its plasma concentration decreases, allowing water to
move into other areas of the body, which leads to swelling known as edema. Edema is commonly
observed in the feet and legs, in the belly or abdomen (ascites), and around the eyes, but can occur

anywhere, especially in response to gravity. Additionally, because of this extra fluid that stays in the body, people often gain weight, experience fatigue and may find that they urinate less often

Many conditions are categorized as nephrotic syndromes, including minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN) (also called
 5 membranous glomerulonephritis, MGN), and membranoproliferative glomerulonephritis (MPGN). For years pathologists found no changes in MCD tissue when viewing specimens under light microscopy, hence the name minimal change disease. With the advent of electron microscopy, the changes now known as the hallmarks for the disease include diffuse loss of podocyte foot processes, vacuolation of the podocyte foot processes, and growth of microvilli on the visceral epithelial cells.
 10 Diabetic nephropathy is the most common cause of nephrotic syndrome.

Hypertriglyceridemia may occur due to changes in the activity of enzymes that degrade triglycerides, such as lipoprotein lipase (LPL) (2-4). Certain proteins involved in the etiology of nephrotic syndrome and proteinuria, such as angiotensin-like 4 (Angptl4), inhibit the activity of LPL.

The molecular basis of nephrotic syndrome is not known. Increased levels of Angptl4 have
 15 been noted in nephrotic syndrome, such as MCD, MN/MGN, and MPGN, but increased circulating levels of Angptl4 have not been associated with causation of proteinuria in nephrotic syndrome. However, the role of Angptl4 in nephrotic syndrome, such as but not limited to, MCD, FSGS, MN/MGN, and MPGN, and related conditions, such as, but not limited to, proteinuria have not been previously reported. Furthermore, the association of proteinuria and glucocorticoid sensitivity in
 20 nephrotic syndrome and the link between proteinuria and hypertriglyceridemia, two key components of nephrotic syndrome, have yet to be established. Therapy designed to reduce proteinuria further complicates the study of disease mechanisms. For example, glucocorticoids used to treat proteinuria in MCD independently raise plasma triglyceride levels (5), and normalization of plasma triglyceride levels lags behind the response of proteinuria to glucocorticoids in certain forms
 25 of nephrotic syndrome, such as MCD (6).

The present disclosure show that increased circulating levels of Angptl4 reduce the severity of nephrotic syndrome and conditions associated therewith, such as but not limited to, proteinuria. As a result, the present disclosure provides method for treating and/or preventing nephrotic syndrome, such as but not limited to, MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy as
 30 well as methods of alleviating symptoms associated with nephrotic syndrome, including, but not limited to, proteinuria and edema. The present disclosure further provides methods for reducing proteinuria and edema.

BRIEF DESCRIPTION OF THE FIGURES

FIG 1 shows the development and characterization of aP2-Angptl4 TG rats.

FIG.1A shows a 2D gel analysis of 200 µg human plasma (n = 4 patients/group, cropped representative blots shown) and demonstrates the presence of increased circulating levels of Angptl4 in patients with minimal change disease (MCD) in relapse and in patients with membranous nephropathy (MN) (indicated by arrows), compared to patients with MCD in remission (i. e. non proteinuric patients).

FIG. 1B shows a transgenic (TG) rat model for adipose tissue specific over expression of Angptl4 (aP2-Angptl4 TG)..

FIG. 1C shows tissue specific over expression of Angptl4 mRNA (n = 3 rats/group) in aP2-Angptl4 TG rats. WAT is white adipose tissue, BAT is brown adipose tissue. *** P < 0.001.

FIG. 1D shows 2D gel electrophoresis of 200 µg plasma, followed by Western blot for Angptl4 and demonstrates that heterozygous aP2-Angptl4 TG rats had higher circulating Angptl4 levels than wild type rats (age 3 months, n = 3 blots/group).

FIG. 1E shows 2D gel electrophoresis of 200 µg plasma, followed by Western blot with the anti-V5 and anti-Angptl4 antibodies and demonstrates the presence of adipose tissue secreted V5-tagged Angptl4 in the plasma of aP2-Angptl4 TG rats.

FIG. 1F shows 2D gel electrophoresis of anti-N-terminal Angptl4 immunoprecipitates from aP2-Angptl4 TG rat plasma followed by Western blotting using lectin SNA I and anti-Angptl4 antibodies and confirmed the presence of circulating sialylated Angptl4 in the aP2-Angptl4.

FIG. 1G shows PAS stained sections from 3 month old heterozygous aP2-Angptl4 TG rats (n = 3 rats/group) and demonstrates normal glomerular morphology (magnification 400x).

FIG. 1H shows immunogold EM with anti-V5 antibody to specifically detect transgenic protein in 3 month heterozygous aP2-Angptl4 TG male rats and demonstrated gold particles selectively on the endothelial surface in aP2-Angptl4 TG rats (indicated by arrows).

FIG. 2 shows the relationship of increased circulating levels of Angptl4 with proteinuria/albuminuria.

FIG. 2A shows assessment of urinary protein excretion (3 µg / lane, except MCD remission) in different human and experimental disease conditions by GelCode blue stained SDS PAGE and demonstrated the absence of significant proteinuria in aP2-Angptl4 TG rats (lane marked with *, arrow shows intact albumin at around 70 kDa).

FIG. 2B shows assessment of albuminuria by ELISA and revealed that heterozygous female aP2-Angptl4 TG rats had lower albuminuria than wild type littermates (n = 6 rats/group).

FIG. 2C shows assessment of albuminuria by ELISA and revealed that heterozygous male aP2-Angptl4 TG rats had lower albuminuria than wild type littermates (n = 6 rats/group).

FIG. 2D shows induction of puromycin nephrosis (PAN), a model of nephrotic syndrome, in wild type and aP2-Angptl4 TG rats and demonstrates less proteinuria in aP2-Angptl4 TG rats compared to wild

type littermates (n = 8 rats/group). * P < 0.05, ** P < 0.01 compared to corresponding controls

FIG. 2E shows recombinant Angptl4 had protective effects on cultured glomerular endothelial cells (GEnCs). ** P < 0.01, *** P < 0.001 compared to corresponding controls

FIG. 2F shows upregulation of Angptl4 in wild type rats in disease models like PAN on Day 6 was exclusively glomerular, while upregulation of Angptl4 in adipose tissue was noted on Day 10 when proteinuria and glomerular Angptl4 expression are on the decline (n = 3 rats / sample). ** P < 0.01, *** P < 0.001 compared to corresponding controls

FIG. 2G shows increased circulating levels of Angptl4 at baseline and after induction of PAN in aP2-Angptl4 TG rats results in increased plasma triglyceride levels compared to wild type rats. * P < 0.05 compared to corresponding controls

FIG. 2H shows increased circulating levels of Angptl4 at baseline and after induction of PAN in aP2-Angptl4 TG rats results in reduced post-heparin lipoprotein lipase (LPL) activity compared to wild type rats. * P < 0.05 compared to corresponding controls

FIG. 3 shows the primers and probes used for Taqman real time PCR (SEQ ID NOS. 11-22).

FIG. 4 shows recombinant Angptl4 reduces proteinuria in animal models of human glomerular disease. FIG. 4(A) shows reduction of proteinuria in Thy1.1 nephritis, a short term model of mesangial injury. Thy1.1 nephritis was induced in male Wistar rats (n=4 rats/group). After assessment of baseline proteinuria (Day 1), concentrated supernatant protein from Angptl4 stable or control cell lines were injected intra-peritoneally on two consecutive days (Days 1 & 2, arrows) into Buffalo Mna rats (n=4 rats/group) followed by assessment of proteinuria. Proteinuria was lower in Angptl4 treated rats throughout, and was statistically significant on Day 5. * P<0.05; ** P<0.01. all values are mean $\pm$ SE. FIG. 4(B) shows reduction of proteinuria in Thy1.1 nephritis, a short term model of mesangial injury. Thy1.1 nephritis was induced in male Wistar rats (n=4 rats/group, injected on Day 0). After confirming the induction of proteinuria (Day 1), concentrated supernatant protein from Angptl4 stable or control cell lines were injected intravenously on two consecutive days (Days 1 & 2, arrows) followed by assessment of proteinuria. Proteinuria was lower in Angptl4 treated rats throughout, and was statistically significant on Day 5. * P<0.05; ** P<0.01. all values are mean $\pm$ SE

FIG. 5 shows the amino acid and cDNA sequences of Angptl4 from various species. SEQ ID NOS. 1 and 2 show amino acid and cDNA sequence from human (Protein Variant 1 isoform a, long form; underlined amino acid sequences at a position 40 and 161-164); SEQ ID NOS. 3 and 4 show amino acid and cDNA sequence from human (Protein Variant 3 isoform b, short form; underlined amino acid sequences at a position 40 and 161-164); SEQ ID NOS. 5 and 6 show amino acid and cDNA sequence from rat; SEQ ID NOS: 7 and 8 show amino acid and cDNA from mouse; underlined are

forward sequencing primers. Bold are reverse sequencing primers.

FIG. 6 shows that elevated circulating Angptl4 levels are required for the development of hypertriglyceridemia in nephrotic syndrome. (A) ELISA for plasma Angptl4 levels in patients with nephrotic syndrome due to primary glomerular disease. Number of patients analyzed are shown in brackets (B) ELISA for plasma Angptl4 levels at pre-nephrotic and nephrotic stages in passive Heymann nephritis (PHN, a model of membranous nephropathy), Buffalo Mna (B. Mna, spontaneously develop focal and segmental glomerulosclerosis) and single dose intravenous puromycin aminonucleoside nephrosis (PAN, a model of minimal change disease), all rat models of nephrotic syndrome. Proteinuria, hypertriglyceridemia and LPL activity in PHN (C to E), Buffalo Mna rats (F to H), and PAN rats (I to K). (L, M) Plasma triglyceride levels and LPL activity in adipose tissue specific Angptl4 overexpressing rats (aP2-Angptl4), that have elevated circulating Angptl4 levels, and 3 month old podocyte specific Angptl4 overexpressing rats (NPHS2-Angptl4), in which transgene expressed Angptl4 does not enter the circulation. (n) Plasma triglyceride levels in Angptl4 $-/-$ and $+/-$ mice 48 hours after induction of nephrotic syndrome using γ 2-NTS. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

FIG. 7 shows the source of circulating Angptl4 in nephrotic syndrome: Multi-organ Angptl4 mRNA expression relative to control in (A) passive Heymann nephritis (PHN), (B) Buffalo Mna rats and (C) puromycin aminonucleoside nephrosis (PAN). (D) Representative 2-dimensional gel electrophoresis and Western blot of plasma showing circulating Angptl4 levels in proteinuric NPHS2-Angptl4 transgenic rats before and after the induction of mild PAN. (E) Densitometry analysis of 2-dimensional gels in d (F) 2-dimensional gel electrophoresis and Western blot of plasma from NPHS2-Angptl4 transgenic rats with PAN to demonstrate the presence of V5-tagged transgene expressed Angptl4 in the circulation. (G and H) Plasma triglyceride levels (G) and lipoprotein lipase (LPL) activity (H) six days after induction of PAN in wild type Sprague Dawley, aP2-Angptl4 and NPHS2-Angptl4 transgenic rats. Empty bars correspond to data from Fig 6(l) and 6(m) included here for comparison. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. In panels G and H, statistical significance is shown for difference between transgenic rats and corresponding wild type controls. $P<0.001$ for each rat type before and after induction of PAN. In panels A to C, 3-fold change in expression (horizontal line) was taken as significant.

FIG. 8 shows urinary loss of Angptl4 and LPL in nephrotic syndrome. (A) Representative reducing Western blots of urine from normal Sprague Dawley (SD), PAN, PHN, and Buffalo Mna rats. Black arrows point towards Angptl4 bands. Albumin blush is also noted in PAN, PHN and Buffalo Mna rats between 65 and 70 kDa. (B) Non-reducing Western blot of urine from nephrotic rats using goat anti LPL antibody to assess for urinary loss of LPL (arrow). (C) Non-reducing western blot of nephrotic rat urine using anti-LPL monoclonal antibody 5D2 to identify active LPL (arrow). (D) Multi-organ mRNA

expression profile for LPL in Sprague Dawley rats with PAN. (E) mRNA expression profile of major organs that express LPL in aP2-Angptl4 transgenic rats. In panels D and E, 3-fold change in expression (horizontal line) was taken as significant.

FIG. 9 shows effect of circulating Angptl4 on proteinuria. Red arrows indicate time points when an antibody or recombinant protein, as appropriate, were injected. (A) Proteinuria after induction of puromycin aminonucleoside nephrosis (PAN) in wild type Sprague Dawley and aP2-Angptl4 transgenic rats (B) The effect of depleting circulating Angptl4 using an anti-Angptl4 Ab. on proteinuria in Sprague Dawley rats with PAN. (C) Proteinuria in Buffalo Mna rats after injection of concentrated supernatant from recombinant rat Angptl4 secreting stable cell lines or control stable cell lines. (D) Effect of injecting recombinant rat Angptl4 or control protein from the above cell lines on proteinuria in severe anti-Thy1.1 nephritis, a model of mesangial injury. * $P < 0.05$, ** $P < 0.01$

FIG. 10 shows dissociation of effects of Angptl4 on triglycerides and proteinuria using mutant recombinant human Angptl4. (A) Schematic representation of wild type and mutant human Angptl4 proteins showing mutations in areas important for LPL binding (amino acid 40, and adjacent amino acid 39) and protein cleavage (amino acids 161 to 164). (B) Western blot of recombinant tagged proteins using mouse anti V5 antibody and control mouse IgG to demonstrate the expected size of the intact protein and reduced cleavage in the mutant proteins (arrows). (C) Plasma levels of wild type or mutant human Angptl4 after injecting 55 μ g of recombinant human protein in Buffalo Mna rats, a model of focal and segmental glomerulosclerosis (FSGS) as assessed by OD 450 using reagents from the human Angptl4 ELISA kit. (D) Effect of wild type and mutant Angptl4 on proteinuria in Buffalo Mna rats. * $P < 0.05$, shown where all 3 study groups were individually different from control. (E) Effect of wild type and mutant Angptl4 on plasma triglyceride levels in Buffalo Mna rats. * $P < 0.05$ for wild type values compared to baseline. # $P < 0.05$ for 6 hour mutant values compared to wild type.

FIG. 11 shows that circulating Angptl4 reduces proteinuria via its interaction with glomerular endothelial α v β 5 integrin. Red arrows indicate time points when anti β 5 integrin antibody or pre-immune serum were injected. (A) Confocal image of a glomerulus from an aP2-Angptl4 transgenic rat demonstrates co-localization of Angptl4-V5 (anti-V5 antibody, red) secreted from adipose tissue with glomerular endothelium (anti Von Willebrand factor antibody, green) (B) Immunogold electron micrograph of a glomerulus from aP2-Angptl4 transgenic rats using anti-V5 antibody to show glomerular endothelial cell surface binding of adipose tissue secreted Angptl4-V5. (C) Interaction of purified α v β 5 integrin with sialylated Angptl4 or control in vitro. Linear regression slope (black) is superimposed. (D) Development of proteinuria after induction of nephrotic syndrome using γ 2-nephrotoxic serum (NTS) in β 5 integrin -/- and +/- mice to demonstrate the protective effects of the endothelial β 5 integrin – circulating Angptl4 interaction on proteinuria during the peripheral phase

of Angptl4 secretion. (E) Effect of blocking the endothelial $\beta 5$ integrin – Angptl4 interaction using anti $\beta 5$ integrin antibodies on recovery from peak proteinuria (corresponds with the peripheral phase of Angptl4 secretion) in Sprague Dawley rats with PAN. (F) Effect of blocking the endothelial $\beta 5$ integrin – Angptl4 interaction using anti $\beta 5$ integrin antibodies on recovery from peak proteinuria in aP2-Angptl4 transgenic rats with PAN. (G) Induction of nephrotic syndrome using $\gamma 2$ -NTS in Angptl4 $-/-$ and $+/+$ mice to determine whether the lack of Angptl4 affects recovery from peak proteinuria during the peripheral phase of Angptl4 expression. The findings of the glomerular phase are consistent with our previously published study (ref. 6 from working example 4). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

10 FIG. 12 shows a two-dimensional gel electrophoresis of human plasma. (A) Cropped image of representative Western blots with anti-Angptl4 antibody to show elevated circulating Angptl4 levels in membranous nephropathy (MN), focal and segmental glomerulosclerosis (FSGS) and minimal change disease (MCD). Angptl4 spots are enclosed in green circles / ovals. (B) Ponceau red stained images of nitrocellulose membranes corresponding to Western blots.

15 FIG. 13 (A) Albuminuria in *Angptl4* $+/+$ and *Angptl4* $-/-$ mice 48 hours after injection of $\gamma 2$ -NTS, corresponds to Figure 6(n). Image is reproduced from the on line supplement of Reference 6 from Working Example 4. Similar results seen on Day 2 of study shown in Figure 6(h). (B) Ponceau red stained images of nitrocellulose membranes used for Western blot in Figure 2d. (C) Overexposed Western blot of stripped membrane from Figure 3b using normal goat serum. (D) Overexposed Western blot of stripped membrane from Figure 3c using normal mouse IgG. (E) Fasting plasma triglyceride levels in Buffalo Mna rats from 12 hours to 17 days after injection of recombinant human wild type Angptl4, human mutant Angptl4, and control protein. * $P < 0.05$

20 FIG. 14: (A) Elevated plasma Angptl4 levels during the peripheral phase of Angptl4 expression (Days 5 and 7) in $\gamma 2$ -NTS injected *Itgb* $+/+$ and $-/-$ mice shown in Figure 11E. (B) Multiorgan mRNA expression profile for Angptl4 in *Itgb* $+/+$ mice shown in Figure 11E, 7 days after injection of $\gamma 2$ -NTS. (C) Plasma Angptl4 levels in Sprague Dawley PAN rats shown in Figure 11F. (D) Plasma triglyceride levels in Sprague Dawley PAN rats shown in Figure 11F. All comparisons in panels A, C and D were made with Day 0 values. * $P < 0.05$

25 FIG. 15 shows a schematic representation of the origin and biological effects of circulating Angptl4 in nephrotic syndrome. Following the establishment of moderate to severe proteinuria, skeletal muscle, adipose tissue and heart upregulate and secrete Angptl4 into the circulation. Some of this Angptl4 binds to $\alpha \beta 5$ integrin on the glomerular endothelial surface and reduces proteinuria, while some binds to, and converts active lipoprotein lipase (LPL) into inactive LPL, that are lost in urine. Reduced LPL mediated triglyceride uptake results in hypertriglyceridemia. Some circulating Angptl4

is also lost in the urine. In minimal change disease (MCD), podocyte secreted hyposialylated Angptl4 exerts local pro-proteinuric effects within the glomerulus, whereas podocyte secreted sialylated protein binds the glomerular endothelium and leaks into the circulation to induce hypertriglyceridemia. In membranous nephropathy (MN), focal and segmental glomerulosclerosis (FSGS) and non-HIV collapsing glomerulopathy (CG), podocytes do not contribute significant amounts of Angptl4 to the circulation.

FIG. 16 (A) Standard curve for human Angptl4 ELISA (B) Standard curve for rodent Angptl4 ELISA (C) Characterization of anti- β 5 integrin antibody 8472A by Western blot. (D) Confocal image of a rat glomerulus that shows binding of anti- β 5 integrin antibody (red, detected using donkey anti rabbit IgG) to glomerular endothelium (blue, labeled with mouse anti-PECAM1 antibody) six hours after intravenous injection, resulting in a magenta overlap pattern.

FIG. 17 shows amino acid and nucleic acid substitutions for four mutant forms of human Angptl4.

FIG. 18 show a Western blot of recombinant proteins shown in FIG. 17 tagged using mouse anti- V5 antibody and control mouse IgG to demonstrate the expected size of the intact protein and reduced cleavage in the mutant proteins (arrows).

FIG. 19 shows peak levels for the mutant 8511 of change in proteinuria after a single intravenous injection into proteinuric Buffalo Mna rats.

FIG. 20 shows decline in proteinuria after injection of 2 mutant human Angptl4 proteins (15 μ g) into Zucker Diabetic Fatty rats, a model of diabetic nephropathy and diabetic kidney disease.

FIG. 21 shows levels of proteinuria of two mutant human Angptl4 proteins after injection (15 μ g) into Zucker Diabetic Fatty rats. All * values are relative to baseline Day 0 values.

FIG. 22 shows no increase in plasma triglyceride levels after injection of 2 mutant forms of human Angptl4 protein (15 μ g) in Zucker Diabetic Fatty rats. For Figs. 20-22: * $P < 0.05$, ** $P < 0.01$. # $P < 0.05$.

SUMMARY OF THE DISCLOSURE

In a first aspect, the present disclosure provides methods of treatment and/or prevention of nephrotic syndrome. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN or diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is characterized as MSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4 or

an Angptl4 polypeptide derivative. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration treats nephrotic syndrome by providing Angptl4 function. In an alternate embodiment, such administration treats nephrotic syndrome by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In a second aspect, the present disclosure provides methods of treatment and/or prevention of MCD. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration treats MCD by providing Angptl4 function. In an alternate embodiment, such administration treats MCD by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In a third aspect, the present disclosure provides methods of alleviating one or more symptoms of nephrotic syndrome, such as, but not limited to, proteinuria, hypercholesterolemia, hypertriglyceridemia and edema. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is

a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one
5 embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration alleviates one or more symptoms of nephrotic syndrome by providing Angptl4 function. In an alternate embodiment,
10 such administration alleviates one or more symptoms of nephrotic syndrome by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In a fourth aspect, the present disclosure provides methods for reducing proteinuria in a subject. In one embodiment, the subject is suffering from nephrotic syndrome. In one embodiment,
15 the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In another embodiment, the subject is suffering from a disorder characterized by proteinuria. In another embodiment, the subject is suffering from a diabetic condition. In a further embodiment, the proteinuria is caused by FSGS. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus
20 nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a
25 derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical
30 composition or in combination with a secondary agent. In one embodiment, such administration reduces proteinuria by providing Angptl4 function. In an alternate embodiment, such administration reduces proteinuria by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In a fifth aspect, the present disclosure provides methods of reducing edema in a subject. In

one embodiment, the subject is suffering from nephrotic syndrome. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN, and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by FSGS. In a specific embodiment, the edema is caused by decreased circulating levels of plasma proteins such as albumin. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. Reduction of proteinuria through the administration of an Angptl4 polypeptide or Angptl4 polypeptide derivative will reduce proteinuria, raise plasma protein levels and thereby reduce edema. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration reduces edema by providing Angptl4 function. In an alternate embodiment, such administration reduces edema by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In a sixth aspect, the present disclosure provides methods of reducing hypercholesterolemia and/or hypertriglyceridemia in a subject. In one embodiment, the subject is suffering from nephrotic syndrome. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be

resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration reduces hypercholesterolemia and/or hypertriglyceridemia by providing Angptl4 function. In an alternate embodiment, such administration reduces hypercholesterolemia and/or hypertriglyceridemia by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In a seventh aspect, the present disclosure provides methods of treatment and/or prevention of a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration treats the foregoing conditions by providing Angptl4 function. In an alternate embodiment, such administration treats the foregoing conditions by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

In an eighth aspect, the present disclosure provides a pharmaceutical composition for use in the methods of the first through sixth aspects. The composition comprises one or more Anptl4 polypeptides or polypeptide derivatives. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one

embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein.

DETAILED DESCRIPTION

In the following discussion certain articles and methods will be described for background and introductory purposes. Nothing contained herein is to be construed as an “admission” of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the articles and methods referenced herein do not constitute prior art under the applicable statutory provisions.

While investigating nephrotic syndrome, it was noted that Angptl4 secreted from podocytes induced proteinuria. More importantly, as described herein, circulating Angptl4 reduced the proteinuria in a transgenic animal model. Increased levels of Angptl 4 have been noted in nephrotic syndrome, such as MCD and MN, but increased circulating levels of Angptl4 have not been associated with causation of nephrotic syndrome.

While increased Angptl4 levels are shown to treat nephrotic syndrome and reduce associated proteinuria, increased Angptl4 in the circulation has been observed to induce hyperlipidemia (hypertriglyceridemia), such as, but not limited to, through inhibition of LPL. It would be advantageous to provide the benefits of increased circulating Angptl4 levels without the negative consequences of hyperlipidemia. Such an approach is possible using the Angptl4 polypeptide derivatives as disclosed herein.

Angiopoietin-like proteins have been implicated in the development of hypertriglyceridemia and tumor metastasis, and are functionally distinct from the angiopoietins. Angptl4 is a PPAR (8) and PPAR (9) target gene highly expressed in the liver and adipose tissue, strongly induced by fasting in white adipose tissue and liver, and is an apoptosis survival factor for vascular endothelial cells under normoxic conditions (10). Angptl4 is a potent inhibitor of LPL (11), inducing significant hypertriglyceridemia following intravenous injection or adenovirus-mediated expression (12, 13). Other studies showed lesser expression of Angptl4 in cardiomyocytes and skeletal muscle, and low level expression in whole kidney on Northern blot analysis (8). Recent population based studies of the *ANGPTL4* gene reveals variants that affect triglyceride levels in humans (14, 15).

The present disclosure shows a conclusive role for circulating Angptl4 in the reduction of proteinuria observed in nephrotic syndrome, such as, but not limited to, MCD, FSGS, MN, MPGN and diabetic nephropathy.

Definitions

The terms “prevention”, “prevent”, “preventing”, “suppression”, “suppress” and “suppressing” as used herein refer to a course of action (such as administering a compound or pharmaceutical composition) initiated prior to the onset of a symptom, aspect, or characteristics of a

disease or condition so as to prevent or reduce such symptom, aspect, or characteristics. Such preventing and suppressing need not be absolute to be useful.

The terms “treatment”, “treat” and “treating” as used herein refers a course of action (such as administering a compound or pharmaceutical composition) initiated after the onset of a symptom, aspect, or characteristics of a disease or condition so as to eliminate or reduce such symptom, aspect, or characteristics. Such treating need not be absolute to be useful.

The term “in need of treatment” as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, but that includes the knowledge that the patient is ill, or will be ill, as the result of a disease or condition that is treatable by a method or compound of the disclosure.

The term “in need of prevention” as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from prevention. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, but that includes the knowledge that the patient will be ill or may become ill, as the result of a disease or condition that is preventable by a method or compound of the disclosure.

The term “individual”, “subject” or “patient” as used herein refers to any animal, including mammals, such as mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, and humans. The term may specify male or female or both, or exclude male or female.

The term “therapeutically effective amount” as used herein refers to an amount of a compound, either alone or as a part of a pharmaceutical composition, that is capable of having any detectable, positive effect on any symptom, aspect, or characteristics of a disease or condition. Such effect need not be absolute to be beneficial. When referring to an Angptl4 polypeptide or Angptl4 polypeptide derivative, the term “therapeutically effective amount” refers to an amount of such polypeptide sufficient to reduce proteinuria in a subject.

The term “pharmaceutically acceptable derivative” means any pharmaceutically acceptable salt, ester, salt of an ester, solvate or other derivative of an Angptl4 polypeptide or polypeptide derivative of the present disclosure that, upon administration to a subject, is capable of providing (directly or indirectly) the function of wild type Angptl4; in certain embodiment, the Angptl4 polypeptide or polypeptide derivative shows decreased LPL inhibitory activity of a resistance to cleavage. Particularly favored derivatives are those that increase the bioavailability of an Angptl4 polypeptide or polypeptide derivative of the disclosure when such polypeptides are administered to a subject (e.g., by allowing an orally administered compound to be more readily absorbed into the blood), enhance delivery of such polypeptides to a given biological compartment, increase solubility

to allow administration by injection, alter metabolism or alter rate of excretion. In one embodiment, the derivative is a prodrug.

The term “pharmaceutically acceptable salt(s)”, unless otherwise indicated, includes salts of acidic or basic groups that may be present in the Angptl4 polypeptide or polypeptide derivative of the present disclosure.

The terms “about” and “approximately” shall generally mean an acceptable degree of error or variation for the quantity measured given the nature or precision of the measurements. Typical, exemplary degrees of error or variation are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. For biological systems, the term “about” refers to an acceptable standard deviation of error, preferably not more than 2-fold of a give value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term “about” or “approximately” can be inferred when not expressly stated.

Methods of Treatment and Prevention

The present disclosure provides methods of treatment and/or prevention of nephrotic syndrome. The present disclosure further provides methods of treatment and/or prevention of MCD, FSGS, and/or conditions with mesangial injury (such as diabetes mellitus). The present disclosure further provides methods of treatment and/or prevention of a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The present disclosure additionally provides methods of alleviating one or more symptoms of nephritic syndrome, such as, but not limited to, proteinuria, hypercholesterolemia, hypertriglyceridemia and edema. Still further, the present disclosure provides for methods of reducing proteinuria. Further still, the present disclosure provides methods of reducing edema. The present disclosure additionally provides for pharmaceutical compositions comprising one or more Angptl4 polypeptides of Angptl4 polypeptide derivatives. The nature of the Angptl4 polypeptide derivatives is described in further detail below.

In one embodiment, the teachings of the present disclosure provide for the treatment and/or prevention of nephrotic syndrome in a subject in need of such treatment or prevention. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, and MPGN. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In

an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration treats nephrotic syndrome by providing Angptl4 function. In an alternate embodiment, such administration treats nephrotic syndrome by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage. Such method may further comprise identifying a subject in need of such treatment and/or prevention.

In an alternate embodiment, the teachings of the present disclosure provide for the treatment and/or prevention of MCD in a subject in need of such treatment or prevention. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration treats MCD by providing Angptl4 function. In an alternate embodiment, such administration treats MCD by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage. Such method may further comprise identifying a subject in need of such treatment and/or prevention.

In further embodiment, the teachings of the present disclosure provide for methods of alleviating one or more symptoms of nephrotic syndrome, such as, but not limited to, proteinuria, hypercholesterolemia, hypertriglyceridemia and edema. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN, and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the

nephrotic syndrome is caused by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one
5 embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or
10 polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration alleviates one or more symptoms of nephrotic syndrome by providing Angptl4 function. In an
15 alternate embodiment, such administration alleviates one or more symptoms of nephrotic syndrome by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage. Such method may further comprise identifying a subject in need of such treatment and/or prevention.

In still a further embodiment, the teachings of the present disclosure provide methods for
20 reducing proteinuria in a subject. In one embodiment, the subject is suffering from nephrotic syndrome. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the
25 diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further
30 embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose,

either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration reduces proteinuria by providing Angptl4 function. In an alternate embodiment, such administration reduces proteinuria by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage. Such method may further comprise identifying a subject in need of such treatment and/or prevention.

In yet a further embodiment, the teachings of the present disclosure provide methods for reducing edema in a subject. In one embodiment, the subject is suffering from nephrotic syndrome. In one embodiment, the nephrotic syndrome is characterized as MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. In a specific embodiment, the edema is caused by decreased circulating levels of plasma proteins such as albumin. Reduction of proteinuria through the administration of an Angptl4 polypeptide or a Angptl4 polypeptide derivative will raise reduce proteinuria, raise plasma protein levels and thereby reduce edema. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration reduces edema by providing Angptl4 function. In an alternate embodiment, such administration reduces edema by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage. Such method may further comprise identifying a subject in need of such treatment and/or prevention.

In still a further embodiment, the teachings of the present disclosure provide methods for reducing hypercholesterolemia and/or hypertriglyceridemia in a subject. In one embodiment, the subject is suffering from nephrotic syndrome. In one embodiment, the nephrotic syndrome is

characterized as MCD, FSGS, MN/MGN, and MPGN. In another embodiment, the nephrotic syndrome is characterized as MCD. In a further embodiment, the nephrotic syndrome is caused by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration reduces proteinuria by providing Angptl4 function. In an alternate embodiment, such administration reduces proteinuria by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage. Such method may further comprise identifying a subject in need of such treatment and/or prevention.

In still a further embodiment, the teachings of the present disclosure provide methods for treatment and/or prevention of a nephrotic syndrome that is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. The methods comprise the step of administering to a subject an Angptl4 polypeptide or an Angptl4 polypeptide derivative. In one embodiment, the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 10. In an alternate, the amino acid sequence is a fragment of any of the foregoing sequences having an activity comparable to wild type Angptl4. In still a further embodiment, the Angptl 4 polypeptide derivative is a derivative described herein and has been modified to have decreased LPL inhibitory activity, to be resistant to cleavage, or a combination of the foregoing. The Angptl4 polypeptide or polypeptide derivative, in one embodiment, is sialylated. Such derivative may be based on any of the Angptl4 polypeptides described herein. The Angptl4 polypeptide or polypeptide derivative may be administered at a therapeutically effective dose, either alone, as a part of a pharmaceutical composition or in combination with a secondary agent. In one embodiment, such administration treats the foregoing conditions by providing Angptl4 function. In an alternate embodiment, such administration treats

the foregoing conditions by providing a modified Angptl4 function, such as, but not limited to, an Angptl4 function that display reduced LPL inhibition or is resistant to cleavage.

Some embodiments of administering the Angptl4 polypeptide or derivative involve a form of administration that delivers the polypeptide to the blood. In one example the polypeptide is administered intravenously. Given the appropriate dosage form, such administration may be performed orally, subcutaneously, or by other means as is known in the art. The Angptl4 polypeptide or derivative may be administered in a therapeutically effective amount; this amount will generally be within a certain range of ratios of mass of compound to mass of subject. In some embodiments of the method the polypeptide is administered at a dosage of about 0.005-150,000 $\mu\text{g/kg}$, 0.5-15,000 $\mu\text{g/kg}$, 5-1500 $\mu\text{g/kg}$, or 50-150 $\mu\text{g/kg}$. Thus, for a typical 70 kg human adult, the dosage may be 0.0035-11,000 mg, 0.035-1100 mg, 0.35-110 mg, or 3.5-11 mg. Administration may occur on a regular schedule. In some embodiments of the method the polypeptide is administered about once per 14 days. In other embodiments the polypeptide is administered about twice per month. In still other embodiments the polypeptide is administered from about once per month to about twice per month. In further embodiments, the polypeptide is administered once per a given time period selected from the group consisting of: a day, two days, three days, a week, ten days, two weeks, three weeks, four weeks, and a month.

Methods of Screening

The present disclosure also relates to a method for identifying a compound effective for treating or preventing nephrotic syndrome or a condition associated therewith, such as, but not limited to, proteinuria, hypercholesterolemia, hypertriglyceridemia or edema. In one embodiment, the nephrotic syndrome is characterized as MCD or MN. In another embodiment, the nephrotic syndrome is characterized as MCD. In another embodiment, the nephrotic syndrome is characterized by FSGS. In a further embodiment, the nephrotic syndrome is caused by a diabetic condition. In one embodiment, the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. Such compounds may be useful as active ingredients included in pharmaceutical compositions or for administration alone. In one embodiment, the methods include determining the level a polypeptide involved in the etiology of nephrotic syndrome, such as, but not limited to, Angptl4.

In general, such screening methods comprises the steps of providing an assay system (as described in more detail below) that expresses a polypeptide involved in the etiology of nephrotic syndrome, such as, but not limited to, Angptl4, introducing into the assay system a test compound to be tested and determining whether the effect of the test compound on the level the polypeptide. The methods involve the identification of candidate or test compounds or agents (polypeptides,

functional nucleic acids, carbohydrates, antibodies, small molecules or other molecules) which effect the the level of sialylation of the polypeptide. Such compounds may then be further tested in appropriate systems (such as, but not limited to, the animal models systems described herein) to determine the activity of the identified compounds.

5 Candidate compounds are identified using a variety of assays, such as, but not limited to, assays that employ cells which express a polypeptide involved in the etiology of nephrotic syndrome, such as, but not limited to, Angptl4 or in assays with isolated polypeptides. The various assays can employ a variety of variants of such polypeptides (e. g., full-length, a biologically active fragment, or a fusion protein which includes all or a portion of the desired polypeptide). Moreover, such
10 polypeptides can be derived from any suitable mammalian species (e. g., human, rat or murine); in a specific embodiment, the polypeptide is derived from a human.

 Where the assay involves the use of a whole cell, the cell may either naturally express a polypeptide involved in the etiology of nephrotic syndrome, such as, but not limited to, Angptl4, or may be modified to express the same. In the latter case, cells can be modified to express a desired
15 polypeptide through conventional molecular biology techniques, such as by infecting the cell with a virus comprising such polypeptide. The cell can also be a prokaryotic or an eukaryotic cell that has been transfected with a nucleotide sequence encoding such polypeptide. In the foregoing, full length polypeptides, fragments or fusion proteins containing at least a part of such polypeptide may be used. Exemplary assay systems are described in the current specification.

20 The various screening assays may be combined with an *in vivo* assay entailing measuring the effect of the test compound on the symptoms the disease states and conditions discussed herein. In such an embodiment, the compounds may be evaluated to determine if they impact a parameter associated with nephrotic syndrome or a condition related thereto, such as, but not limited to, proteinuria or edema. Such parameters include, but are not limited to, determining 1) the level of a
25 polypeptide involved in the etiology of nephrotic syndrome and related conditions, such as, but not limited to Angptl4 and 2) determining the level of protein excretion, either total or with regard to specific components.

 In one embodiment, such a screening assay can be performed, for example, by determining the level of a polypeptide, such as, but not limited to, Angptl4 and detecting a difference in the level
30 of such polypeptide in the presence of as compared to the absence of a test compound. Such screening assay may be *in vitro*, *in vivo* or *ex vivo* and may be cell culture based (either with whole cells or lysates) or may be based on an animal model. Any assay of the present disclosure may be used in the foregoing method.

 Suitable test compounds for use in the screening methods can be obtained from any suitable

source, such as conventional compound libraries. The test compounds can also be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the “one-bead one-compound” library method and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds. Examples of methods for the synthesis of molecular libraries can be found in the art. Libraries of compounds may be presented in solution or on beads, bacteria, spores, plasmids or phage.

The present disclosure also provides kits for carrying out any method of the present disclosure, which can contain any of the compounds and/or compositions disclosed herein or otherwise useful for practicing a method of the disclosure.

Creation and Selection of Angptl4 polypeptide derivatives

Angiopoietin-related protein 4 is a polypeptide that in humans is encoded by the *ANGPTL4* gene. This gene is a member of the angiopoietin/angiopoietin-like gene family and encodes a glycosylated, secreted protein with a N-terminal signal sequence (amino acid residues 1-22 of SEQ ID NO:1), a coiled-coil domain (amino acid residues 23-170 of SEQ ID NO:1), a linker region (amino acid residues 171-185 of SEQ ID NO:1) and a fibrinogen C-terminal domain (amino acid residues 186-406 of SEQ ID NO:1). This gene is induced under hypoxic conditions in endothelial cells and is the target of peroxisome proliferation activators. The encoded protein is a serum hormone directly involved in regulating glucose homeostasis, lipid metabolism, and insulin sensitivity and also acts as an apoptosis survival factor for vascular endothelial cells. Alternatively spliced transcript variants encoding different isoforms have been described. This gene was previously referred to as *ANGPTL2* but has been renamed *ANGPTL4*.

Angptl4 inhibits LPL by breaking the LPL dimer molecule. *Angptl4* has been unambiguously established as potent inhibitors of blood plasma triglyceride (TG) clearance, causing elevation of plasma TG levels. Recent evidence indicates that variations in the sequence of the *Angptl4* polypeptide impact the effect on triglycerides, with certain mutations conferring reduced triglyceride levels implying a decreased inhibition of LPL.

Furthermore, it has been reported that *Angptl4* polypeptides exist in oligomeric forms and that oligomerization is required for inhibition of LPL activity. Once secreted from the cell, the oligomeric form is cleaved at a cleavage site (R₁₆₁RKR₁₆₄ of SEQ ID NOS: 1 and 3) to provide monomeric C-terminal forms and oligomeric N-terminal forms (34). The N-terminal residues 1-187 of the *Angptl4* peptide were found to be sufficient to inhibit LPL (33).

The amino acid and cDNA sequences of the human, rat and mouse are provided in FIG. 5 and designated SEQ ID NOS: 1-8. The present disclosure contemplates the use of Angptl4 polypeptides and polypeptide derivatives in the methods disclosed herein, such as but not limited to, methods of treatment and prevention. As defined herein an Angptl4 polypeptide derivative refers to an Angptl4 polypeptide that includes one or more insertions, deletions and/or substitutions as determined from the amino acid sequence of the human polypeptides shown in SEQ ID NOS: 1 or 3 or the polypeptides shown in SEQ ID NOS: 5 or 7.

Some embodiments of the Angptl4 derivative comprise a core structure that is a consensus sequence between any two or more of SEQ ID NOS: 1, 3, 5, or 7, with one or more substitutions as described herein. One embodiment of the Angptl4 derivative comprises the consensus sequence between SEQ ID NOS: 1 and 3 (both version of human Angptl4); the consensus sequence comprising:

$$A-B-C$$

in which A is at least 80% homologous to SEQ ID NO: 26, B is an oligopeptide of 0-38 residues (an optional linking region), and C is at least 80% homologous to SEQ ID NO: 27. The level of homology of A to SEQ ID NO: 26 and of C to SEQ ID NO: 27 may of course be higher than 80%. These levels of homology may be independently selected from 80-100%, for example 85%, 90%, 95%, 99%, 99.5%, and 100%. The sequence of oligopeptide B may be any sequence. Some embodiments of oligopeptide B are at least 50% homologous to positions 184-222 of SEQ ID NO: 1. In such embodiments the level of homology may be selected from any point in the range of 50-100%, including for exemplary purposes 60%, 70%, 80%, 85%, 90%, 95%, 99%, 99.5%, and 100%.

Another embodiment of the Angptl4 derivative comprises a consensus sequence between all of SEQ ID NOS: 1, 3, 5, AND 7 (human variant, rat and mouse); the consensus sequence comprising:

$$V-W-X-Y-Z$$

in which V has at least 80% homology to SEQ ID NO: 23, W is an oligopeptide of 0-5 residues, X has at least 80% homology to SEQ ID NO: 24, Y is an oligopeptide of 0-38 residues (an optional linking region), and Z has at least 80% homology to SEQ ID NO: 25. The levels of homology of V to SEQ ID NO: 23, X to SEQ ID NO 22, and Z to SEQ ID NO: 25 may be higher than 80%. These levels of homology may be independently selected from 80-100%, for example 85%, 90%, 95%, 99%, 99.5%, and 100%. The sequence of oligopeptide Y may be any sequence. Some embodiments of oligopeptide B are at least 50% homologous to positions 184-222 of SEQ ID NO: 1. In such embodiments the level of homology may be selected from any point in the range of 50-100%, including for exemplary purposes 60%, 70%, 80%, 85%, 90%, 95%, 99%, 99.5%, and 100%.

These consensus sequences allow for substitutions at positions corresponding to positions 39, 40, 46, 50, and 53 of SEQ ID NO: 1, which as taught in this disclosure may serve to reduce LPL inhibitory activity. They also allow for substitutions at positions corresponding to positions 63-66 of SEQ ID NO: 1, which as taught in this disclosure may serve to increase the protein's resistance to cleavage. They also allow for substitutions at positions corresponding to SEQ ID NO: 1 positions 5, 67, 72, 77, 167, 174, 190, 230, 233, 237, 251, 266, 278, 291, 293, 296, 307, 308, 336, 338, 349, 361, 371, and 384.

Specific embodiments of the Angptl14 derivative comprise one or more of the following substitutions at these positions: P5L, S67R, R72L, G77R, E167K, P174S, E190Q, E196K, R230C, G233R, F237V, P251T, T266M, R278Q, V291M, L293M, E296V, P307S, V308M, R336C, D338E, W349C, G361R, G361S, R371Q, and R384W. Such naturally occurring substitutions would be expected to preserve the function of the protein.

In one embodiment, amino acid residues of the Angptl4 polypeptide are removed and replaced with different amino acid residues. The variants may be constructed as described herein or as known in the art. The variants so constructed may be evaluated using the methods and assays described herein to screen for activity.

When used herein, single letters when used to refer to amino acids have the following meanings:

G	Glycine	P	Proline	W	Tryptophan	H	Histidine
A	Alanine	V	Valine	K	Lysine	R	Arginine
L	Leucine	I	Isoleucine	Q	Glutamine	N	Asparagine
M	Methionine	C	Cysteine	E	Glutamic Acid	D	Aspartic Acid
F	Phenylalanine	Y	Tyrosine	S	Serine	T	Threonine

In one embodiment, the variant comprises a change in the amino acid sequence of an Angptl4 polypeptide that decreases the ability of Angptl4 to inhibit LPL or to or to be resistant to cleavage. The change may be a replacement, deletion and/or substitution of one or more residues in this region. Such changes have been described in the art.

In one embodiment, such change occurs in residues 1-187 with respect to SEQ ID NO: 1, residues 1-182 of SEQ ID NO: 3, residues 1-182 of SEQ ID NO: 26, any residues in SEQ ID NO: 23, and residues 1-79 in SEQ ID NO: 24.

Some embodiments of the derivative of the Angptl4 polypeptide derivative differ from the human wild-type sequence at positions 39-55 of SEQ ID NO: 1 (DEMNVLAHGLLQLGQGL); this region corresponds to positions 39-55 of SEQ ID NOS: 1, 3, 5, 7, 22, and 24. Additional embodiments of the

derivative comprise a sequence at positions 39-55 that is neither DEMNVLAHGLLQLGQGL (positions 39-55 of SEQ ID NO: 1) nor DKMNVLAHGLLQLGQGL (SEQ ID NO: 28). Further embodiments of the Angptl4 polypeptide derivative have at least one substitution at positions 39, 40, 46, 50, and 53, such that positions 39-40 of V is not DE, position 46 of V is not H, position 50 of V is not Q, and position 53 of V is not Q.

In some embodiments, such change occurs at position 40 with respect to SEQ ID NOS: 1, 3, 5, 7, 22, or 24. In one embodiment, the amino acid at position 40 (a negatively charged glutamic acid residue in wild-type Angptl4) is replaced with a neutral amino acid or a positively charged amino acid. In a particular embodiment, the change is an E40K substitution. In another particular embodiment, the change is an E40A substitution. The E40K and E40A substitutions have been shown to reduce LPL inhibition by Angptl4, but not interfere with expression, secretion, processing and other functions of the polypeptide. In a further particular embodiment, the change at position 40 is selected from those shown in Table 1 below. In yet a further embodiment, the amino acid at position 39 of SEQ ID NOS: 1, 3, 5, 7, 21, or 24 (a negatively charged aspartic acid residue in wild-type Angptl4) is replaced with a neutral or positively charged amino acid. In one embodiment, the substitution is a D39K substitution or a D39A substitution. In a further particular embodiment, the change at position 39 of SEQ ID NOS: 1, 3, 5, 7, 21, or 24 is selected from those shown in Table 1 below. In certain embodiments, a polypeptide variant may contain one of the aforementioned changes at position 40, one of the aforementioned changes at position 39 or a combination of the foregoing. In a particular embodiment, the polypeptide contains a D39K substitution and a E40K substitution, a D39A substitution and a E40K substitution or a D39K substitution and an E40A substitution. In a further specific embodiment the polypeptide derivative the sequence at positions 39-40 is selected from the group consisting of: DK, KE, DA, and AE. In yet another embodiment the polypeptide derivative the sequence at positions 39-40 is not DE.

Table 1: Modifications of D₃₉ and E₄₀ in the human Angptl4 protein

G	P	V	L	I	M	C	F	Y
W	H	R	Q	N	S	T		

In another embodiment, the derivative comprises one or more changes in a region of the Angptl4 polypeptide responsible for cleavage of the polypeptide. In one embodiment, this region is the R₁₆₁RKR₁₆₄ region of Angptl4 (corresponding to positions 161-164 of SEQ ID NOS: 1, 3, 5, 7, and 26; and positions 63-66 of SEQ ID NO: 24). The change may be a replacement, deletion and/or substitution of one or more residues in this region. The R₁₆₁RKR₁₆₄ region has been shown to be responsible for cleavage of the oligomeric forms of Angptl4, releasing oligomers of the N-terminal sequences and monomers of the C-terminal sequence. Forms of Angptl4 with a mutated cleavage

site were shown to accumulate at higher levels in the circulation than wild-type polypeptide. Furthermore, preventing cleavage of the Angptl4 polypeptide stabilizes the oligomeric forms of Angptl4 observed to be efficacious in the present disclosure. In one embodiment, all 4 amino acid residues of the R₁₆₁RKR₁₆₄ region are changed, such that the sequence is not RRKR; in an alternate embodiment, any 1, 2 or 3 amino acid residues of the R₁₆₁RKR₁₆₄ region are changed. In a further embodiment, the arginine residues at positions 161, 162 or 164 are independently substituted with glycine, alanine, valine or serine and the lysine residue at position 163 is substituted with glycine, alanine, valine or serine. In a specific embodiment the R₁₆₁RKR₁₆₄ sequence is replaced with a sequence selected from the group consisting of: GAAG (SEQ ID NO: 29), GSGS (SEQ ID NO: 80), GVVA (SEQ ID NO: 49), SGGG (SEQ ID NO: 87), and VAVA (SEQ ID NO: 90). In a further specific embodiment the R₁₆₁RKR₁₆₄ sequence is replaced with AAVV. Exemplary amino acid sequences for replacement of the entire R₁₆₁RKR₁₆₄ region of SEQ ID NOS: 1 or 3 is provided in Table 2 below.

TABLE 2: Modifications of ₁₆₁ RRKR ₁₆₄ in the Angptl4 protein or derivatives													
SEQ ID		SEQ ID		SEQ ID		SEQ ID		SEQ ID		SEQ ID		SEQ ID	
29	GAAG	38	GAAG	47	GAVV	56	GVVV	65	GAAA	74	AVVV	83	SGSG
30	GAGA	39	GAVA	48	GVAV	57	VGVV	66	AGAA	75	VAVV	84	S GGS
31	GGAA	40	GVAA	49	GVVA	58	VVVG	67	AAAG	76	VVVV	85	SSGG
32	AGGA	41	AGVA	50	AGVV	59	VVGV	68	AAGA	77	SSSS	86	GS GG
33	AGAG	42	AGAV	51	AVVG	60	VAVG	69	AAVV	78	GGGG	87	S GGG
34	AAGG	43	AAVG	52	AVGV	61	VVGA	70	AAVA	79	AAAA	88	GGSG
35	VGAA	44	AAGV	53	VGAV	62	VVAG	71	AAAV	80	GSGS	89	GGGS
36	VAAG	45	AVAG	54	VGVA	63	VVVA	72	AVAA	81	GSSG	90	VAVA
37	VAGA	46	AVGA	55	VAGV	64	VVAV	73	VAAA	82	GGSS		

In a further embodiment, one or more of the amino acids in the R₁₆₁RKR₁₆₄ sequence is altered to remove a consensus binding site of an enzyme capable of cleaving Angptl4, such that Angptl4 is resistant to cleavage. In one embodiment, the enzyme is a proprotein convertase and the consensus binding site is RXKR, RXRR, RR or KR, where X is any amino acid. In making such alternations, one or more amino acids may be deleted or substituted with glycine, alanine, valine or serine or with any of the other substitutions discussed herein.

In still a further embodiment, the variant comprises one or more changes in a region of the Angptl4 polypeptide responsible for oligomerization of the polypeptide. In one embodiment, this region is the C₇₆ and/or C₈₀ region of Angptl4. The C₇₆ and/or C₈₀ region has been shown to be involved in oligomerization of the Angptl4 polypeptide.

The change may be a replacement, deletion and/or substitution of one or more residues in this region. In a particular embodiment, only one of the cysteine residues at positions 76

and 80 is substituted; in an alternate embodiment, both cysteine residues at positions 76 and 80 are both substituted. In one embodiment, at least one of the cysteine residues at position 76 and 80 are substituted independently with alanine or serine; in another embodiment, both cysteine residues are substituted with alanine or serine.

5 In a further embodiment, the variant comprises one or more changes in the R₁₆₁RKR₁₆₄ region of Angptl4 that inhibits the cleavage of the Angptl4 polypeptide oligomer and a change at position 40 that reduces inhibition of LPL activity by Angptl4. Any of the changes discussed herein are included.

In one embodiment, the present disclosure provides for Angptl4 polypeptide variants having
10 the amino acid sequence of SEQ ID NOS: 9 or 10. SEQ ID NO: 9 is shown in FIG.4 and includes the wild type sequence of Angptl4 from SEQ ID NO: 1, with the exception of substitutions at positions 39, 40, 76, 80 and 161-164 indicated by X₃₉, X₄₀, X₇₆, X₈₀, X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄, respectively. SEQ ID NO: 10 is shown in FIG.4 and includes the wild type sequence of Angptl4 from SEQ ID NO: 3, with the exception of substitutions at positions 39, 40, 76, 80 and 161-164 indicated by X₃₉, X₄₀, X₇₆, X₈₀, X₁₆₁,
15 X₁₆₂, X₁₆₃ and X₁₆₄, respectively.

In SEQ ID NOS: 9 and 10, X₃₉ may be A, G, P, V, L, I, M, C, F, Y, W, H, R, Q, N, S, T or K. In one embodiment, X₃₉ is a neutral or positively charged amino acid. In a further embodiment, X₃₉ may be A or K. In still a further embodiment, X₃₉ may be D.

In SEQ ID NOS: 9 and 10, X₄₀ may be A, G, P, V, L, I, M, C, F, Y, W, H, R, Q, N, S, T or K. In one
20 embodiment, X₄₀ is a neutral or positively charged amino acid. In a further embodiment, X₄₀ may be A or K. In still a further embodiment, X₄₀ may be E. In yet a further embodiment, X₄₀ may be E when X₃₉ is not D and X₃₉ may be D when X₄₀ is not E.

In SEQ ID NOS: 9 and 10, at least one of X₇₆ and X₈₀ may be substituted. In one embodiment, X₇₆ and X₈₀ are independently A or S or C. In one embodiment, one of X₇₆ and X₈₀ may be A or S and
25 the other of X₇₆ and X₈₀ is C. In a further embodiment, both of X₇₆ and X₈₀ may be independently A or S. In still a further embodiment, both of X₇₆ and X₈₀ may C.

In SEQ ID NOS: 9 and 10, at least one of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ may be substituted. In one embodiment, all 4 of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are substituted; in an alternate embodiment, 1, 2 or 3 of
30 X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are substituted. In a further embodiment, X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are independently D, R, K, G, A, V or S. In still a further embodiment, all 4 of are substituted with the combinations recited in Table 2.

The present disclosure contemplates combinations of the foregoing in any form. Furthermore, the designated residues in SEQ ID NOS: 9 and 10 may be substituted with conservative amino acid substitutions as designated in Table 3, or with residues having a difference in hydrophatic

index of +/- 1 or less or with residues having a difference in hydrophilicity values of +/- 1 or less.

In a one embodiment, X₃₉ is D, X₄₀ is A or K, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X₃₉ is D, X₄₀ is A or K, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are selected from the combinations shown in Table 2. In still another embodiment, X₃₉ is D, X₄₀ is A or K, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are GSGS or GAAG.

In an additional embodiment, X₃₉ is D, X₄₀ is A or K, one of X₇₆ and X₈₀ is A or S and the other of X₇₆ and X₈₀ is C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ is an amino acid not found in SEQ ID NOS: 1 or 3. In a further embodiment, X₃₉ is D, X₄₀ is A or K, one of X₇₆ and X₈₀ is A or S and the other of X₇₆ and X₈₀ is C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are selected from the combinations shown in Table 2. In still a further embodiment, X₃₉ is D, X₄₀ is A or K, one of X₇₆ and X₈₀ is A or S and the other of X₇₆ and X₈₀ is C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are GSGS or GAAG.

In one embodiment, X₃₉ is A or K, X₄₀ is E, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X₃₉ is A or K, X₄₀ is E, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are selected from the combinations shown in Table 2. In still another embodiment, X₃₉ is A or K, X₄₀ is E, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are GSGS or GAAG.

In one embodiment, X₃₉ is D, X₄₀ is K, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X₃₉ is D, X₄₀ is K, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are selected from the combinations shown in Table 2. In still another embodiment, X₃₉ is D, X₄₀ is K, X₇₆ and X₈₀ are C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are GSGS or GAAG.

In one embodiment, X₃₉ is D, X₄₀ is K, one of X₇₆ and X₈₀ is A or S and the other of X₇₆ and X₈₀ is C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X₃₉ is D, X₄₀ is K, one of X₇₆ and X₈₀ is A or S and the other of X₇₆ and X₈₀ is C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are selected from the combinations shown in Table 2. In still another embodiment, X₃₉ is D, X₄₀ is K, one of X₇₆ and X₈₀ is A or S and the other of X₇₆ and X₈₀ is C and X₁₆₁, X₁₆₂, X₁₆₃ and X₁₆₄ are GSGS or GAAG.

In one embodiment, the Angptl4 derivative is based on a fragment of Angptl4. Suitable

fragments include any fragment that retains the activity of wild type Angptl4 or any fragment of 100 or more consecutive amino acids. In one embodiment, such fragment is based on amino acids 1-187 SEQ ID NO: 1 or amino acids 1-182 of SEQ ID NO: 3. Such fragments may have the amino acid substitutions described in the preceding paragraphs.

5 The Angptl4 polypeptide derivative may have an activity that is comparable to or increased (in one embodiment, 50% or more) as compared to the wild-type Angptl4 polypeptide activity; alternatively, the Angptl4 polypeptide derivative may have an activity that is decreased (in one embodiment, less than 50%) as compared to the wild-type Angptl4 polypeptide activity. In a specific embodiment, the Angptl4 polypeptide derivative has a decreased ability to inhibit LPL and shows an
10 increased resistance to cleavage.

 The deletions, additions and substitutions can be selected, as would be known to one of ordinary skill in the art, to generate a desired Angptl4 polypeptide derivative. For example, conservative substitutions or substitutions of amino acids with similar properties are expected to be tolerated. In addition, specific deletions, insertions and substitutions may impact, positively or
15 negatively, a certain Angptl4 polypeptide activity but not impact a different Angptl4 polypeptide activity.

 Conservative modifications to the amino acid sequence of any of SEQ ID NOS: 1 or 3 or 5 or 7, including combinations thereof (and the corresponding modifications to the encoding nucleotides) will produce Angptl4 polypeptide derivatives having functional and chemical characteristics similar
20 to those of naturally occurring Angptl4 polypeptides while minimizing undesirable properties such as LPL inhibitory activity. In contrast, substantial modifications in the functional and/or chemical characteristics of Angptl4 polypeptides may be accomplished by selecting substitutions in the amino acid sequence of any of SEQ ID NOS: 1 or 3 or 5 or 7, including combinations thereof, that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of
25 the substitution.

 For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine.

30 Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties. It will be appreciated by those of skill in the art that nucleic acid and polypeptide molecules described herein may be chemically synthesized as well as produced by recombinant

means.

Naturally occurring residues may be divided into classes based on common side chain properties: 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile; 2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; 3) acidic: Asp, Glu; 4) basic: His, Lys, Arg; 5) residues that influence chain orientation: Gly, Pro; and 6) aromatic: Trp, Tyr, Phe.

For example, non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of the Angptl4 polypeptide derivatives that are homologous with non-human Angptl4 polypeptide orthologs, or into the non-homologous regions of the molecule.

In making such changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art (Kyte et al., J. Mol. Biol., 157:105-131, 1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity.

In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within +/- 2 may be used; in an alternate embodiment, the hydropathic indices are with +/- 1; in yet another alternate embodiment, the hydropathic indices are within +/- 0.5.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The greatest local average hydrophilicity of a polypeptide as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

The following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0+-.1); glutamate (+3.0+-.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5+-.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within +/- 2 may be used; in an alternate embodiment, the

hydrophilicity values are with +/- 1; in yet another alternate embodiment, the hydrophilicity values are within +/- 0.5.

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of the Angptl4 polypeptide, or to increase or decrease the affinity of the Angptl4 polypeptide with a particular binding target in order to increase or decrease an Angptl4 polypeptide activity.

Exemplary amino acid substitutions are set forth in Table 3.

Table 3: Amino Acid Substitutions		
Original Amino Acid	Exemplary substitution	Preferred substitution
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Glu	Glu
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Ile, Val, Met, Ala, Phe, Norleucine	Ile
Lys	Arg, 1,4-diaminobutyric acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala, Gly	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

A skilled artisan will be able to determine suitable variants of the polypeptide as set forth in any of SEQ ID NOS: 1, 3, 5, 7, 9, 10, and 20-24, including combinations thereof, using well known techniques. For identifying suitable areas of the molecule that may be changed without destroying activity, one skilled in the art may target areas not believed to be important for activity. For example, when similar polypeptides with similar activities from the same species or from other species are known, one skilled in the art may compare the amino acid sequence of an Angptl4 polypeptide to such similar polypeptides. With such a comparison, one can identify residues and portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of an Angptl4 polypeptide that are not conserved relative to such similar polypeptides would be less likely to adversely affect the biological activity and/or structure of the Angptl4 polypeptide. One skilled in the art would also know that, even in relatively conserved

regions, one may substitute chemically similar amino acids for the naturally occurring residues while retaining activity (conservative amino acid residue substitutions). Therefore, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

Additionally, one skilled in the art can review structure-function studies identifying residues in similar polypeptides that are important for activity or structure. In view of such a comparison, one can predict the importance of amino acid residues in an Angptl4 polypeptide that correspond to amino acid residues that are important for activity or structure in similar polypeptides. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted important amino acid residues of an Angptl4 polypeptide.

One skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In view of that information, one skilled in the art may predict the alignment of amino acid residues of an Angptl4 polypeptide with respect to its three dimensional structure. One skilled in the art may choose not to make radical changes to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules. Moreover, one skilled in the art may generate test Angptl4 polypeptide derivatives containing a single amino acid substitution at each desired amino acid residue. The derivatives can then be screened using activity assays known to those skilled in the art and as disclosed herein. Such derivatives could be used to gather information about suitable substitution. For example, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, derivatives with such a change would be avoided. In other words, based on information gathered from such routine experiments, one skilled in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

Numerous scientific publications have been devoted to the prediction of secondary structure from analyses of amino acid sequences (see Chou et al., *Biochemistry*, 13(2):222-245, 1974; Chou et al., *Biochemistry*, 113(2):211-222, 1974; Chou et al., *Adv. Enzymol. Relat. Areas Mol. Biol.*, 47:45-148, 1978; Chou et al., *Ann. Rev. Biochem.*, 47:251-276, 1979; and Chou et al., *Biophys. J.*, 26:367-384, 1979). Moreover, computer programs are currently available to assist with predicting secondary structure of polypeptides. Examples include those programs based upon the Jameson-Wolf analysis (Jameson et al., *Comput. Appl. Biosci.*, 4(1):181-186, 1998; and Wolf et al., *Comput. Appl. Biosci.*, 4(1):187-191, 1988), the program PepPlot.RTM. (Brutlag et al., *CABS*, 6:237-245, 1990; and Weinberger et al., *Science*, 228:740-742, 1985), and other new programs for protein tertiary

structure prediction (Fetrow. et al., *Biotechnology*, 11:479-483, 1993).

Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins which have a sequence identity of greater than 30%, or
5 similarity greater than 40% often have similar structural topologies. The recent growth of the protein structural data base (PDB) has provided enhanced predictability of secondary structure, including the potential number of folds within a polypeptide's or protein's structure (see Holm et al., *Nucl. Acid. Res.*, 27(1):244-247, 1999).

Additional methods of predicting secondary structure include "threading" (Jones, D., *Curr.*
10 *Opin. Struct. Biol.*, 7(3):377-87, 1997; Suppl et al., *Structure*, 4(1):15-9, 1996), "profile analysis" (Bowie et al., *Science*, 253:164-170, 1991; Gribskov et al., *Meth. Enzym.*, 183:146-159, 1990; and Gribskov et al., *Proc. Nat. Acad. Sci.*, 84(13): 4355-4358, 1987), and. "evolutionary linkage" (See Home, supra, and Brenner, supra).

Any of the polypeptide forms discussed herein may also contain a sequence useful in the
15 identification or purification of the polypeptide; an example of such a sequence is the C-terminal V5 tag. The foregoing also includes nucleic acid sequences (such as, but not limited to cDNA sequences) coding for such polypeptides, including polypeptide derivatives as described herein.

Compositions

Useful compositions of the present disclosure may comprise one or more polypeptides of
20 the present disclosure useful in the treatment and prevention methods of the present disclosure; useful compositions also include one or more nucleic acids coding for one or more polypeptides of the present disclosure useful in the treatment and prevention methods of the present disclosure. The compositions disclosed may comprise one or more of such compounds, in combination with a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be
25 found in Remington: The Science and Practice of Pharmacy (20th Ed., Lippincott, Williams & Wilkins, Daniel Limmer, editor). To form a pharmaceutically acceptable composition suitable for administration, such compositions will contain an therapeutically effective amount of compound.

The pharmaceutical compositions of the disclosure may be used in the treatment and prevention methods of the present disclosure. Such compositions are administered to a subject in
30 amounts sufficient to deliver a therapeutically effective amount of the compound(s) so as to be effective in the treatment and prevention methods disclosed herein. The therapeutically effective amount may vary according to a variety of factors such as, but not limited to, the subject's condition, weight, sex and age. Other factors include the mode and site of administration. The pharmaceutical compositions may be provided to the subject in any method known in the art. Exemplary routes of

administration include, but are not limited to, subcutaneous, intravenous, topical, epicutaneous, oral, intraosseous, and intramuscular. The compositions of the present disclosure may be administered only one time to the subject or more than one time to the subject. Furthermore, when the compositions are administered to the subject more than once, a variety of regimens may be used, such as, but not limited to, one per day, once per week or once per month. The compositions may also be administered to the subject more than one time per day. The therapeutically effective amount and appropriate dosing regimens may be identified by routine testing in order to obtain optimal activity, while minimizing any potential side effects. In addition, co-administration or sequential administration of other agents may be desirable.

The therapeutically effective amount may be a range of ratios between the mass of the compound and the mass of the subject. In some embodiments of the compositions the therapeutically effective amount is about 0.005-150,000 µg/kg, 0.5-15,000 µg/kg, 5-1500 µg/kg, or 50-150 µg/kg. Thus, for a typical 70 kg human adult, the therapeutically effective amount may be 0.0035-11,000 mg, 0.035-1100 mg, 0.35-110 mg, or 3.5-11 mg. Administration may occur on a regular schedule.

The compositions of the present disclosure may be administered systemically, such as by intravenous administration, or locally such as by subcutaneous injection or by application of a paste or cream. In some embodiments of the composition containing an Angptl4 polypeptide or derivative, the pharmaceutical will be suitable for delivery of the polypeptide to the blood. Such suitable types of pharmaceuticals include intravenous formulations, intramuscular formulations, transdermal pastes or creams, transdermal patches, suppositories, and oral dosage forms that protect the polypeptide from digestion.

In one embodiment, a nucleic acid, which may be in the form of a suitable plasmid or vector, is provided that codes for an Angptl4 polypeptide or Angptl4 polypeptide variant of the present disclosure. Such nucleic acid is introduced into a cell, which may be obtained from the subject, by suitable methods known in the art (for example, electroporation). In one embodiment, the cell is an adipose cell. The cells may be assayed for expression of the Angptl4 polypeptide or polypeptide derivative (in one embodiment, expression of the polypeptide can be determined by the presence of a tag on the polypeptide as discussed herein). The cells expressing an Angptl4 polypeptide or polypeptide derivative may then be introduced into the subject. In one embodiment, the cells are administered to the subject by subcutaneous injection; other methods of administration may also be used, including those discussed herein. The cells then express Angptl4 polypeptide or an Angptl4 polypeptide derivative, which is taken up into the circulation.

The compositions of the present disclosure may further comprise agents which improve the

solubility, half-life, absorption, etc. of the compound(s). Furthermore, the compositions of the present disclosure may further comprise agents that attenuate undesirable side effects and/or or decrease the toxicity of the compounds(s). Examples of such agents are described in a variety of texts, such a, but not limited to, Remington: The Science and Practice of Pharmacy (20th Ed.,
5 Lippincott, Williams & Wilkins, Daniel Limmer, editor).

The compositions of the present disclosure can be administered in a wide variety of dosage forms for administration. For example, the compositions can be administered in forms, such as, but not limited to, tablets, capsules, sachets, lozenges, troches, pills, powders, granules, elixirs, tinctures, solutions, suspensions, elixirs, syrups, ointments, creams, pastes, emulsions, or solutions for
10 intravenous administration or injection. Other dosage forms include administration transdermally, via patch mechanism or ointment. Any of the foregoing may be modified to provide for timed release and/or sustained release formulations.

In the present disclosure, the pharmaceutical compositions may further comprise a pharmaceutically acceptable carriers include, but are not limited to, vehicles, adjuvants, surfactants,
15 suspending agents, emulsifying agents, inert fillers, diluents, excipients, wetting agents, binders, lubricants, buffering agents, disintegrating agents and carriers, as well as accessory agents, such as, but not limited to, coloring agents and flavoring agents (collectively referred to herein as a carrier). Typically, the pharmaceutically acceptable carrier is chemically inert to the active compounds and has no detrimental side effects or toxicity under the conditions of use. The pharmaceutically
20 acceptable carriers can include polymers and polymer matrices. The nature of the pharmaceutically acceptable carrier may differ depending on the particular dosage form employed and other characteristics of the composition.

For instance, for oral administration in solid form, such as but not limited to, tablets, capsules, sachets, lozenges, troches, pills, powders, or granules, the compound(s) may be combined
25 with an oral, non-toxic pharmaceutically acceptable inert carrier, such as, but not limited to, inert fillers, suitable binders, lubricants, disintegrating agents and accessory agents. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage
30 forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthum gum and the like. Tablet forms can include one or more of the following: lactose, sucrose, mannitol, corn starch, potato starch, alginic acid, microcrystalline cellulose, acacia, gelatin, guar gum, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium

stearate, calcium stearate, zinc stearate, stearic acid as well as the other carriers described herein. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, and gels containing, in addition to the active ingredient, such carriers as are known in the art.

For oral liquid forms, such as but not limited to, tinctures, solutions, suspensions, elixirs, syrups, the nucleic acid molecules of the present disclosure can be dissolved in diluents, such as water, saline, or alcohols. Furthermore, the oral liquid forms may comprise suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methylcellulose and the like. Moreover, when desired or necessary, suitable and coloring agents or other accessory agents can also be incorporated into the mixture. Other dispersing agents that may be employed include glycerin and the like.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the patient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The compound(s) may be administered in a physiologically acceptable diluent, such as a sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol, isopropanol, or hexadecyl alcohol, glycols, such as propylene glycol or polyethylene glycol such as poly(ethyleneglycol) 400, glycerol ketals, such as 2,2-dimethyl-1,3-dioxolane-4-methanol, ethers, an oil, a fatty acid, a fatty acid ester or glyceride, or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant, such as, but not limited to, a soap, an oil or a detergent, suspending agent, such as, but not limited to, pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agents and other pharmaceutical adjuvants.

Oils, which can be used in parenteral formulations, include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils include peanut, soybean, sesame, cottonseed, corn, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include polyethylene sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol, oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters. Suitable soaps for use in parenteral formulations include fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents include (a) cationic detergents such as, for example, dimethyldialkylammonium halides, and alkylpyridinium halides, (b)

anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylene polypropylene copolymers, (d) amphoteric detergents such as, for example, alkylbeta-aminopropionates, and 2-alkylimidazoline quaternary ammonium salts, and (e) mixtures thereof.

Suitable preservatives and buffers can be used in such formulations. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations ranges from about 5% to about 15% by weight.

Topical dosage forms, such as, but not limited to, ointments, creams, pastes, emulsions, containing the nucleic acid molecule of the present disclosure, can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions, and shampoos in cream or gel formulations. Inclusion of a skin exfoliant or dermal abrasive preparation may also be used. Such topical preparations may be applied to a patch, bandage or dressing for transdermal delivery or may be applied to a bandage or dressing for delivery directly to the site of a wound or cutaneous injury.

The compound(s) of the present disclosure can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines. Such liposomes may also contain monoclonal antibodies to direct delivery of the liposome to a particular cell type or group of cell types.

The compound(s) of the present disclosure may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include, but are not limited to, polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxyethylaspartamidephenol, or polyethyl-eneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

WORKING EXAMPLES

In the following results, the methods used were those methods specified in the Methods section of the present disclosure and the references cited therein. Some of the following results are described in Clement LC et. al., "Podocyte secreted Angiopoietin-like 4 mediates proteinuria in

glucocorticoid-sensitive nephrotic syndrome,” *Nature Medicine*, January 2011

for the disclosure contained therein regarding the use of Angptl4 polypeptides).

1. Patients with Nephrotic Syndrome Have Increased Levels of Circulating Angptl4

5 Patients with Nephrotic syndrome have increased circulating levels of Angptl4 polypeptide. 200 µg human plasma from patients (n = 4 patients/group) with diagnosed with MCD and MN and patients in MCD relapse were analyzed by 2D gel electrophoresis and Western blots were prepared using anti-Angptl4 antibodies (FIG. 1A). FIG. 1A shows that only patients with MCD relapse and MN had increased levels of Angptl4 (indicated by arrows). This form of Angptl4 exists as a neutral pl
10 form and is present as monomers and oligomers.

2. aP2-Angptl4 TG Rats Have Increased Circulating Levels of Angptl4

A transgenic rat models for adipocyte specific Angptl4 overexpression was developed and is shown in FIG. 1B (aP2-Angptl4 TG). Analysis of mRNA expression in organs that normally express Angptl4 confirmed specificity of expression, with Angptl4 being detected in brown adipose tissue
15 (BAT) and white adipose tissue (WAT) (FIG. 1C).

2D gel electrophoresis of 200 µg plasma, followed by Western blotting using an anti- Angptl4 antibody revealed that heterozygous aP2-Angptl4 TG rats had higher circulating Angptl4 levels than wild type rats (FIG. 1D) (age 3 months, n = 3 blots/group). FIG. 1E shows 2D gel electrophoresis of 200 µg plasma, followed by Western blotting using anti-Angptl4 and anti-V5 antibodies show the
20 presence of adipose tissue secreted V5-tagged Angptl4 in the plasma of aP2-Angptl4 TG rats. 2D gel electrophoresis of immunoprecipitated Angptl4 from aP2-Angptl4 TG rat plasma (using an antibody specific for the N-terminus of Angptl4), followed by Western blotting using anti-Angptl4 or anti-lectin. SNA I antibodies revealed the presence of sialylated Angptl4 polypeptide in the circulation.

The aP2-Angptl4 TG rats had morphologically normal glomeruli by light (FIG. 1G) and
25 electron microscopy (not shown), and glomerular Angptl4 expression was unchanged. This is in contrast to podocyte specific expression of Angptl4, where such expression resulted in glomerular defects, including progressive development of foot process effacement between age one to five months (see U.S. Provisional application No. 61/351,865 (filed 5 June 2010)).

30 Immunogold EM using anti-V5 antibody to specifically detect transgene expressed protein in 3 month old heterozygous aP2-Angptl4 TG male rats demonstrated detection selectively on the endothelial surface, indicating that circulating Angptl4 middle and high order oligomers do not enter the GBM and have receptors on the endothelial surface. The effects of circulating Angptl4 is relevant to both human and experimental nephrotic syndrome, since adipose tissue upregulation of Angptl4

is noted in later stages of nephrotic syndrome, when proteinuria is on the decline.

3. Relationship of Increased Circulating Levels of Angptl4 with Proteinuria and Albuminuria

To examine the relationship between circulating levels of Angptl4 proteinuria, including albuminuria, proteinuria was analyzed in aP2-Angptl4 TG rats. FIG. 2A shows that that aP2-Angptl4
5 TG do not exhibit proteinuria as determined by analysis of urinary proteins. In FIG. 2A urinary proteins were analyzed by GelCode blue stained SDS PAGE (3 μ g / lane, except MCD remission) (densitometry readings are provided under each lane). The intact albumin band is observed at 70 kDa (indicated by arrow). As can be seen, WT rats, aP2-Angptl4 TG rats and MCD patients in remission showed little or no intact albumin in the analysed urinary samples, wherein NPHS2-
10 Angptl4 TG rats (a rat transgenic model having podocytes specific Angptl4 expression and shown to develop MCD with proteinuria

MCD relapse, MN relapse and PAN rats (a rat model of nephrotic syndrome) showed strong albumin staining indicative of albuminuria. FIG. 2B shows that female heterozygous aP2-Angptl4 female TG rats had decreased albuminuria as compared to WT littermate
15 controls. FIG. 2C shows the same results for aP2-Angptl4 heterozygous male TG rats. FIG. 2D shows that aP2-Angptl4 TG rats exhibited reduced proteinuria in the puromycin nephrosis (PAN model; a rat model of nephrotic syndrome) as compared to WT littermates. As demonstrated above, aP2-Angptl4 TG rats have higher circulating Angptl4 levels that migrate at or around neutral isoelectric point, and is sialylated. These results show a role for circulating Angptl4 in reducing proteinuria and
20 nephrotic syndrome.

Since endothelial binding of adipose tissue secreted Angptl4 bound to glomerular endothelium, experiments were conducted to determine the effect of recombinant Angptl4 on glomerular epithelial cells (GEnCs) to investigate whether lower baseline albuminuria and less PAN induced proteinuria in this rat model were mediated by glomerular endothelial protection. GEnCs
25 were subject to oxidative injury by addition of hydrogen peroxide and into the culture media and incubated with concentrated supernatant (600 μ g / well) from the control stable cell line, Angptl4-HEK293 cell line (secreting high isoelectric point (pI), hyposialylated Angptl4) or Angptl4-HEK293 cell line incubated with ManNAc (neutral pI, normally sialylated Angptl4). It should be noted that the high pI form of Angptl4 is secreted in large amounts from podocytes in MCD. Release of LDH was
30 assessed as a marker of cell injury. Control cells without hydrogen peroxide injury were given a relative score of 1. High pI Angptl4 increased GEnC injury, whereas neutral pI Angptl4 (which comprises most of circulating Angptl4) was significantly protective at all measured time points. (n = 3 readings/condition).

Upregulation of Angptl4 in wild type rats on PAN Day 6 was exclusively glomerular, whereas

upregulation in adipose tissue was noted on Day 10 when proteinuria and glomerular Angptl4 expression are on the decline (n = 3 rats / sample) (FIG. 2F). Therefore, increases in circulating Angptl4 levels are coincident with the protective effect of circulating Angptl4 in nephrotic syndrome and reduction of proteinuria. The effects of circulating Angptl4 are likely to be relevant to both human and experimental MCD, since adipose tissue upregulation of Angptl4 is noted in later stages of PAN when proteinuria is on the decline. Furthermore, increased circulating Angptl4 levels at baseline and after induction of PAN in aP2-Angptl4 TG rats resulted in increased plasma triglyceride levels (FIG. 2G) and reduced post-heparin lipoprotein lipase activity (FIG. 2H) as compared to wild type rat.

In order to demonstrate the effectiveness of the therapeutic delivery of Angptl4 into the circulation, wild type Angptl4 or a control protein was administered to Buffalo/Mna rats, a model of FSGS, or to Wistar rats in which Thy1.1 nephritis, a short term model of mesangial injury, was induced (FIGS. 4A and B). Wild-type recombinant Angptl4 polypeptide was generated by harvesting of recombinant protein. *Angptl4*-HEK293 stable or pcDNA3.1-HEK293 control stable cell lines were grown to confluence in 15 cm dishes, washed twice with warm PBS, and incubated with serum free DMEM without Phenol Red, with or without 25 mM ManNAc, for 48 hours. Cells were harvested and the supernatant concentrated. Concentrated supernatant from one 15 cm dish was used at each injection time point.

Buffalo/Mna rats spontaneously develop lesions mimicking human FSGS at around age 2 months, including focal and segmental lesions on light microscopy, effacement of podocyte foot processes on electron microscopy, and proteinuria. The rats develop progressive increase in proteinuria as they age. The rats used in the above studies were male and 5 months old. Anti-Thy1.1 nephritis was induced by injection of 150 µg of anti-Thy1.1 (Ox-7 hybridoma) or control IgG IV into different groups of male Wistar rats (100-125 gm, n = 4 rats/group).

In the Buffalo/Mna rat model, assessment of baseline proteinuria was made on Day 0. Angptl4 or control protein were injected intra-peritoneally on two consecutive days (Days 1 & 2, arrows) into Buffalo Mna rats (n=4 rats/group). Proteinuria was assessed on alternate days, and expressed as a percentage of baseline values. Significant reduction in proteinuria was noted in recombinant Angptl4 treated rats.

In the Thy1.1 nephritis model, proteinuria confirmed on Day 1. Rats were injected intravenously with either recombinant Angptl4 or control protein on two consecutive days (Days 1 & 2, arrows). Proteinuria was then assessed. As shown in FIG. 4B, proteinuria was lower in Angptl4 treated rats throughout, and was statistically significant on Day 5.

These results show that therapeutic delivery of Angptl4 into the circulation are an effective

treatment for nephrotic syndrome, such as but not limited to minimal change disease, focal segmental glomerulosclerosis, membranous nephropathy/membranous glomerulonephritis, membranoproliferative glomerulonephritis or a diabetic condition, such as, but not limited to, diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease. Furthermore, these results show that therapeutic delivery of Angptl4 into the circulation are an effective treatment for and conditions related to nephrotic syndrome, such as but not limited to, proteinuria, hypercholesterolemia, hypertriglyceridemia and edema. In one embodiment, the Angptl4 polypeptide is a derivative with decreased LPL inhibitory activity, resistance to cleavage or a derivative described herein. Administration of such a derivative would retain the beneficial effects of Angptl4 treatment without the negative effects associated with inhibition of LPL activity, such as increased plasma triglyceride levels.

METHODS FOR EXAMPLES 1-3

Cloning of full length rat Angptl4, and generation of antibody against full length recombinant Angptl4

The full length rat Angptl4 open reading frame of 1218 bp from our previous experiments (7), excluding the stop codon, was cloned into pcDNA3.1/V5-HisB for eukaryotic expression, and into pET28a for prokaryotic expression. The E. Coli expressed purified full length protein was used to generate a polyclonal antibody in rabbits (Proteintech group, Inc. Chicago IL USA) that was tested by ELISA and Western blot. Antibody reactive bands were excised from GelCode blue stained gels, trypsin digested and presence of Angptl4 peptide sequences confirmed by MALDI-TOF/TOF. Part of the antiserum was affinity purified to the antigen. Unless otherwise specified, all studies described used this antibody. An additional polyclonal antibody against the N-terminal part of rat Angptl4 (amino acids 7 – 86 excluding signal peptide) was similarly raised in rabbits.

Induction of proteinuria in animal models of human glomerular disease

All animal studies were approved by the institutional IACUC. Induction of animal models of proteinuria (n = 4 rats/group) in WT rats are described in previous publications in parenthesis: PAN (7), PHN (7), PAN with glucocorticoids (20), non-HIV collapsing glomerulopathy (18), nephrotoxic serum induced heterologous phase proteinuria (7). Anti-Thy1.1 nephritis was induced by injection of 200 mcg of anti-Thy1.1 (Ox-7 hybridoma) or control IgG IV into different groups of male Wistar rats (100-125 gm, n = 4 rats/group), and rats euthanized after 24 and 72 hours.

The following techniques are described in prior publications: Taqman real time PCR (26), confocal imaging (7), in situ hybridization (27), immunogold EM (26), glomerular extraction and processing for Western blot (26), assessment of charge by PEI method (28). For alcian blue staining, the pH of the staining solution was adjusted to 2.5 using acetic acid, and 0.1% nuclear fast red

solution was used as a counterstain. Densitometry of glomerular basement membrane alcian blue stain (20 glomeruli/rat, 3 rats/group) was assessed using Image-Pro software (Media Cybernetics, Inc., Bethesda MD, USA). Densitometry of 2D gel Western blots was assessed using Gel-Pro Analyzer software (Media Cybernetics, Inc.). Taqman real time PCR primers and probes are listed in FIG. 3. For *in situ* hybridization, the digoxigenin labeled probe for rat Angptl4 included bp 1 to 548 of the ORF.

To obtain samples for post heparin LPL activity, rats were injected intravenously with 10 units/100 gm weight of porcine heparin 15 minutes prior to euthanasia, and activity measured using an assay from Roar Biomedical, Inc (New York NY). Serum triglycerides were measured in the fasting state.

10 Injection of NTS into Angptl4 -/- mice

Angptl4 -/- mice were provided to Sander Kersten as a kind gift from Eli Lilly Corporation (Indianapolis IN USA). The study protocol was approved by the Animal Studies Committee at Wageningen University. Eleven week old male Angptl4 -/- or +/+ mice (n = 4 mice/group) were injected intravenously with 1.5 mg γ 2-NTS or normal sheep serum (Sigma Aldrich St. Louis MO USA), spot urine samples collected at 48 hours, mice euthanized at 72 hours, plasma collected for biochemical measurements, and kidneys preserved for histological analysis. Urine albumin was assessed by ELISA (Bethyl laboratories, Montgomery TX USA) and urine creatinine measured by mass spectrometry. To assess for foot process effacement, the mean width of foot processes was first measured in control treated Angptl4 +/+ mouse transmission electron micrographs (10 equally spaced readings/loop, 3 loops/glomerulus, 3 glomeruli/kidney, 3 kidneys/group). Effacement was described as an over 2.5 fold increase in mean width. Total and effaced foot processes were counted in NTS treated or control treated Angptl4 -/- mice.

Studies with archived human samples

Immunostaining of archived human kidney biopsies (n = 5 biopsies per condition) was conducted on samples obtained via IRB approved protocols at the Instituto Nacional de Cardiologia, Mexico City. Control kidney biopsies used for these studies were sex and age matched protocol pre-transplant biopsies. Archival human sera for 2D gel electrophoresis and Western blot (n = 4 samples / condition) were obtained from a previously published study (29).

Generation of transgenic rats

30 aP2-Angptl4 TG rats (adipose tissue specific) construct was generated in the vector that contained the 5.4 Kb mouse aP2 promoter construct (30) (purchased from Addgene Inc. Cambridge MA USA) by cloning the rat Angptl4 cDNA (including the signal sequence) with a C-terminal V5 tag at the NotI site just upstream of the polyA tail.

Transgenic rats were generated by microinjection of the digested DNA constructs into

fertilized Sprague Dawley eggs (conducted at University of Michigan), implantation into pseudopregnant host Sprague Dawley females, and the resulting offsprings were genotyped by routine PCR and TaqMan genomic DNA real time PCR strategy using construct specific and control genomic prolactin primer and probe combinations (FIG. 3). Three founder lines for adipose tissue specific expression were generated. Data from aP2-Angptl4 TG rat line 375 (3 copies), both stable over 4 generations, are presented. Urinary total protein was assessed using the Bradford method (Biorad laboratories, Hercules CA USA), and albuminuria by ELISA (Bethyl laboratories, Montgomery TX USA).

In vitro studies with GEnCs

For GEnC studies, cultured rat GEnCs (32) were grown to 75% confluence in 6 well plates (n = 3 wells / condition), washed twice with warm PBS, serum free RPMI containing 200 μ M H₂O₂, along with 600 μ g / well of control stable cell line supernatant, or Angptl4-HEK293 stable cell line supernatant, or supernatant from ManNAc treated Angptl4-HEK293 cell line. Wells were sampled at 24, 36 and 48 hours. LDH release was measured using the cytotoxicity detection kit (Roche Diagnostics, Mannheim Germany). OD 492 values were expressed as a ratio of readings from wells in which no H₂O₂ or stable cell line supernatant was added.

Statistical analysis

Analysis of difference in proteinuria or gene expression involving three or more groups was conducted by ANOVA with post analysis testing using GraphPad InStat software, Version 3.05. For comparison of two groups, the unpaired Students t test in Microsoft Excel 2003 was used.

4. Circulating Angiopoietin-like-4 links proteinuria with hypertriglyceridemia in nephrotic syndrome

A molecular basis for the relationship between proteinuria and hyperlipidemia (hypertriglyceridemia and hypercholesterolemia) in nephrotic syndrome is not known. In this study, it is shown that increased plasma levels of the glycoprotein Angptl4 link proteinuria with hypertriglyceridemia in nephrotic syndrome due to membranous nephropathy (MN), focal and segmental glomerulosclerosis (FSGS), and minimal change disease (MCD). Circulating Angptl4 had a near neutral isoelectric point (pI), and was mostly secreted from skeletal muscle, adipose tissue and heart after the establishment of moderate to severe proteinuria. In MCD, additional early podocyte expression of high pI Angptl4, that induces proteinuria, and neutral pI Angptl4 were previously shown. Using adipose tissue overexpressing Angptl4 transgenic rats (aP2-Angptl4) and recombinant Angptl4, it was shown that circulating Angptl4 reduced proteinuria by binding to glomerular endothelial α v β 5 integrin, while also inducing hypertriglyceridemia by blocking lipoprotein lipase (LPL) mediated triglyceride uptake. Hypertriglyceridemia was absent in nephrotic *Angptl4* $-/-$ mice.

Nephrotic *Angptl4* ^{-/-} and *Itgb5* ^{-/-} mice, and nephrotic rats injected with an anti-β5 integrin antibody had delayed recovery from peak proteinuria. Moreover, recombinant human Angptl4 with mutations at the LPL binding site could reduce proteinuria without affecting plasma triglyceride levels. In summary, circulating Angptl4 reduces proteinuria while also inducing hypertriglyceridemia, and is mostly produced from peripheral organs as a systemic response to nephrotic range proteinuria.

Background

Molecular pathways that link proteinuria with hyperlipidemia, two key hallmarks of nephrotic syndrome, are not known. Hyperlipidemia has two components, hypercholesterolemia and hypertriglyceridemia¹. In the past, hypercholesterolemia has been attributed to increased hepatic synthesis of lipoproteins in response to proteinuria and hypoalbuminemia (2). However, the precise molecular link between proteinuria and increased hepatic lipoprotein synthesis remains unknown. The development of hypertriglyceridemia has received much less attention. A major determinant of plasma triglyceride levels is the activity of endothelium bound lipoprotein lipase (LPL), that regulates tissue uptake of triglycerides from the circulation (3). Mice that lack LPL develop very high triglyceride levels and die soon after birth (4). Prior studies show that the activity and expression of LPL protein, but not mRNA, are reduced in nephrotic syndrome (5). The molecular basis of this reduction in LPL protein activity and expression, or its relationship to proteinuria in nephrotic syndrome has not been determined.

A recent study from our laboratory showed increased expression of Angptl4 in podocytes and in the circulation in minimal change disease (MCD) (6, 7). To study the biological role of podocyte-secreted Angptl4, two types of transgenic rat models were generated. NPHS2-Angptl4 transgenic rats, that selectively overexpress Angptl4 within the glomerulus from podocytes, develop massive albuminuria without increasing circulating Angptl4 levels. By contrast, aP2-Angptl4 transgenic rats, that selectively overproduce and secrete Angptl4 from adipose tissue, develop high circulating Angptl4 levels, but are not proteinuric. Further studies showed that podocytes secrete two distinct forms of Angptl4 in nephrotic syndrome: a high pI form that is hyposialylated, and neutral pI form that is sialylated. Treatment with the sialic acid precursor N-acetyl-D-mannosamine (ManNAc) converts high pI Angptl4 to neutral pI Angptl4 in vivo, and significantly reduces albuminuria / proteinuria. By contrast, circulating Angptl4 in normal and nephrotic rats and humans is comprised almost entirely of sialylated neutral pI Angptl4.

Angptl4 is believed to block LPL activity (8) by inactivating LPL, which reduces triglyceride update and results in hypertriglyceridemia (9). Population based sequencing studies of the human ANGPTL4 gene revealed low plasma triglyceride levels in about 3% of the European – American

population that has an E40K variant (10). Subsequent studies showed that recombinant Angptl4 with the E40K variant is unable to inhibit LPL activity *in vitro* (11). Angptl4 in circulation tends to cleave into an N-terminal fragment (contains LPL inhibiting region and a coiled coil domain, forms oligomers) and a C-terminal fragment (contains fibrinogen-like domain, remains monomeric), and

5 mutating the Angptl4 cleavage region between amino acids 161 and 164 improves the stability of the full length protein (11). We utilized these properties of Angptl4 to develop mutants of potential therapeutic significance.

In the present study, the biological role of circulating Angptl4 in nephrotic syndrome was investigated. We noted elevated levels of Angptl4 and triglycerides, and reduced LPL activity in MN,

10 FSGS and MCD. In addition, Angptl4 $-/-$ mice with nephrotic syndrome did not develop hypertriglyceridemia. In rat models of nephrotic syndrome, elevated circulating Angptl4 originated primarily from skeletal muscle, adipose tissue and heart after severe proteinuria had developed. In experimental MCD, some circulating Angptl4 also originated from podocytes. Elevated circulating Angptl4, whether by transgenic expression or injection of recombinant protein, increased

15 triglyceride levels and reduced LPL activity, but also reduced proteinuria in nephrotic rodents by binding to glomerular endothelial $\alpha\beta 5$ integrin. Absence of $\beta 5$ integrin, or its *in vivo* blockage using specific antibodies, or absence of circulating Angptl4, all slowed recovery from peak proteinuria. Angptl4 is therefore the first direct molecular link between proteinuria and hypertriglyceridemia. It is likely that peripheral Angptl4 secretion is stimulated primarily to help reduce ongoing proteinuria

20 in nephrotic syndrome, but also ends up binding to LPL and inducing hypertriglyceridemia.

Results

Increased circulating Angptl4 levels determine the development of hypertriglyceridemia in nephrotic syndrome:

When compared with normal healthy volunteers, significantly elevated fasting plasma

25 Angptl4 levels were noted by ELISA in untreated patients with nephrotic syndrome due to MCD, focal and segmental glomerulosclerosis (FSGS), non-HIV collapsing glomerulopathy (CG), and membranous nephropathy (MN) (Fig. 6(a), Fig. 12). To determine whether elevated plasma Angptl4 levels can be correlated with hypertriglyceridemia and LPL activity in nephrotic syndrome, we studied plasma Angptl4, triglycerides and post-heparin LPL activity in passive Heymann nephritis

30 (PHN, a model of MN) (12, 13), Buffalo Mna rats, that spontaneously develop FSGS (14,15), and puromycin aminonucleoside nephrosis (PAN, a model of MCD)(12) (Fig. 6(b)). Fasting plasma Angptl4 were elevated in these models after, but not before, they developed moderate to severe proteinuria. In PHN and Buffalo Mna rats, significant hypertriglyceridemia was noted when plasma Angptl4 levels were elevated and plasma LPL activity was reduced (Fig. 6(c)-(h)). In PAN,

hypertriglyceridemia was present throughout proteinuria, persisted after proteinuria had normalized, and correlated well with decline in LPL activity (Fig. 6(i)-(k)). Overexpression of Angptl4 from adipose tissue in aP2-Angptl4 transgenic rats, that develop increased circulating Angptl4 levels but no proteinuria⁶, also induced hypertriglyceridemia and reduced LPL activity (Fig. 6(l) and (m)). By contrast, 3 month old NPHS2-Angptl4 transgenic rats, in which Angptl4 overexpressed from podocytes causes proteinuria but no leakage into the circulation, did not develop elevated triglyceride levels or reduced LPL activity. These overexpression studies suggest that entry of Angptl4 into the circulation is required for the development of hypertriglyceridemia. To study the relative importance of Angptl4 in the development of hypertriglyceridemia in nephrotic syndrome, severe heterologous phase complement- and leukocyte-independent proteinuria was induced in Angptl4 – /– and +/+ mice using γ 2-nephrotoxic serum (NTS) (Fig. 6(n)). When compared with *Angptl4* +/+ mice, hypertriglyceridemia was absent in *Angptl4* – /– mice injected with NTS, despite these mice having significant proteinuria (Fig 13(a)). These studies show that circulating Angptl4 is a critical mediator of hypertriglyceridemia in nephrotic syndrome.

15 Origin of elevated circulating Angptl4 in nephrotic syndrome

To determine the origin of increased circulating Angptl4, we conducted multi-organ Angptl4 mRNA expression profiles in rat models of nephrotic syndrome. On PHN Day 9 (Fig. 7(a)), corresponding with elevated circulating Angptl4 levels (Fig. 6(b)) and heavy proteinuria (Fig. 6(c)), prominent upregulation was noted in skeletal muscle, white adipose tissue (WAT), brown adipose tissue (BAT), and heart. Transient mild upregulation noted in glomeruli and liver earlier on Day 5 had subsided. In 4.5 month old Buffalo Mna rats (Fig. 7(b)) with elevated Angptl4 levels (Fig. 6(b)) and moderate to severe proteinuria (Fig. 6(f)), prominent upregulation was noted in the heart. Glomerular upregulation of Angptl4 is not seen in this model. In PAN, a self-limiting acute model of nephrotic syndrome, elevated circulating Angptl4 levels occur throughout disease (Fig. 6(b)). Prominent upregulation of glomerular / podocyte Angptl4 during the crescendo phase of proteinuria (Days 6 and 10) (Fig. 7(c)) was also previously described (6). This “glomerular” phase of Angptl4 upregulation (Day 6 and 10) was followed by a “peripheral” phase (Days 14 and 21) during which glomerular upregulation was absent, and prominent upregulation was noted in skeletal muscle, WAT and BAT. Since monogenic overexpression of Angptl4 from podocytes in NPHS2-Angptl4 transgenic rats does not increase circulating Angptl4 levels, 2-dimensional gel western blot studies of rat plasma were performed during the “glomerular” phase after mild PAN was induced in these rats (Figs. 7(d), 7(e), 13(b)). Significantly higher circulating levels of Angptl4 were noted in NPHS2-Angptl4 transgenic PAN rats than wild type PAN rats. This circulating protein was reactive with the anti-V5 antibody (Fig. 7(f)), thereby confirming its podocyte origin, since the transgene expressed protein is

V5-tagged at its C-terminal end. In keeping with increased circulating transgene overexpressed Angptl4, aP2-Angptl4 and NPHS2-Angptl4 transgenic rats with PAN had higher plasma triglycerides and lower LPL activity than wild type PAN rats (Fig. 7(g) and (h)).

Urinary loss of LPL and Angptl4 in nephrotic syndrome

5 Unlike normal Sprague Dawley rats, nephrotic rats lost Angptl4 in the urine (Fig. 8(a)), thereby suggesting that circulating Angptl4 levels underestimate total Angptl4 production in nephrotic states. Whereas normal rats degrade Angptl4 released LPL by hepatic uptake from the circulation (16), additional loss was noted in the urine in nephrotic syndrome (Fig. 8(b), control serum blot in Fig. 13(c)). Reactivity with 5D2 (Fig. 8(c), control serum blot in Supplementary Fig. 10 7(d)), a monoclonal antibody that selectively binds active LPL, suggests that active LPL is also lost in urine during heavy proteinuria. Also, nephrotic PAN rats were unable to increase LPL mRNA expression until proteinuria was greatly diminished (Fig. 8(d)). By comparison, non-proteinuric aP2-Angptl4 transgenic rats could upregulate LPL expression in skeletal muscle and heart in response to elevated Angptl4 levels (Fig. 8(e)). These factors may contribute towards the maintenance of 15 Angptl4-mediated hypertriglyceridemia in nephrotic syndrome.

Increased circulating Angptl4 reduces proteinuria

Induction of PAN in aP2-Angptl4 transgenic and wild type Sprague Dawley rats revealed significantly lower proteinuria in transgenic rats (Fig. 9(a)), suggesting that circulating Angptl4 has an anti-proteinuric effect. To test whether this anti-proteinuric effect was specifically induced by 20 circulating Angptl4, previously characterized rabbit anti-rat Angptl4 antibodies (6) were injected into wild type PAN rats after the onset of proteinuria to partially deplete circulating Angptl4 levels, and noted increased proteinuria (Fig. 9(b)). Next, recombinant sialylated rat Angptl4 was injected intravenously into Buffalo Mna rats (Fig. 9(c)), and rats with anti-Thy1.1 nephritis, a model of mesangial injury (Fig. 9(d)). In both cases, significant reduction of proteinuria was noted.

Human Angptl4 mutants with reduced LPL inactivation reduce proteinuria

25 In order to dissociate the LPL mediated effects of Angptl4 on triglyceride uptake from its effects on proteinuria, pcDNA3.1 V5 His B constructs of human Angptl4 with mutations at two sites were generated (Fig. 10(a)). One set of mutations were made at or near a site known to be important for binding to LPL (amino acids 40 or 39). Another set of mutations were made in a region 30 known to be involved in the cleavage of full length Angptl4 (amino acids 161 to 164).

Next, HEK 293 based stable cell lines were developed for these mutant and wild type plasmids, and recombinant protein containing supernatant harvested in serum free conditions. To ensure adequate sialylation of Angptl4, ManNAc (*N*-acetyl-*D*-mannosamine) was added to the culture media. Wild type and recombinant Angptl4 were then assessed by Western blot using anti-

V5 antibody, and migration of the mutant proteins at the appropriate size and reduced cleavage were noted (Fig. 10(b)). After single intravenous injection of equal amounts of human recombinant Angptl4 into proteinuric Buffalo Mna rats, higher peak levels were noted for the mutant proteins (Fig. 10(c)). Significant reduction of proteinuria was noted for 2 weeks after a single injection in wild
 5 type and mutant proteins (mean $\pm$ SE of nadir as a percentage of baseline: wild type 8525, 53.8 ± 6.3 ; mutant 8501, 35.9 ± 12.1 ; mutant 8515, 41.2 ± 7.2) (Fig. 10(d)). Plasma triglyceride levels were significantly higher compared to baseline in wild type, but not in mutant, Angptl4 injected rats at 3 and 6 hours after injection (Fig. 10(e)). Triglyceride levels were significantly lower in mutant protein than in wild type protein injected rats at 6 hours. Fasting triglyceride levels between 12 hours and
 10 Day 17 were indistinguishable between wild type and mutant Angptl4 injected rats, and were similar to controls (Fig. 13(e)).

Circulating Angptl4 reduces proteinuria by binding to glomerular endothelial $\alpha\beta 5$ integrin:

Confocal imaging of aP2-Angptl4 TG rat kidney using anti-V5 antibodies showed that adipose tissue secreted Angptl4-V5 colocalized with the glomerular endothelium (Fig. 11(a)). Using
 15 immunogold EM, this Angptl4-V5 was noted on the glomerular endothelial surface, mostly in the region of the endothelial cell - glomerular basement membrane interface (Fig. 11(b)). Using recombinant rat Angptl4 secreted from stable cell lines, we showed that sialylated Angptl4 protein, which mimics circulating Angptl4 in nephrotic states, protected cultured rat glomerular endothelial cells (GEnCs) from oxidative injury, whereas hyposialylated Angptl4, a pro-proteinuric form that
 20 comprises about half of podocyte-secreted Angptl4 in PAN and NPHS2-Angptl4 TG rats, increases the effects of oxidative injury (Fig. 11(c)).

Since Angptl4 was recently shown to bind $\beta 5$ integrin (17), it was determined whether the protective effects of circulating Angptl4 on proteinuria were mediated via binding to $\alpha\beta 5$ integrin present in glomerular endothelium. This protein : protein interaction was confirmed using
 25 recombinant rat Angptl4 and plates coated with purified human $\alpha\beta 5$ integrin, and noted strong dose dependent binding (R^2 0.996) (Fig. 11(d)). Induction of nephrotic syndrome using 2-NTS in $\beta 5$ integrin knockout (*Itgb5* $-/-$) and wild type (*Itgb5* $+/+$) mice revealed much higher levels of proteinuria in the knockout mice during the recovery phase (Days 5 and 7) (Fig. 11(e)), which corresponds with the peripheral phase of circulating Angptl4 production from skeletal muscle and
 30 adipose tissue in this model (Supplementary Fig. 14(a) and (b)). This suggests the decline from peak proteinuria (beyond Day 3) was influenced by the presence of circulating proteins like Angptl4 that exert anti-proteinuric effects by binding $\alpha\beta 5$ integrin. To block the $\alpha\beta 5$ integrin – Angptl4 interaction, an antibody against the extracellular part of $\beta 5$ integrin (anti- $\beta 5$ integrin antibodies) or preimmune serum was injected into wild type (Sprague Dawley) rats (Fig. 11(f), Supplementary Fig.

14(c) and (d)) and aP2-Angptl4 transgenic rats (Fig. 11(g)) during recovery from peak proteinuria (beyond Day 10, corresponds to peripheral phase of circulating Angptl4 production) in PAN. A significant delay in recovery was noted in both models. Finally, nephrotic syndrome was induced in *Angptl4* $+/+$ and *Angptl4* $-/-$ mice and noted a significant delay in recovery from peak proteinuria (Day 7) during the peripheral phase of circulating Angptl4 production, which is absent in *Angptl4* $-/-$ mice (Fig. 11(h)). The lower level of proteinuria in *Angptl4* $-/-$ mice during the glomerular phase is consistent with our previously published description of podocyte secreted hyposialylated Angptl4 (6) as being one of several causes of proteinuria in this model.

Additional Mutants

Four addition mutant proteins were studied: 8496, 8506, 8511, and 8520. Each has at least one amino acid substitution at positions 39, 40, or 161-164 as shown in Fig. 17. HEK 293 based stable cell lines were developed and cultured as described above to express mutant protein. All four mutant proteins were tagged with V5, with anti-V5 antibody, then assessed by Western blot using anti-V5 antibody, and migration of the mutant proteins at the appropriate size and reduced cleavage were noted. Results show that the amount of cleaved protein is significantly reduced in the mutants compared to the wild type via Western blot (Fig. 18). After single intravenous injection of equal amounts of mutant 8511 protein into proteinuric Buffalo Mna rats, higher peak levels were noted for the mutant 8511 (Fig. 19). Red arrows indicate single time point when recombinant protein was injected. Two mutant human Angptl4 proteins (15 μ g) were injected into Zucker Diabetic Fatty rats (a model of diabetic nephropathy and diabetic kidney disease, n = 4 rats / group), after which increased circulating levels of the mutant proteins (Fig. 20) were noted, along with reduction in proteinuria (Fig. 21), but without significant increase in plasma triglyceride levels (Fig. 22). * $P < 0.05$, ** $P < 0.01$. # $P < 0.05$. All * values are relative to baseline Day 0 values.

Discussion

Without wishing to be bound by any given hypothetical model, this study shows that circulating Angptl4 is a key molecular mediator in nephrotic syndrome (Fig. 15). It describes, for the first time, how two key components of this syndrome, proteinuria and hypertriglyceridemia, are linked at a molecular level. The glomerulus is central to the development of proteinuria by mechanisms that vary among different diseases. Using animal models of MN and FSGS, two conditions commonly associated with sub-acute onset of proteinuria and edema, it was demonstrated that peripheral organs, especially skeletal muscle, adipose tissue and heart, respond to increasing proteinuria by upregulating Angptl4 expression. Circulating Angptl4, derived at this stage exclusively from these peripheral organs, has two potent effects. First, it binds to $\alpha v \beta 5$ integrin in the glomerular endothelium and reduces proteinuria, thereby suggesting that the primary

purpose of increasing circulating Angptl4 is an attempt to reduce proteinuria. Second, and perhaps an unintended consequence of increasing circulating Angptl4 levels, is its binding to LPL (its physiological function), reduced triglyceride uptake, and hypertriglyceridemia. It is important to clarify that the link between proteinuria and hypertriglyceridemia discussed here does not relate to the primary pathogenesis of proteinuria, but to its reduction / modification by circulating Angptl4. The lag between the onset of proteinuria and the development of hypertriglyceridemia in human nephrotic syndrome is also explained by peripheral production of Angptl4, since it requires moderate to severe proteinuria (at least 3.5 grams / 24 hours in humans) to develop this response. Early, mild proteinuria in these two animal models was not associated with a peripheral organ Angptl4 response, elevated plasma Angptl4 or increased triglyceride levels. Since increased Angptl4 secretion from peripheral tissues is also a physiological response to fasting (18), it is possible that increased peripheral production of Angptl4 is part of a fasting-like response being used by the body to curb excessive urinary protein loss in nephrotic syndrome.

Additional interesting lessons are learnt from PAN rats, a model of MCD, in which onset of edema and proteinuria is acute. Prior studies (6) show that podocytes in MCD produce two distinct forms of Angptl4: a high pl, hyposialylated form that induces proteinuria, and a neutral pl sialylated form identical to circulating Angptl4. Among other factors, inadequate sialic acid substrate plays a major role in the production of high pl Angptl4 by podocytes in MCD, and conversion of high pl Angptl4 to neutral pl Angptl4 in vivo using the sialic acid precursor N-acetyl-D-mannosamine reduces proteinuria. Unlike FSGS and MN, glomerular upregulation of hyposialylated Angptl4 plays a key role in the development of proteinuria in this disease. Circulating sialylated Angptl4 remains elevated throughout the duration of the PAN model, with the source being the glomerulus in the initial part (i.e. glomerular phase), and skeletal muscle and adipose tissue (i.e. peripheral phase) in the later stages. An in vitro study in this paper show that high pl Angptl4 increases, and neutral pl Angptl4 reduces endothelial injury in the setting of oxidative stress. Further studies on high pl Angptl4 are beyond the scope of this paper. All recombinant Angptl4 used for in vivo studies in this paper was the neutral pl sialylated form. As with rat models of MN and FSGS, PAN rats also develop a peripheral phase of Angptl4 upregulation that contributes significantly to the decline of proteinuria after Day 10.

A significant mediator to reduction in proteinuria in all models is the Angptl4 – $\alpha\beta 5$ integrin interaction in glomerular endothelium, since absence of $\beta 5$ integrin or Angptl4 in knockout mice, or blockage of this interaction using antibodies directed against the extracellular part of $\beta 5$ integrin reduces the rate of decline of proteinuria. Another effect of the peripheral phase of Angptl4 production in PAN is the persistence of mild hypertriglyceridemia (Day 21) even after proteinuria has

subsided. Similar residual hypertriglyceridemia has been previously documented in children with MCD after they go into remission (19). It is possible that circulating Angptl4 may interact with other glomerular cell surface molecules as well to exert its protective effects. There were two reasons for pursuing binding of Angptl4 to glomerular endothelial $\alpha\beta 5$ integrin. First, confocal imaging and immunogold electron microscopy showed that Angptl4-V5 secreted from adipose tissue in aP2-Angptl4 transgenic rats binds specifically to endothelial cells in the glomerulus. Second, $\alpha\beta 5$ is the only integrin expressed on glomerular endothelial cells in vivo shown to interact with Angptl4. The other major glomerular endothelial integrin, $\alpha\beta 3$, does not interact with Angptl4 (17) (confirmed by us, data not shown). The precise mechanism by which Angptl4 binding to endothelial $\alpha\beta 5$ integrin reduces proteinuria will be explored in the future. It is possible that putative endothelial – podocyte feedback loops are affected.

Another interesting observation is that entry of Angptl4 into the circulation after monogenic overexpression is organ dependent. Similar to a heart specific Angptl4 overexpressing transgenic mouse developed in the past (20), monogenic over expression of Angptl4 in podocytes in NPHS2-Angptl4 rats does not automatically allow entry into the circulation. By contrast, overexpression in adipose tissue (aP2-Angptl4 transgenic rats (6), aP2-Angptl4 transgenic mice (18)) reliably increases circulating Angptl4 levels. The entry of podocyte secreted Angptl4 into the circulation, as noted in the Sprague Dawley rat PAN glomerular phase and in NPHS2-Angptl4 transgenic rats with PAN, likely requires the activity of other as yet unidentified proteins produced in the glomerulus. This also fits in well with human glomerular disease, in which expression of multiple genes and proteins is simultaneously affected. Therefore, the systemic availability and effects of circulating Angptl4 is likely to be affected by other genes / proteins altered in multiple organs as part of the disease process, and also by urinary loss of Angptl4 and LPL in the nephrotic state.

The anti-proteinuric effects of circulating Angptl4 may already play a partial role in the efficacy of glucocorticoids used to treat many different forms of glomerular disease. The effects of glucocorticoids on Angptl4 expression are organ dependent. Whereas they reduce Angptl4 expression in podocytes in MCD (6), they have been shown to increase adipose tissue expression of Angptl4 in mice (21). Future studies could explore whether glucocorticoids induce secretion of sufficient amounts of Angptl4 from adipose tissue into the circulation, whether this effect is dose dependent *in vivo*, and whether this also happens in nephrotic syndrome.

Other soluble proteins have been implicated in the pathogenesis of human glomerular disease. Vascular endothelial growth factor, secreted from podocytes and also present in the circulation, is shown to be involved in the development of human thrombotic microangiopathy, and exerts its biological effects via specific receptors expressed on the endothelial and podocyte surface

(22). Soluble fms-like tyrosine kinase 1 (23) and soluble endoglin (24), secreted in excessive amounts from the placenta in pre-eclampsia, are involved in the pathogenesis of glomeruloendotheliosis. These proteins, however, are implicated in disease pathogenesis and are not a systemic response to disease. The soluble urokinase receptor suPAR was recently shown to have pro-proteinuric effects exerted primarily by binding to podocyte $\alpha\beta 3$ integrin (25). A common denominator between anti-proteinuric Angptl4 and pro-proteinuric suPAR is the interaction of both circulating proteins with glomerular integrins. A follow up study by the same group shows that suPAR levels are also increased in FSGS patients with mutations in the *NPHS2* gene (26). This would suggest that in these patients with *NPHS2* mutations, the elevation of suPAR is a systemic response to glomerular disease.

10 In such cases, SuPAR joins a class of circulating proteins exemplified by Angptl4 that are increased in response to glomerular injury or proteinuria, and have potent effects that influence the course of the underlying glomerular disease. This list will grow in the near future once putative circulating proteins that influence the pathogenesis of non-HIV collapsing glomerulopathy (27, 28) are identified.

15 Lastly, mutant forms of human Angptl4 are able to reduce proteinuria very significantly (mean peak reduction around 60%) without significantly affecting plasma triglyceride levels, and are effective for at least two weeks after a single intravenous injection. These recombinant proteins hold promise for further development as therapeutic agents for human glomerular disease. In summary, circulating Angptl4 is an important biological mediator of nephrotic syndrome, and represents a

20 critical link between proteinuria and hypertriglyceridemia.

Methods for Working Example 4

ELISA for human and rodent Angptl4

A sandwich ELISA to measure human Angptl4 from patient and control plasma samples was purchased from R&D Systems (Minneapolis MN, USA). The standard curve was calibrated between

25 1.25 ng/ml and 40 ng/ml, and had a R^2 value of 0.98. (Supplementary Fig. 16(a))

To measure rat and mouse Angptl4 in plasma, a new ELISA assay was developed. A sheep anti-rat Angptl4 antibody (5006B) was raised against amino acids 22 to 101, and characterized for specificity by Western blot using a previously published rabbit anti-rat Angptl4 antibody (6) as positive control. Activity was also absorbed out using recombinant Angptl4 and loss of reactivity by

30 Western blot and immunofluorescence was documented. The assay was standardized using concentrated supernatant from a previously published HEK293 based stable cell line that secretes recombinant rat Angptl4. Wells were coated with between 0.1 and 0.5 mg of concentrated supernatant. After blocking and washes, 10 μ g of sheep anti-rat Angptl4 antibody was added, followed by washes, 16 ng / well of donkey anti sheep Ig HRP (Jackson laboratories), washes, and

TMB system reagents, and measurement at OD 450 nm on an ELISA plate reader. A standard curve with a linear relationship with a R^2 of 0.998 was obtained (Fig. 16(b)). For analysis of rodent plasma, wells were coated with 50 μ l of plasma in duplicate, followed by steps as detailed above. Readings from blank control wells, that contained all reagents minus the study sample, were obtained and subtracted from readings of the study samples. A minimum of 4 samples were measured for each time point.

Human plasma samples used for ELISA assay were obtained from IRB approved studies conducted at UAB (PI Chugh), Instituto Nacional De Cardiologia, Mexico City (PI Avila-Casado), and from previously published studies (6).

10 Animal studies

The generation and characterization of NPHS2-Angptl4 and aP2-Angptl4 transgenic rats was previously published (6). Buffalo Mna rats were obtained via MTA from Dr. Masao Mitsuyama at Kyoto University, Kyoto Japan. Unless otherwise stated, all comparisons for Buffalo Mna rats were made with age and sex matched Sprague Dawley and Wistar rats. Since results were similar, only data from comparisons with Sprague Dawley rats is presented. *Itgb5* $-/-$ and control 129S1/SvImJ mice were purchased from Jackson Laboratories (Bar Harbor ME USA). *Angptl4* $-/-$ mice were provided to Sander Kersten by Eli Lilly Corporation. All studies with *Angptl4* $-/-$ mice were approved by the Animal Ethics Committee at Wageningen University. All other animal studies were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

20 Induction of single intravenous dose PAN (n = 4 rats / group), PHN (n = 4 rats / group) and anti-Thy1.1 nephritis (n = 4 rats / group) was previously described (12, 13). For full dose PAN, puromycin aminonucleoside (Sigma Chemical Company, St. Louis MO USA) 15 mg/100 gram was used. For mild PAN, dose was reduced to 7.5 mg/100 grams. Induction of complement- and leukocyte-independent nephrotic syndrome (n = 4 mice / group) using the γ 2 fraction of sheep anti-rat nephrotoxic serum (NTS, kind gift from David Salant, Boston Medical Center) was previously described (29). For animal studies in which rabbit anti-rat Angptl4 antibody (6) as injected into PAN rats (n = 3 rats / group), 500 μ l of antibody or preimmune serum was injected in each dose. Depletion of circulating Angptl4 by the antibody was confirmed by western blot. The volume of anti- β 5 integrin antibodies or pre-immune serum injected per dose in rat PAN studies was as follows:

30 Sprague Dawley rat PAN (250 μ l); aP2-Angptl4 transgenic rat PAN (500 μ l).

For multiorgan gene expression studies, organ samples were snap frozen in liquid nitrogen immediately after euthanasia (3 rats or mice/organ sample, pooled). White adipose tissue was obtained from the abdomen, brown adipose tissue from the interscapular area, skeletal muscle from the thigh, liver frozen intact or samples from both left and right lobe, heart frozen intact, and rat

kidneys frozen and used subsequently for glomerular isolation. In mouse experiments, kidneys were perfused through the heart immediately after euthanasia using dynabeads, and then used for glomerular isolation. Twelve cDNA templates were generated from each pooled organ, and gene expression assessed by Taqman real time PCR.

5 Injection of recombinant rat and human Angptl4

Harvesting of sialylated rat Angptl4 from a HEK293 cell based stable cell line was previously described (6). Concentrated HEK293 Angptl4 or empty pcDNA 3.1 V5 His vector stable cell line supernatant (1.8 mg, derived from approximately 200 ml of media) containing rat Angptl4 was injected per dose in the Buffalo Mna rat (n = 4 rats / group) and the Thy1.1 rat (n = 4 rats / group) studies. For studies in which recombinant human wild type Angptl4, mutant Angptl4 and control protein were injected into Buffalo Mna rats (n = 3 rats / group), 55 µg of recombinant protein (quantified by ELISA) in concentrated supernatant, or equal amounts of control stable cell line supernatant (equalized by protein assay) was used per dose.

Post heparin LPL activity

15 Rats were injected intravenously with 10 units / 100 g weight of porcine heparin 15 minutes prior to euthanasia, and activity measured using an assay from Roar Biomedical, Inc (New York NY USA) (30). Serum triglycerides were measured in the fasting state using an autoanalyzer (some studies) or a kit from Cayman Chemical Company (Ann Arbor MI USA).

The following techniques have been previously described: 18 hour urine collection in metabolic cages, measurement of proteinuria, mouse urine albumin and creatinine, 2D gel electrophoresis and Western blot, confocal imaging, immunogold electron microscopy, extraction of total RNA, generation of cDNA templates (2 µg total RNA / template), real time PCR (6, 12, 13, 31). In real time PCR studies, a three-fold change in mRNA expression was taken as significant and has been validated by us in prior publications (13, 31). Western blot for LPL was conducted using goat anti-LPL antibody (Santa Cruz Biotechnology, Santa Cruz CA USA), and a 5D2 monoclonal antibody (gift from John Brunzell, University of Washington) that specifically identifies active dimeric LPL (32). Densitometry of 2D gel Western blots was conducted using Image Quant TL 7.0 software (GE Healthcare, Waukesha WI USA). Mouse anti-PECAM1 antibody was purchased from BD Pharmingen (San Diego CA USA). All secondary antibodies used were purchased from Jackson ImmunoResearch laboratories (West Grove PA USA), and had minimal background reactivity to non-target species.

Development of human Angptl4 mutant constructs and stable cell lines

A human Angptl4 clone was mutated using PCR based mutagenesis. Wild type and mutant human Angptl4 clones in pcDNA 3.1 V5 His vector were used to develop HEK 293 based stable cell lines as previously described (6). When used for harvesting protein, the serum free DMEM included

25 mM N-acetyl-D-mannosamine (ManNAc), a precursor of sialic acid, to ensure adequate sialylation of recombinant proteins secreted into the media. Proteins were harvested and supernatant concentrated as previously described (6). Recombinant human Angptl4 was quantified using ELISA.

Endothelial cell study

5 Cultured rat GEnCs grown in serum free conditions for 24 hours were subjected to H₂O₂ (200 μ M) induced stress, and co-incubated with equal amounts of concentrated supernatant from Angptl4-HEK293 or control-HEK293 stable cell lines. LDH levels were measured at 24, 48, and 72 hours in the supernatant as a measure of cell injury using a cytotoxicity kit (Roche Applied Science Indianapolis IN USA).

α v β 5 integrin plate assay

10 96 well plates were coated with 5 ng human purified α v β 5 integrin / well, blocked with TBST with 1% BSA, followed by incubation with increasing amounts of concentrated supernatant from stable cell lines that secrete rat Angptl4-V5. The V5 tag was detected using an anti-V5 HRP antibody (Life Technologies, Grand Island NY USA, 1:2500), the reaction developed using the TMB peroxidase
15 substrate and solution (KPL, Inc., Gaithersburg MD USA), and read on a Labsystems Multiscan MCC340 (Thermo Fisher Scientific, Waltham MA USA) at 450 nm.

Generation and characterization of anti- β 5 integrin antibodies

Fusion proteins were generated against parts of the extracellular segment of human β 5 integrin to generate two polyclonal antibodies in rabbits (antibody 8472A, amino acids 35-460,
20 includes integrin beta domain; antibody 8472B, amino acids 461 to 719, includes integrin beta tail). Both antibodies were tested for specificity by Western blot before and after absorbing out reactivity to recombinant human β 5 integrin. Pilot studies were conducted by inducing PAN in Sprague Dawley rats (n = 3 rats / group), and injecting two doses each antibody intravenously during the recovery phase to assess for in vivo blockage of β 5 integrin (i. e. slower recovery of proteinuria). Since the
25 efficacy of 8472A was several times higher than 8472B, 8472A (Fig. 16(c)) was used for further studies (n = 4 rats / group). Injection of this antibody intravenously, followed by confocal imaging of glomeruli, showed localization to the endothelium in glomeruli (Fig. 16(d)).

Statistical analysis

30 Analysis of difference between two groups was conducted using the unpaired Students t-test in Microsoft Excel 2010. For three or more groups, ANOVA with post analysis testing using GraphPad InStat software, Version 3.10 was used.

References (except for Working Example 4)

1. Falk R, Jennette C, Nachman PH. Primary glomerular disease. In The Kidney, Brenner BM, editor, 6th edition, 1263-1349 (2000).

2. Gutman, A. & Shafrir, E. Adipose tissue in experimental nephrotic syndrome. *Am. J. Physiol.* **205**, 702-706 (1963).
3. Vaziri, N.D. Molecular mechanisms of lipid disorders in nephrotic syndrome. *Kidney Int.* **63**, 1964-1976 (2003).
- 5 4. Shearer, G.C. & Kaysen GA. Endothelial bound lipoprotein lipase (LpL) depletion in hypoalbuminemia results from decreased endothelial binding, not decreased secretion. *Kidney Int.* **70**, 647-653 (2006).
5. Reaven, E.P., Kolterman, O.G. & Reaven, G.M. Ultrastructural and physiological evidence for corticosteroid-induced alterations in hepatic production of very low density lipoprotein particles. *J. Lipid Res.* **15**, 74-83 (1974).
- 10 6. Tsukamoto, Y., Kokubo, T., Horii, A., Moriya, R. & Kobayashi, Y. Lipoprotein derangement during steroid treatment in minimal-change nephrotic syndrome. *Nephron* **73**, 606-612 (1996).
7. Liu, G., Clement, L., Kanwar, Y.S., Avila-Casado, C. & Chugh, S.S. ZHX proteins regulate podocyte gene expression during the development of nephrotic syndrome. *J. Biol. Chem.* **281**, 39681-39692 (2006).
- 15 8. Yoon, J.C. et al. Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. *Mol. Cell. Biol.* **20**, 5343-5349 (2000).
9. Kersten, S. et al. Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. *J. Biol. Chem.* **275**, 28488-28493 (2000).
- 20 10. Kim, I. et al. Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis. *Biochem. J.* **346**, 603-610 (2000).
11. Yoshida, K., Shimizugawa, T., Ono, M. & Furukawa, H. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J. Lipid Res.* **43**, 1770-1772 (2002).
- 25 12. Ge, H., et al. Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4. *J. Biol. Chem.* **279**, 2038-2045 (2004).
13. Ge, H., Yang, G., Yu, X., Pourbahrami, T. & Li, C. Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4. *J. Lipid Res.* **45**, 2071-2079 (2004).
- 30 14. Romeo, S., et al. Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. *Nat. Genet.* **39**, 513-516 (2007).
15. Romeo, S. et al. Rare loss-of-function mutations in ANGPTL family members contribute to plasma triglyceride levels in humans. *J. Clin. Invest.* **119**:70-79 (2009).
16. Eremina, V., et al. VEGF inhibition and renal thrombotic microangiopathy. *N. Engl. J. Med.*

- 358**, 1129-1136 (2008).
17. Davis, B., et al. Podocyte-specific expression of angiopoietin-2 causes proteinuria and apoptosis of glomerular endothelia. *J. Am. Soc. Nephrol.* **18**, 2320-2329 (2007).
 18. Avila-Casado, C., et al. Proteinuria in rats induced by serum from patients with collapsing
5 glomerulopathy. *Kidney Int.* **66**, 133-143 (2004).
 19. Mandard, S., et al. The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. *J. Biol. Chem.* **281**:934-944 (2006).
 20. Clement, L., et al. Early changes in gene expression that influence the course of primary
10 glomerular disease. *Kidney Int.* **72**, 337-347 (2007).
 21. Cazes, A. et al. Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. *Circ. Res.* **99**, 1207-1215 (2006).
 22. Malicdan, M.C., Noguchi, S., Hayashi, Y.K., Nonaka, I. & Nishino, I. Prophylactic treatment with sialic acid metabolites precludes the development of the myopathic phenotype in the DMRV-
15 hIBM mouse model. *Nat. Med.* **15**, 690-695 (2009).
 23. Galeano, B. et al. Mutation in the key enzyme of sialic acid biosynthesis causes severe glomerular proteinuria and is rescued by N-acetylmannosamine. *J. Clin. Invest.* **117**, 1585-1594 (2007).
 24. Ruge, T. et al. Lipoprotein lipase in the kidney: activity varies widely among animal species.
20 *Am. J. Physiol. Renal Physiol.* **287**, F1131-F1139 (2004).
 25. Koliwad, S.K. et al. Angiopoietin-like 4 (ANGPTL4/FIAF) is a direct glucocorticoid receptor target and participates in glucocorticoid-regulated triglyceride metabolism. *J. Biol. Chem.* **284**, 25593-25601 (2009).
 26. Liu, G. et al. Nephrin and nephrin interaction in the slit diaphragm is an important
25 determinant of glomerular permeability. *J. Clin. Invest.* **112**, 209-221 (2003).
 27. Dijkman, H.B.P.M., Mentzel, S., de Jong, A.S. & Assmann, K.J.M. RNA in situ hybridization using digoxigenin-labeled cRNA probes. *Biochemica* **2**, 23-27 (1995).
 28. Isogai, S., Mogami, K., Shiina, N. & Yoshino, G. Initial ultrastructural changes in pore size and anionic sites of the glomerular basement membrane in streptozotocin-induced diabetic rats and
30 their prevention by insulin treatment. *Nephron*. **83**, 53-58 (1999).
 29. Bakker, W.W. et al. Altered activity of plasma hemopexin in patients with minimal change disease in relapse. *Pediatr. Nephrol.* **20**, 1410-1415 (2005).
 30. Graves, R.A., Tontonoz, P., Platt, K.A., Ross, S.R. & Spiegelman, B.M. Identification of a fat cell enhancer: analysis of requirements for adipose tissue-specific gene expression. *J. Cell Biochem.*

49, 219-224 (1992).

31. Yoshida, K., Ono, M., Koishi, R. & Furukawa, H. Characterization of the 5' regulatory region of the mouse angiopoietin-like protein 4. *Vet. Res. Commun.* **28**, 299-305 (2004).

32. Zeng, L. et al. HMG CoA reductase inhibition modulates VEGF-induced endothelial cell hyperpermeability by preventing RhoA activation and myosin regulatory light chain phosphorylation. *FASEB J.* **19**, 1845-1847 (2005).

33. Romeo, S. et al. Population-based resequencing of ANGPTL4 uncovers variations that reduce triglyceride and increase HDL. *Nature Genetics*, **39**, 513-517 (2007).

34. Yin, Wu et al. Genetic variation in Angptl4 provides insight into protein processing and function, *J.Biol.Chem.*, 284, 13213-13222 (2009).

References (working example 4)

1. Nachman, P. H, Jennette, J. C. & Falk, R. Primary glomerular disease. in *The Kidney*, 8th edn. (ed. Brenner, B. M.) 987 - 1066 (Elsevier, Philadelphia, 2008).

2. Marsh, J.B. & Drabkin, D.L. Experimental reconstruction of metabolic pattern of lipid nephrosis: key role of hepatic protein synthesis in hyperlipemia. *Metabolism* **9**, 946-955 (1960).

3. Vaziri N.D. Molecular mechanisms of lipid disorders in nephrotic syndrome. *Kidney Int.* **63**, 1964-1976 (2003).

4. Weinstock, P.H. et al. Severe hypertriglyceridemia, reduced high density lipoprotein, and neonatal death in lipoprotein lipase knockout mice. Mild hypertriglyceridemia with impaired very low density lipoprotein clearance in heterozygotes. *J. Clin. Invest.* **96**, 2555-2568 (1995).

5. Shearer, G.C. & Kaysen, G.A. Endothelial bound lipoprotein lipase (LpL) depletion in hypoalbuminemia results from decreased endothelial binding, not decreased secretion. *Kidney Int.* **70**, 647-653 (2006)

6. Clement, L.C. et al. Podocyte – secreted Angiopoietin-like-4 mediates proteinuria in glucocorticoid-sensitive nephrotic syndrome. *Nat Med.* **17**, 117-122 (2011).

7. Chugh, S.S., Clement, L.C. & Macé, C. New insights into human minimal change disease: Lessons from animal models. *Am. J. Kid. Dis.* **59**, 284-292 (2012).

8. Yoshida, K., Shimizugawa, T., Ono, M. & Furukawa, H. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J. Lipid Res.* **43**, 1770-1772 (2002).

9. Sukonina, V., Lookene, A., Olivecrona, T. & Olivecrona, G. Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. *Proc. Natl. Acad. Sci. U S A* **103**, 17450-17455 (2006).

10. Romeo, S. *et al.* Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. *Nat. Genet.* **39**, 513-516 (2007).
11. Yin, W. *et al.* Genetic variation in ANGPTL4 provides insights into protein processing and function. *J. Biol. Chem.* **284**, 13213-13222 (2009).
- 5 12. Clement L. *et al.* Early changes in gene expression that influence the course of primary glomerular disease. *Kidney Int.* **72**, 337-347 (2007).
13. Liu, G., Clement, L., Kanwar, Y.S., Avila-Casado, C. & Chugh, S.S. ZHX proteins regulate podocyte gene expression during the development of nephrotic syndrome. *J. Biol. Chem.* **281**, 39681-39692 (2006).
- 10 14. Nakamura, T. *et al.* Sclerotic lesions in the glomeruli of Buffalo/Mna rats. *Nephron* **43**, 50-55 (1986).
15. Le Berre, L. *et al.* Extrarenal effects on the pathogenesis and relapse of idiopathic nephrotic syndrome in Buffalo/Mna rats. *J. Clin. Invest.* **109**, 491-498 (2002).
16. Neuger, L. *et al.* Effects of heparin on the uptake of lipoprotein lipase in rat liver. *BMC*
- 15 *Physiol.* **4**, 13 (2004).
17. Zhu, P. *et al.* Angiopoietin-like 4 protein elevates the prosurvival intracellular O₂(-):H₂O₂ ratio and confers anoikis resistance to tumors. *Cancer Cell* **19**, 401-415 (2011).
18. Mandard, S. *et al.* The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. *J. Biol. Chem.* **281**, 934-
- 20 944 (2006).
19. Zilleruelo, G., Hsia, S.L., Freundlich, M., Gorman, H.M. & Strauss, J. Persistence of serum lipid abnormalities in children with idiopathic nephrotic syndrome. *J. Pediatr.* **104**, 61-64 (1984).
20. Yu, X. *et al.* Inhibition of cardiac lipoprotein utilization by transgenic overexpression of Angptl4 in the heart. *Proc. Natl. Acad. Sci. U S A.* **102**, 1767-1772 (2005).
- 25 21. Koliwad, S.K. *et al.* Angiopoietin-like 4 (ANGPTL4, fasting-induced adipose factor) is a direct glucocorticoid receptor target and participates in glucocorticoid-regulated triglyceride metabolism. *J. Biol. Chem.* **284**, 25593-25601 (2009).
22. Eremina, V. *et al.* VEGF inhibition and renal thrombotic microangiopathy. *N. Engl. J. Med.* **358**, 1129-1136 (2008).
- 30 23. Maynard, S.E., *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin. Invest.* **111**, 649-658 (2003).
24. Venkatesha, S. *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat.*

- Med.* **12**, 642-649 (2006).
25. Wei, C. *et al.* Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nat. Med.* **17**, 952-960 (2011).
26. Wei, C. *et al.* Circulating suPAR in Two Cohorts of Primary FSGS. *J. Am. Soc. Nephrol.* **23**, 2051-2059 (2012).
27. Avila-Casado, C. *et al.* Proteinuria in rats induced by serum from patients with collapsing glomerulopathy. *Kidney Int.* **66**, 133-143 (2004).
28. Chugh, S.S., & Clement, L.C. Telomerase at the center of collapsing glomerulopathy. *Nat. Med.* **18**, 26-27 (2012).
29. Chugh, S. *et al.* Aminopeptidase A: A nephritogenic target antigen of nephrotoxic serum. *Kidney Int.* **59**, 601-613 (2001).
30. Imamura, S. *et al.* A novel method for measuring human lipoprotein lipase and hepatic lipase activities in postheparin plasma. *J. Lipid Res.* **49**, 1431-1437 (2008).
31. Liu, G., *et al.* Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability. *J. Clin. Invest.* **112**, 209-221 (2003).
32. Chang, S.F., Reich, B., Brunzell, J.D. & Will, H. Detailed characterization of the binding site of the lipoprotein lipase-specific monoclonal antibody 5D2. *J. Lipid Res.* **39**, 2350-2359 (1998).

TABLE 4:

LEGEND TO THE SEQUENCE LISTING

20	SEQ ID NO: 1	Variant 1 of human Angptl4 polypeptide
	SEQ ID NO: 2	cDNA sequence of variant 1 of human Angptl4
	SEQ ID NO: 3	Variant 2 of human Angptl4 polypeptide
	SEQ ID NO: 4	cDNA sequence of variant 2 of human Angptl4
	SEQ ID NO: 5	Rat Angptl4 polypeptide
25	SEQ ID NO: 6	cDNA sequence of rat Angptl4 polypeptide
	SEQ ID NO: 7	Mouse Angptl4 polypeptide
	SEQ ID NO: 8	cDNA sequence of mouse Angptl4 polypeptide
	SEQ ID NO: 9	Variant 1 of human Angptl4 polypeptide with substitutions
	SEQ ID NO: 10	Variant 2 of human Angptl4 polypeptide with substitutions
30	SEQ ID NOS: 11-22	PCR primers and probes (see text)
	SEQ ID NO: 23	N-terminal multispecies consensus sequence with substitutions
	SEQ ID NO: 24	Central multispecies consensus sequence with substitutions
	SEQ ID NO: 25	C-terminal multispecies consensus sequence with substitutions
	SEQ ID NO: 26	N-terminal human consensus sequence with substitutions

	SEQ ID NO: 27	C-terminal human consensus sequence with substitutions
	SEQ ID NO: 28	Mutant variant of human Angptl4 polypeptide DKMNVLAHGLLQLGQGL
	SEQ ID NOS: 29-90	Sequences from Table 2
	SEQ ID NO: 91	Reverse primer for amplification of aP2-Angptl4 construct
5	SEQ ID NO: 92	Probe for identification of aP2-Angptl4 construct
	SEQ ID NO: 93	cDNA encoding peptide of SEQ ID NO: 80
	SEQ ID NO: 94	cDNA encoding the peptide of SEQ ID NO: 87

CLAIMS

What is claimed:

1. An Angptl4 polypeptide derivative having decreased LPL inhibitory activity and increased resistance to cleavage, the Angptl4 polypeptide derivative having a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, a sequence having at least 95% identity to SEQ ID NO: 1, and a sequence having at least 95% identity to SEQ ID NO: 3, wherein the amino acids at positions 39-40 are DA and the amino acids at positions 161-164 are GVVA.
2. The Angptl4 polypeptide derivative of claim 1, wherein the Angptl4 polypeptide derivative is sialylated.
3. A use of the Angptl4 polypeptide derivative of claim 1 or claim 2 in the manufacture of a medicament for the treatment or prevention of nephrotic syndrome.
4. A use of the Angptl4 polypeptide derivative of claim 1 or claim 2 in the treatment or prevention of nephrotic syndrome.
5. An Angptl4 polypeptide derivative of claim 1 or claim 2 for use in the treatment or prevention of nephrotic syndrome.
6. The use according to claim 4 or the Angptl4 polypeptide derivative for use of claim 5, wherein the nephrotic syndrome is minimal change disease, focal segmental glomerulosclerosis, membranous nephropathy/membranous glomerulonephritis, membranoproliferative glomerulonephritis, diabetic nephropathy, diabetes mellitus, lupus nephritis, or primary glomerular disease.
7. The use, or Angptl4 polypeptide derivative for use, according to claim 6, wherein the nephrotic syndrome displays a symptom selected from the group consisting of: edema, hyperlipidemia, hypoproteinemia, hypercholesterolemia, and proteinuria.
8. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the Angptl4 polypeptide derivative of claim 1 or claim 2.
9. The pharmaceutical composition of claim 8, comprising 0.035-1100 mg of the Angptl4 polypeptide derivative.
10. An isolated polynucleotide encoding the Angptl4 polypeptide derivative of claim 1 or claim 2.

11. An isolated cell comprising the polynucleotide of claim 10, wherein the cell is not a fertilized egg or a totipotent stem cell.

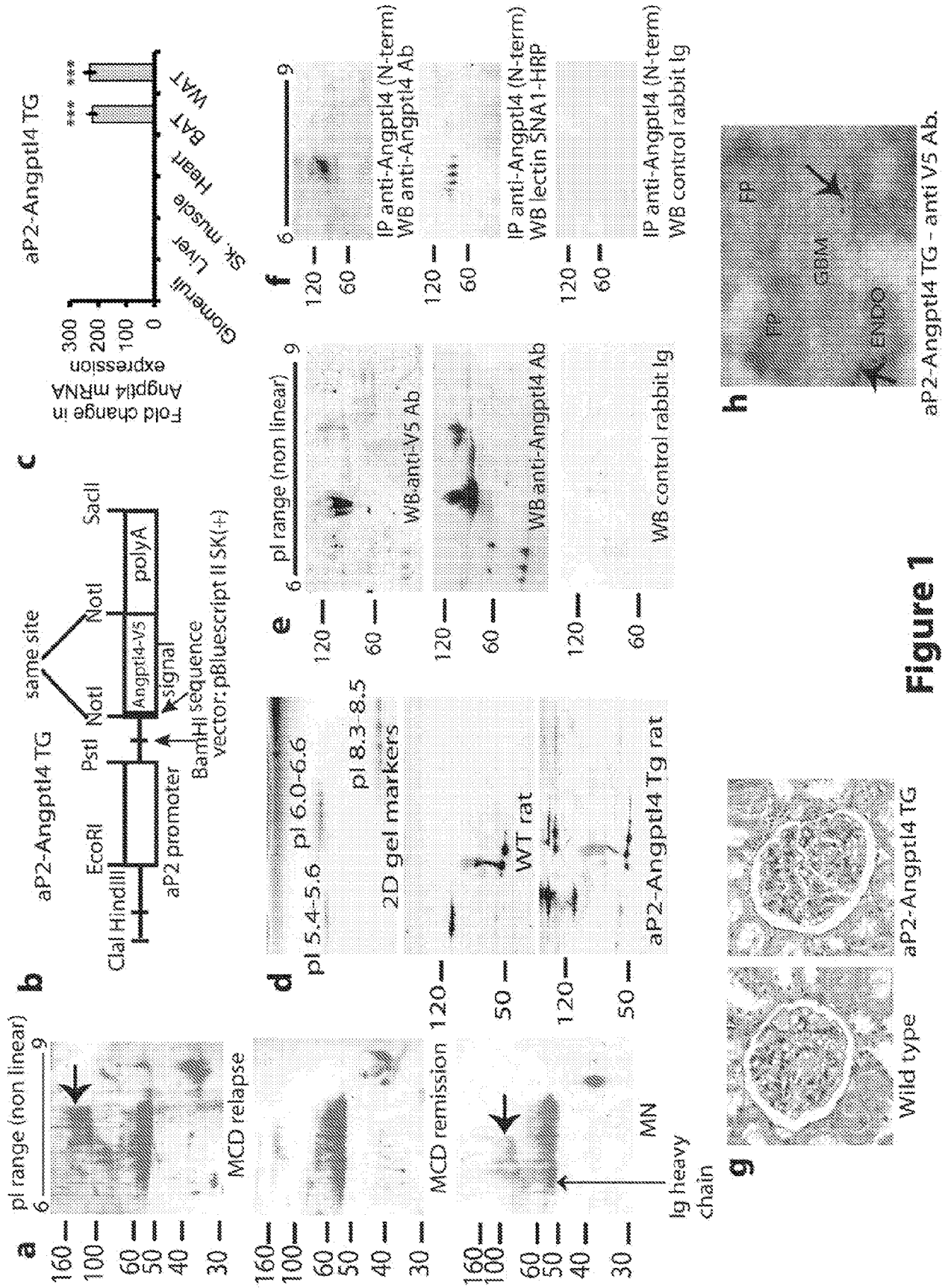


Figure 1

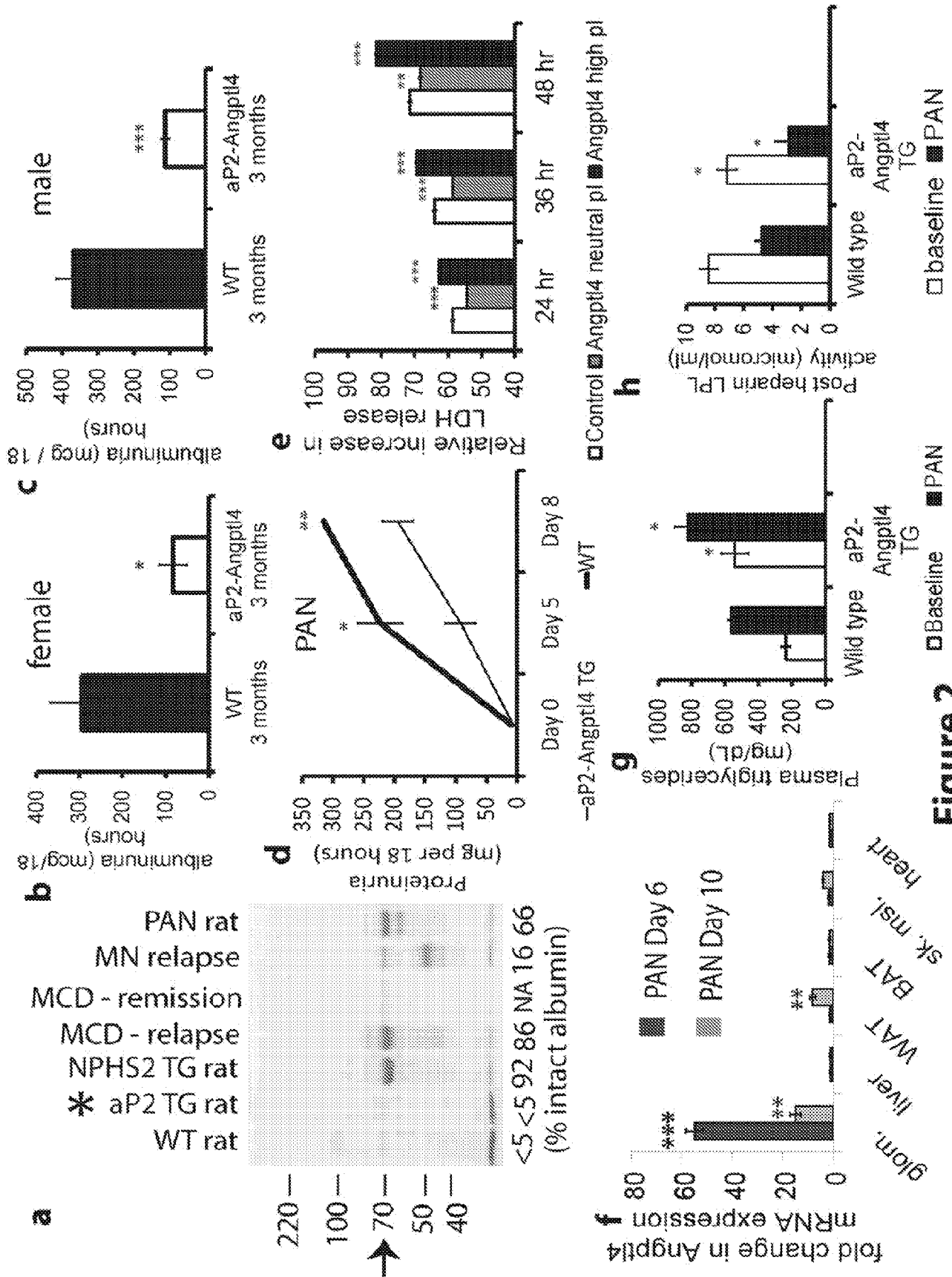


Figure 2

Gene/Transgene	Species	Forward Primer	Reverse Primer	Taqman Probe
Angptl4	Rat	tctgggatct ccaccatttt tg SEQ ID NO: 11	tcaccgtcca gcctccat SEQ ID NO: 12	caactgtgag atgacttc SEQ ID NO: 13
Angptl4	Rat	cgccacccgc ttacaca SEQ ID NO: 14	cagaggctgg atctggaaaa gt SEQ ID NO: 15	tgccaggaac tcttt SEQ ID NO: 16
aP2-Angptl4 construct	Rat	tgttgatcca gcccattgga 1181-1199 of SEQ ID NO: 6	agggataggc ttaccttcca atg SEQ ID NO: 91	cagcagcctc cc SEQ ID NO: 92
Prolactin (genomic)	Rat	cttgaaggga ttgaaaagat aattagc SEQ ID NO: 20	ccatgagtca gaaaagcatt gaac SEQ ID NO: 21	aggtgagcat ttctctg SEQ ID NO: 22

FIGURE 3

FIG. 4

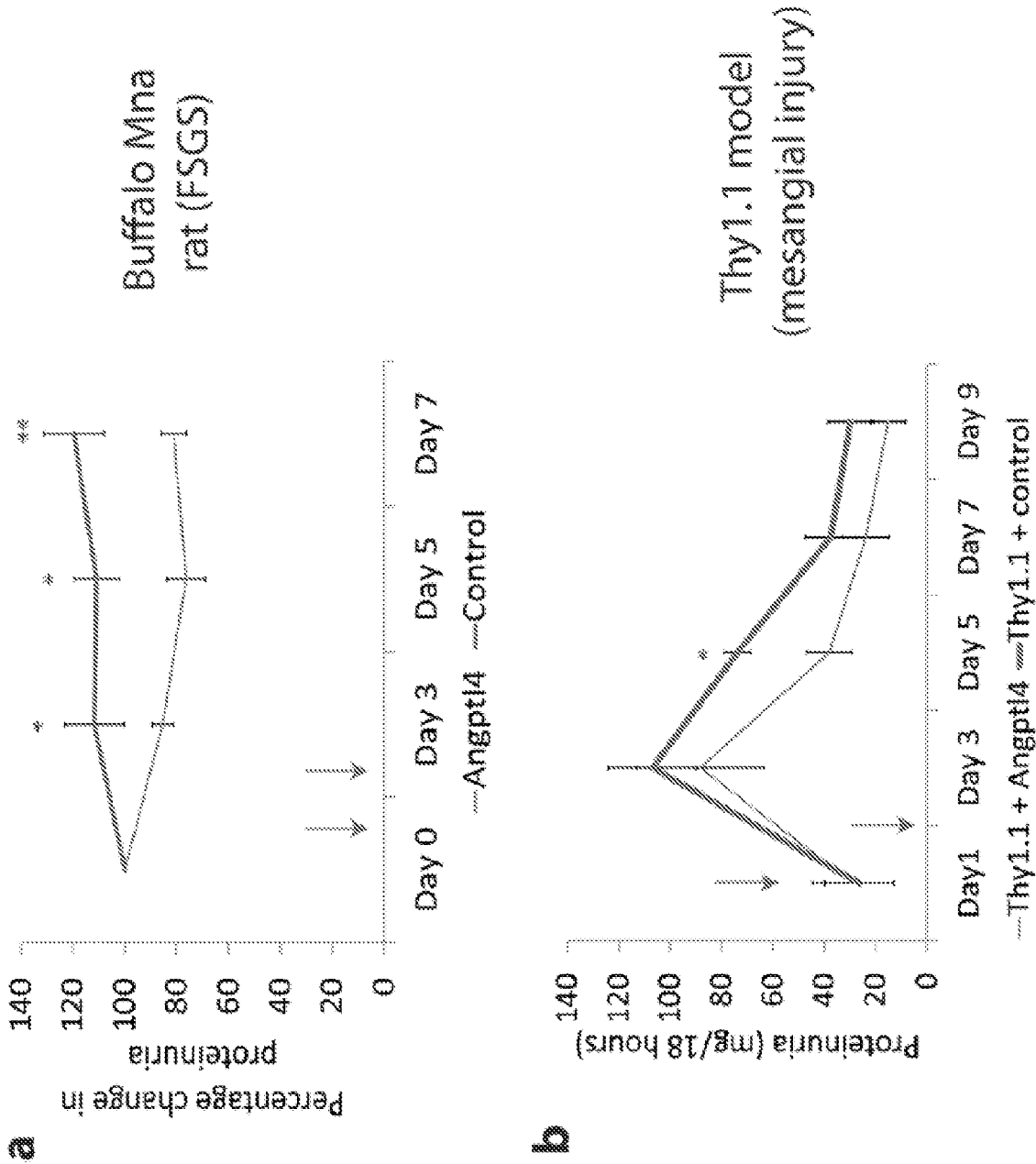


FIG. 5

SEQ ID NO: 1

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWDEMNVLAHGLLQLGQGLREHAERTRSQLSALER
 RLSACGSACQGTEGSTDLPLAPESRVDPEVLHSLQTQLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLSQSFGL
 LDHKHLDHEVAKPARRKRLPEMAQPVDPAHNVSRHLRLPRDCQELFQVGERQSGLFELIQPQGSPPFLVNCKMTSD
 GGWTVIQRHHDGSDVDFNRPWEAYKAGFGDPHGEFWLGLGKLVHSITGDRNSRLAVQLRDWDGNAELLQFSVHLGGE
 DTAYSLQLTAPVAGQLGATTVPVPSGLSVPFSTWDQDHDLLRRDKNCAKSLSGGWVFGTCSHNSNLNGQYFRSIPQQR
 QKLKKGIFWKTWRGRYYPLQATTMLIQPMAAEAAAS
 406 amino acids

SEQ ID NO: 2

1	ataaaaaaccg	tcctcggggcg	cggcgggggag	aagccgagct	gagcgggatcc	tcacacgact
61	gtgatccgat	tctttccagc	ggcttctgca	accaagcggg	tcttaccccc	ggtcctccgc
121	gtctccagtc	ctcgacacctg	gaaccccaac	gtccccgaga	gtccccgaat	ccccgctccc
181	aggctaccta	agaggatgag	cgggtgctccg	acggccgggg	cagccctgat	gctctgcgcc
241	gccaccgccg	tgctactgag	cgctcagggc	ggaccctg	agtcgaagtc	gccgcgcttt
301	gcgtcctggg	acgagatgaa	tgtcctggcg	cacggactcc	tgcagctcgg	ccaggggctg
361	cgcgaacacg	cggagcgcac	ccgcagtcag	ctgagcgcgc	tggagcggcg	cctgagcgcg
421	tgccgggtccg	cctgtcaggg	aaccgagggg	tccaccgacc	tcccgttagc	ccctgagagc
481	cgggtggacc	ctgaggtcct	tcacagcctg	cagacacaac	tcaaggctca	gaacagcagg
541	atccagcaac	tcttccacaa	ggtggcccag	cagcagcggc	acctggagaa	gcagcacctg
601	cgaattcagc	atctgcaaag	ccagtttggc	ctcctggacc	acaagcacct	agaccatgag
661	gtggccaagc	ctgcccgaag	aaagaggctg	cccagatgg	cccagccagt	tgaccgggct
721	cacaatgtca	gccgcctgca	cgggctgccc	agggattgcc	aggagctggt	ccaggttggg
781	gagaggcaga	gtggactatt	tgaatccag	cctcaggggt	ctccgccatt	tttggtgaac
841	tgcaagatga	cctcagatgg	aggctggaca	gtaattcaga	ggcgccacga	tggctcagtg
901	gacttcaacc	ggccctggga	agcctacaag	gcgggggttg	gggatcccca	cggcgagttc
961	tggtctgggtc	tggagaaggt	gcatagcatc	acgggggacc	gcaacagccg	cctggccgtg
1021	cagctgcggg	actgggatgg	caacgccgag	ttgtctcagt	tctccgtgca	cctgggtggc
1081	gaggacacgg	cctatagcct	gcagctcact	gcaccctggg	ccggccagct	ggcgccacc
1141	accgtcccac	ccagcggcct	ctccgtaccc	ttctccactt	gggaccagga	tcacgacctc
1201	cgcagggaca	agaactgcgc	caagagcctc	tctggaggct	ggtgggtttg	cacctgcagc
1261	cattccaacc	tcaacggcca	gtacttccgc	tccatccccc	agcagcggca	gaagcttaag
1321	aagggaatct	tctggaagac	ctggcggggc	cgctactacc	cgctgcaggc	caccaccatg
1381	ttgatccagc	ccatggcagc	agaggcagcc	tcctagcgtc	ctggctgggc	ctggtcccag
1441	gcccacgaaa	gacggtgact	cttggctctg	cccagggatg	tggccggttc	ctgctggggc
1501	aggggctcca	aggaggggcc	atctggaaac	ttgtggacag	agaagaagac	cacgactgga
1561	gaagccccct	ttctgagtgc	aggggggctg	catgcgttgc	ctcctgagat	cgaggctgca
1621	ggatatgctc	agactctaga	ggcgtggacc	aaggggcatg	gagcttccct	ccttgctggc
1681	cagggagttg	gggactcaga	gggaccactt	ggggccagcc	agactggcct	caatggcgga
1741	ctcagtcaca	ttgactgacg	gggaccaggg	ctgtgtggg	tcgagagcgc	cctcatgggtg
1801	ctgggtgctgt	tgtgtgtagg	tcccctgggg	acacaagcag	gcgccaatgg	tatctggggc
1861	gagctcacag	agttcttgga	ataaaagcaa	cctcagaaca	cttaaaaaaa	aaaaaaaaaa
1921	aaaaaaaaaa	aaaaaaaaaa	aaaaaaaaaa	aaaaaaaaaa	aaaaaa	

SEQ ID NO: 3

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWDEMNVLAHGLLQLGQGLREHAERTRSQLSALERRLSA
 CGSACQGTEGSTDLPLAPESRVDPEVLHSLQTQLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLSQSFGLLDHK
 HLDHEVAKPARRKRLPEMAQPVDPAHNVSRHLHGGWTVIQRHHDGSDVDFNRPWEAYKAGFGDPHGEFWLGLGKLVH
 SITGDRNSRLAVQLRDWDGNAELLQFSVHLGGEDTAYSLQLTAPVAGQLGATTVPVPSGLSVPFSTWDQDHDLLRRD
 KNCASLSGGWVFGTCSHNSNLNGQYFRSIPQQRQKLKKGIFWKTWRGRYYPLQATTMLIQPMAAEAAAS
 368 amino acids

SEQ ID NO: 4

1	ataaaaaaccg	tcctcggggcg	cggcgggggag	aagccgagct	gagcgggatcc	tcacacgact
61	gtgatccgat	tctttccagc	ggcttctgca	accaagcggg	tcttaccccc	ggtcctccgc
121	gtctccagtc	ctcgacacctg	gaaccccaac	gtccccgaga	gtccccgaat	ccccgctccc
181	aggctaccta	agaggatgag	cgggtgctccg	acggccgggg	cagccctgat	gctctgcgcc
241	gccaccgccg	tgctactgag	cgctcagggc	ggaccctg	agtcgaagtc	gccgcgcttt

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301      gcgtcctggg acgagatgaa tgtcctggcg cacggactcc tgcagctcgg ccaggggctg
361      cgcgaaacacg cggagcgcac ccgcagtcag ctgagcgcgc tggagcggcg cctgagcgcg
421      tgcgggtccg cctgtcaggg aaccgagggg tccaccgacc tcccgttagc ccctgagagc
481      cgggtggacc ctgaggctct tcacagcctg cagacacaac tcaaggctca gaacagcagg
541      atccagcaac tcttccacaa ggtggcccag cagcagcggc acctggagaa gcagcacctg
601      cgaattcagc atctgcaaag ccagtttggc ctcttggaac acaagcacct agaccatgag
661      gtggccaagc ctgcccgaag aaagaggctg cccgagatgg cccagccagt tgacccggt
721      cacaatgtca gccgcctgca ccatggaggc tggacagtaa ttcagaggcg ccacgatggc
781      tcagtggact tcaaccggcc ctgggaagcc tacaaggcgg ggtttgggga tccccacggc
841      gagttctggc tgggtctgga gaaggtgcat agcatcacgg gggaccgcaa cagccgcctg
901      gccgtgcagc tgcgggactg ggatggcaac gccgagttgc tgcagttctc cgtgcacctg
961      ggtggcgagg acacggccta tagcctgcag ctactgcac ccgtggccgg ccagctgggc
1021     gccaccaccg tcccaccag cggcctctcc gtacccttct ccacttggga ccaggtcac
1081     gacctccgca gggacaagaa ctgcccgaag agcctctctg gaggttggtg gtttggcacc
1141     tgcagccatt ccaacctcaa cggccagtac ttccgctcca tcccacagca gcggcagaag
1201     cttaagaagg gaatcttctg gaagacctgg cggggccgct actaccgct gcaggccacc
1261     accatgttga tccagcccat ggcagcagag gcagctcct agcgtcctgg ctgggcctgg
1321     tcccaggccc acgaaagacg gtgactcttg gctctgcccg aggatgtggc cgttccctgc
1381     ctgggcaggg gctccaagga ggggccatct ggaaacttgt ggacagagaa gaagaccacg
1441     actggagaag cccctttctt gagtgcaggg gggctgcatg cgttgccctc tgagatcgag
1501     gctgcaggat atgctcagac tctagaggcg tggaccaagg ggcattggagc ttcactcctt
1561     gctggccagg gatttgggga ctcagaggga ccacttgggg ccagccagac tggcctcaat
1621     ggcggaactca gtcacattga ctgacgggga ccagggcttg tgtgggtcga gacgcccctc
1681     atggtgctgg tgctgttggt tgtagggtccc ctggggacac aagcaggcgc caatgggtatc
1741     tgggcgaggc tcacagagtt cttggaataa aagcaacctc agaactta aaaaaaaaaa
1801     aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaa

```

SEQ ID NO: 5

MRCAPTAGAALVLCAATAGLLSAQGRPAQPEPPRFASWDEMNL LAHGLLQLGHGLREHVERTRGQLGALERMAA
CGNACQGFPGTDPKDRVPEQAPETLQSLQTLKAQNSKIQQLFQKVAQQQRYLSKQNLRIQNLSQIDLLTPH
LDNGVDKTSRGKRLPKMAQLIGLTPNATRLHRPPRDCQELFQEGERHSGLFQIQPLGSPFVLNCEMTSDGGWTV
IQRRLLNGSVDFNQSWEAYKDGFGDPQGEFWLGLLEKMHSTITGDRGSQLAVQLQDWDGNAKLLQFPFIHLGGEDTAYS
LQLTEPTANELGATNVSPNGLSLPFSTWDQDHDRLGDLNCAKSLSGGWVFGTCSHSNLNGQYFHSIPRQRQQRKK
GIFWKTKGRYYPLOATTLLIQPMEATAAS
405 amino acids

SEQ ID NO: 6

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1      atgcgctgcg ctccgaccgc aggcgctgct ctagtgtctat gcgcagctactcgggggctg
61      ctgagcgcgc aagggcgccc tgcacagccg gagccgcgcg gcttcgcctcctgggatgaa
121     atgaacttgc tggctcacgg gctgtctgag ctcggtcacg ggctgcgggaacacgtggag
181     cgcaccctgt gacagctggg cgcgctggaa cgcgcctgag ctgcctgcggtaacgcttgt
241     caggggccca aggggacaga cccgaaggat agagtccccg aaggccaggctcctgagact
301     ctgcagagtt tacagactca actcaaggct cagaacagca agatccagcaactgttccag
361     aaggtagccc agcagcagag atacctatca aagcagaatc tgagaatacagaatcttcag
421     agccagattg acctcttgac cccacacac ctagacaatg gggtagacaagacttcgagg
481     ggaaagaggc ttccaagat gggccagctc attggcttga ctccaacgccaccgcctta
541     cacaggcctc cccgggactg ccaggaactc tttcaagaag gggagcggcacagtggactt
601     ttccagatcc agcctctggg atctccacca tttttggtca actgtgagatgacttcagat
661     ggaggctgga cggtgattca gagacgcctg aacggctctg tggacttcaatcagttcttg
721     gaagcctaca aagatggctt cggagatccc caaggcgagt tctggctggcctagagaag
781     atgcacagca tcacagggga ccaggaagc cagttggctg tgcagctccaggactgggat
841     ggcaatgcc aattgtctca atttctatc catttggggg gtgaggacacagcctacagc
901     ctgcagctca ccgagcccac ggccaatgag ctgggtgcc acaatgtttcccccaatggc
961     ctttccctgc cttctctac ctgggaccaa gaccacgacc tccgaggggaccttaactgt
1021    gccaaagacc tctctggtgg ctggtggttt ggcacctgca gccattccaatctaaatgga
1081    caatacttcc actctattcc acggcaacgg cagcagcgta aaaaggggatcttctggaaa
1141    acatggaagg gccgctacta tccactacag gctaccaccc tgttgatccagcccatggag
1201    gctacagcag cctcttag

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SEQ ID NO: 7

MRCAPTAGAALVLC AATAGLLSAQGRPAQPEPPRFASWDEMNL LAHGLLQLGHGLREHVERTRGQLGALERRMAA
CGNACQGPKGKDAPFKDSEDRVPEGQTPETLQSLQTLKAQNSKIQQLFQKVAQQQRYLSKQNLRIQNLSQSIDL
LAPTHLDNGVDKTSRGRKRLPKMTQLIGLTPNATHLHRPPRDCQELFQEGERHSGLFQIQPLGSPFFLVNCEMTSD
GGWTVIQRRNLNGSVDFNQSW EAYKDGFGDPQGEFWLGLEKMH S ITGNRGSQ L AVLQDWDGNAKLLQFPIHLGGE
DTAYSLQLTEPTANELGATNVSPNGLSLPFSTWDQDHDRLRGDLNCAKSLSGGWVFGTCSHSNLNGQYFHSIPRQR
QERKKGIFWK TWKGRYYPLQATTLLIQPMEATAAS

410 amino acids

SEQ ID NO: 8

1	acgggctcca	gatcttcttc	tgcaccagag	caagtctaag	tctgagccggtccccaga
61	actccagctg	ctgggtcttg	aactcctgcg	ttccggagtc	ctagcgttgctgcacccaag
121	gccaccccc	gaatcatgcg	ctgcgctccg	acagcaggcg	ctgccctggtgctatgcgcg
181	gctactgcgg	ggcttttgag	cgcgcaagg	cgccctgcac	agccagagccaccgcgcttt
241	gcatacctggg	acgagatgaa	cttgctggct	cacgggctgc	tacagctcgcccatggctg
301	gcggaacacg	tggagcgcac	ccgtgggcag	ctgggcgcgc	tggagcgcgcgatggctgcc
361	tgtggtaacg	ctgtcagg	gcccaggga	aaagatgcac	ccttcaaagactccaggat
421	agagtccttg	aaggccagac	tcctgagact	ctgcagagtt	tgcagactcagctcaaggct
481	caaaacagca	agatccagca	attgttccag	aagtggtccc	agcagcagagatacctatca
541	aagcagaatc	tgagaataca	gaatcttcag	agccagatag	acctcttggccccacgcac
601	ctagacaatg	gagtagacaa	gacttcgagg	ggaaagaggc	ttcccaagatgaccagctc
661	attggcttga	ctcccaacgc	caccacttta	cacaggccgc	cccgggactgccagggaactc
721	ttccaagaag	gggagaggca	cagtggactt	ttccagatcc	agcctctggggtctccacca
781	tttttggtca	actgtgagat	gacttcagat	ggaggctgga	cagtgattcagagacgcctg
841	aacggctctg	tggacttcaa	ccagtcctgg	gaagcctaca	aggatggcttcggagatccc
901	caaggcgagt	tctggctggg	cctggaaaag	atgcacagca	tcacagggaaccgagggaagc
961	caattggctg	tgcagctcca	ggactgggat	ggcaatgcca	aattgctccaatttcccatc
1021	catttggggg	gtgaggacac	agcctacagc	ctgcagctca	ctgagcccacggccaatgag
1081	ctgggtgcca	ccaatgtttc	ccccaatggc	cttccctgc	ccttctctacttgggaccaa
1141	gaccatgacc	tccgtgggga	ccttaactgt	gccaagagcc	tctctggtggctggtggttt
1201	ggtacctgta	gccattccaa	tctcaatgga	caatacttcc	actctatcccacggcaacgg
1261	caggagcgta	aaaagggtat	cttctggaaa	acatggaagg	gccgctactatcctctgcag
1321	gctaccaccc	tgtgatcca	gcccattggg	gctacagcag	cctcttagcctcctcactgg
1381	agcctggttc	caggcctaag	aagacagtga	ctttggttgt	ggccctgagatttggccatt
1441	ctctgctggg	ggcaggagct	ctaagtaggg	ctatctgcgt	cttggtggacaaagaagaagc
1501	ccgtaactgg	agagactgga	ggaccccttt	tccgtgttgg	ggtctgcaagcattgtgtgc
1561	tgaacagtc	agagcaacag	gaaacaaatg	gccagatcc	agaaaacatgggctcgaggg
1621	gcactgaata	tcacttctcg	cctaccagag	aagttgggga	tgcagagggaccactacagt
1681	ccaactagct	gggcccttaa	tggcggactc	agtcataattg	actgactggagacaggtgctc
1741	caggagccct	ggatacactc	atggtgctgt	tgtaggtgct	gtggatgcacaggtgctaac
1801	tgtggttccc	aggcacaact	cacagcattc	ttacaataaa	aacaacctcagaacaaaaaa
1861	aaaaaaaa				

SEQ ID NO: 9

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWX₃₉X₄₀MNVLAHGLLQLGQGLREHAERTRSQLSALERR
LSAX₇₆GSAX₈₀QTEGSTDLPLAPESRVDP EVLHSLQTLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLQSQF
GLLDHKHLDHEVAKPAX₁₆₁X₁₆₂X₁₆₃X₁₆₄LP EMAQPVDP AHNVSR LHR LPRDCQELFQVGERQSGLF EIQ PQSGSPF
FLVNCMTSX₂₂₁GGWTVIQRRHDGSDVFNRPWEAYKAGFGDPHGEFWLGLEKVHSITGDRNSRLAVQLRDWDGNA
ELLQFSVHLGGEDTAYSLQLTAPVAGQLGATTVP P SGLSVPFSTWDQDHDRLRRDKNCAKSLSGGWVFGTCSHSNL
NGQYFRSIPQQRQKLKKGIFWKTRGRYYPLQATTMLIQPMAAEAAAS

406 amino acids

SEQ ID NO: 10

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWX₃₉X₄₀MNVLAHGLLQLGQGLREHAERTRSQLSALERR
LSAX₇₆GSAX₈₀QTEGSTDLPLAPESRVDP EVLHSLQTLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLQSQF
GLLDHKHLDHEVAKPAX₁₆₁X₁₆₂X₁₆₃X₁₆₄LP EMAQPVDP AHNVSR LHHGGWTVIQRRHDGSDVFNRPWEAYKAGFG

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DPHGEFWLGLEKVHSITGDRNSRLAVQLRDWDGNAELLQFSVHLGGEDTAYSLQLTAPVAGQLGATTVPVPSGLSV
PFSTWDQDHDLRDKNCAKSLSGGWWFGTCSHSNLNGQYFRSIPQQRQKLKKGIFWKTWRGRYYPLQATTMLIQP
MAAEAAS

368 amino acids

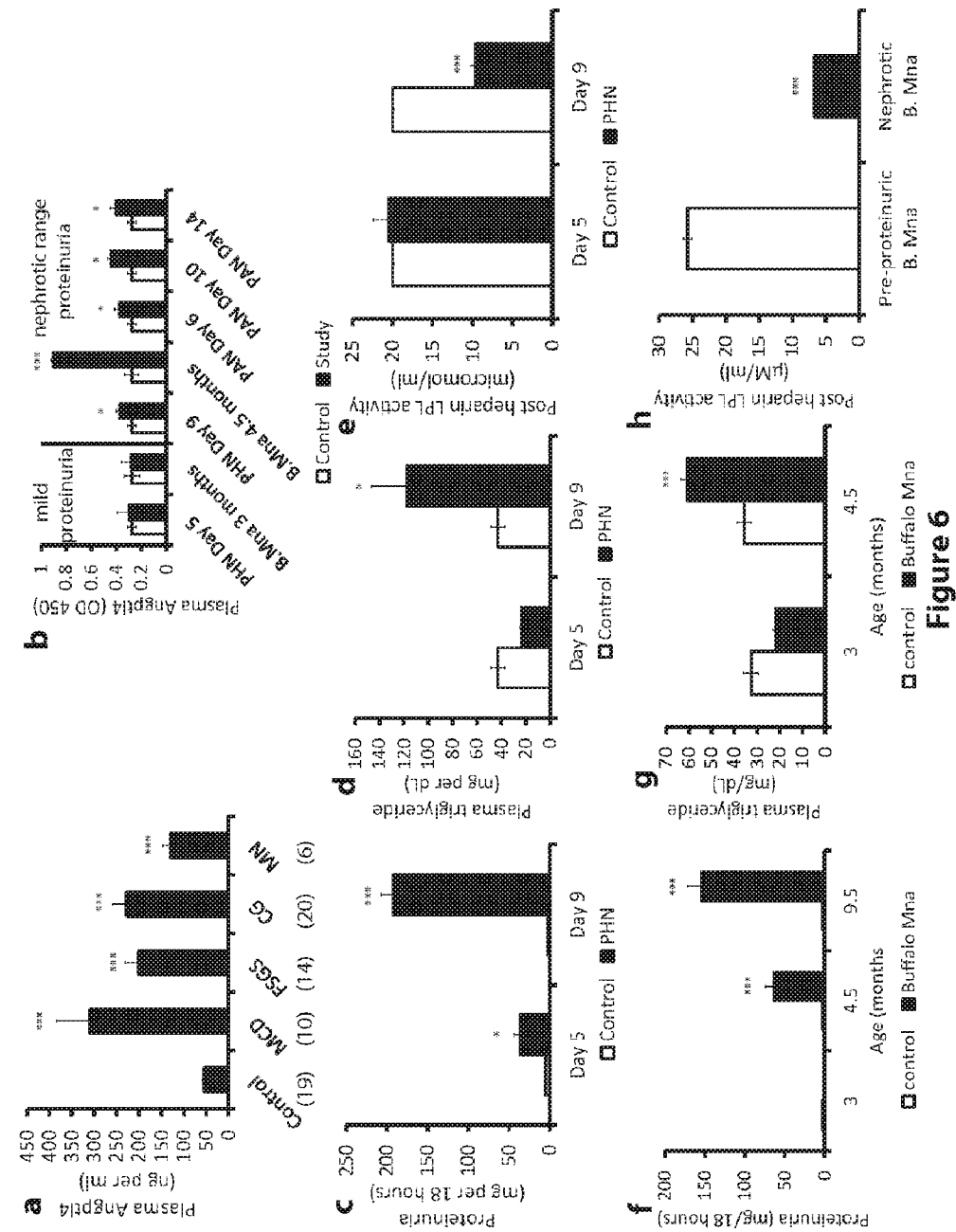


FIG. 6 (cont'd)

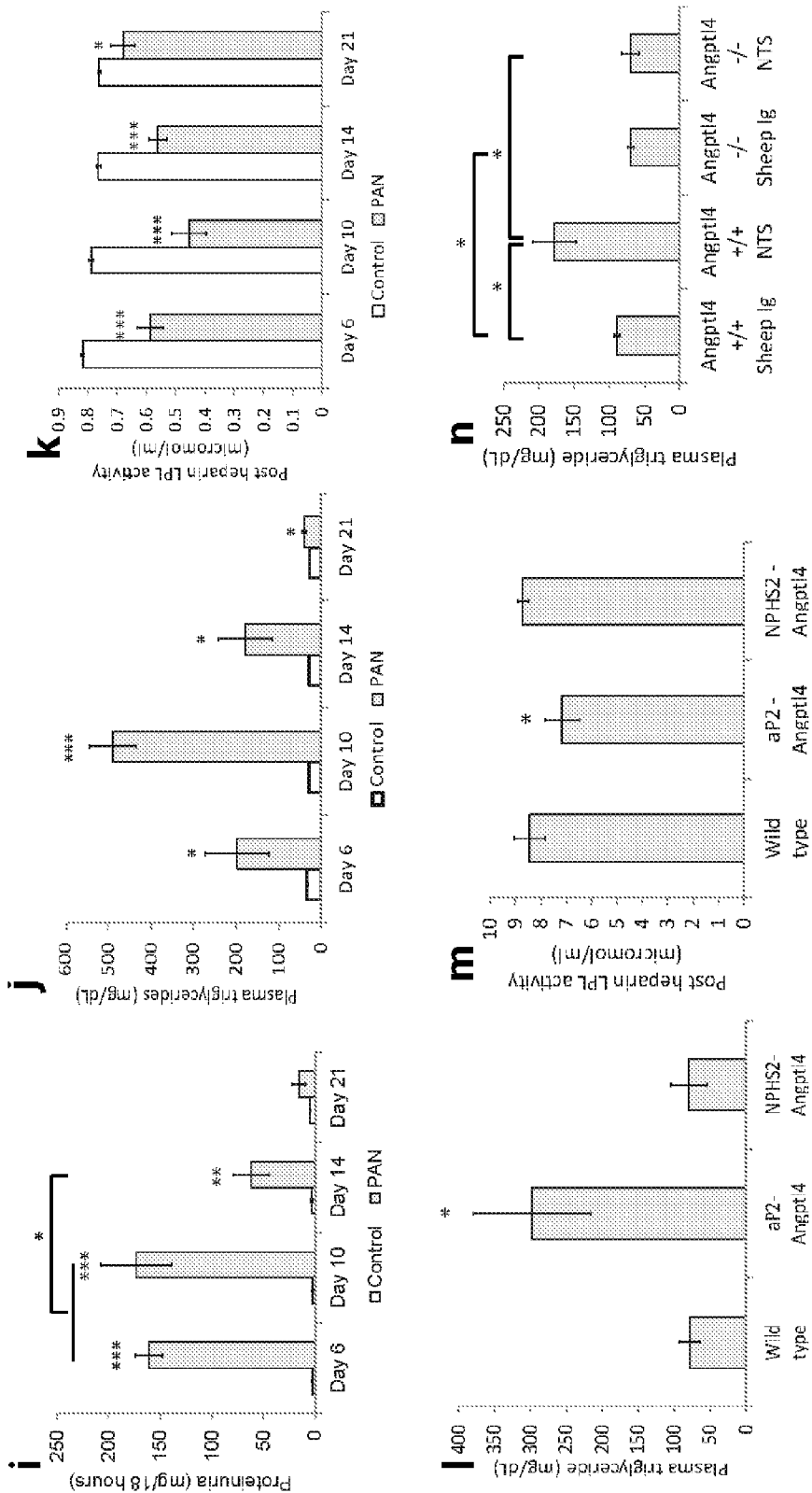
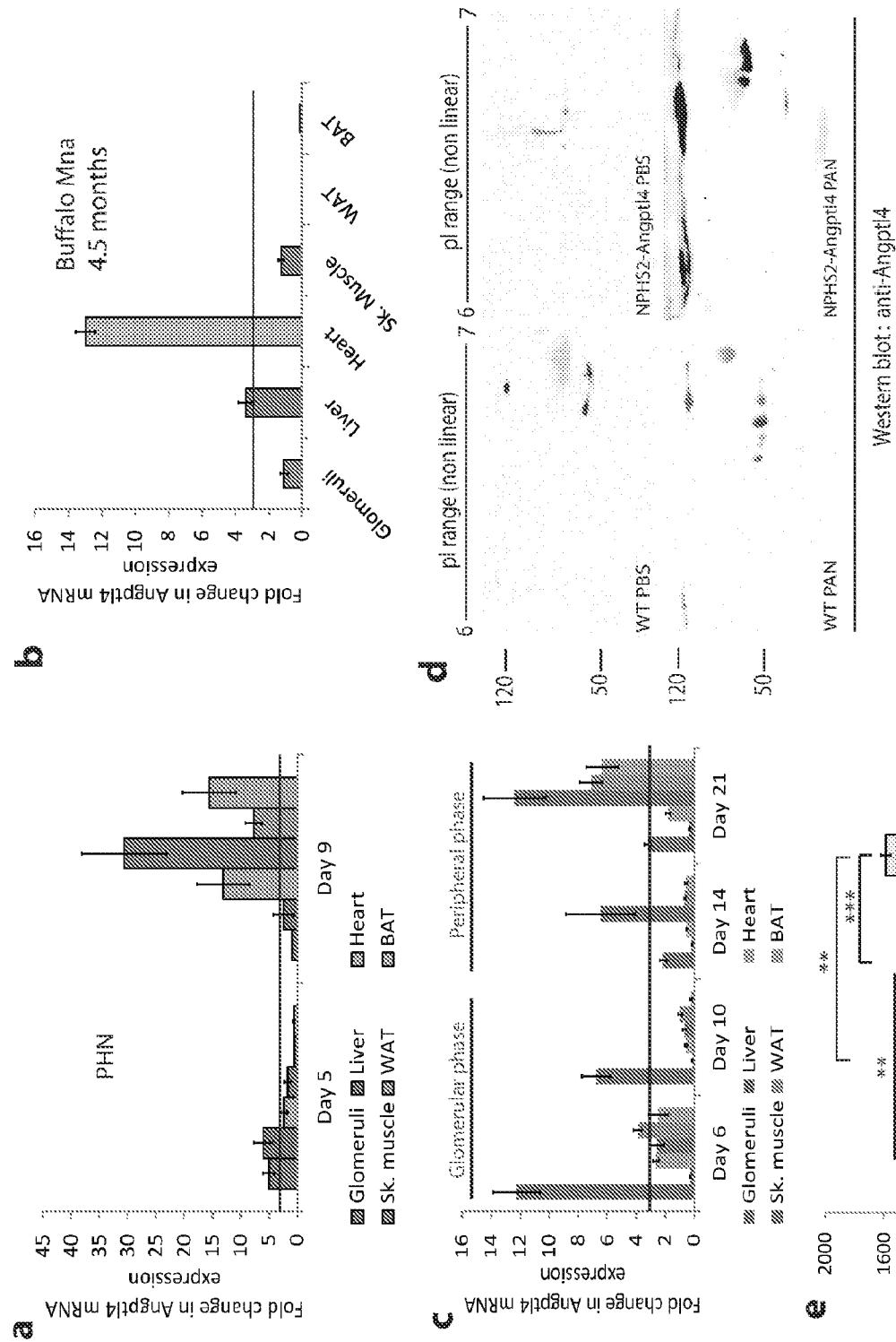


FIG. 7



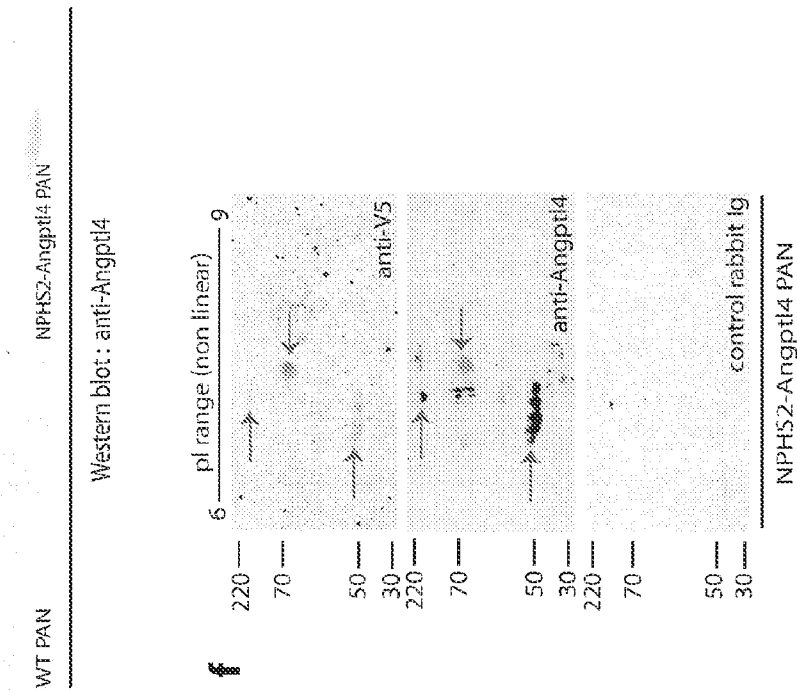
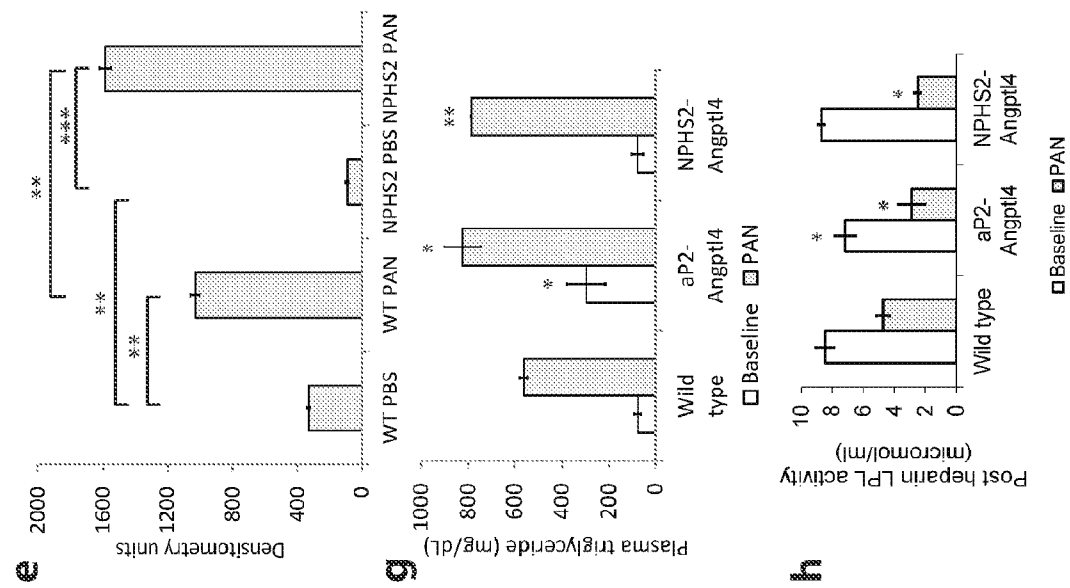


Figure 7 cont'd

FIG. 8

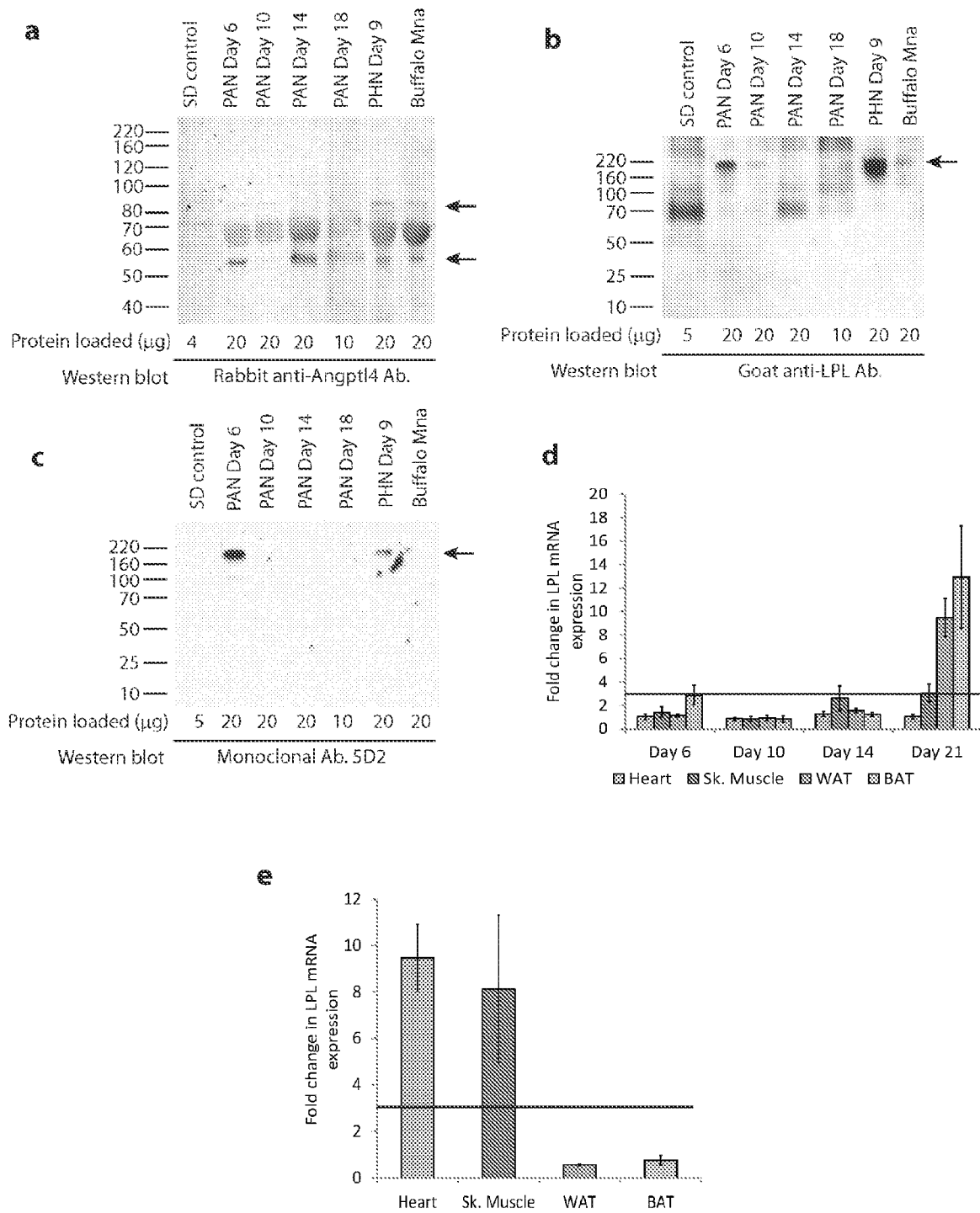
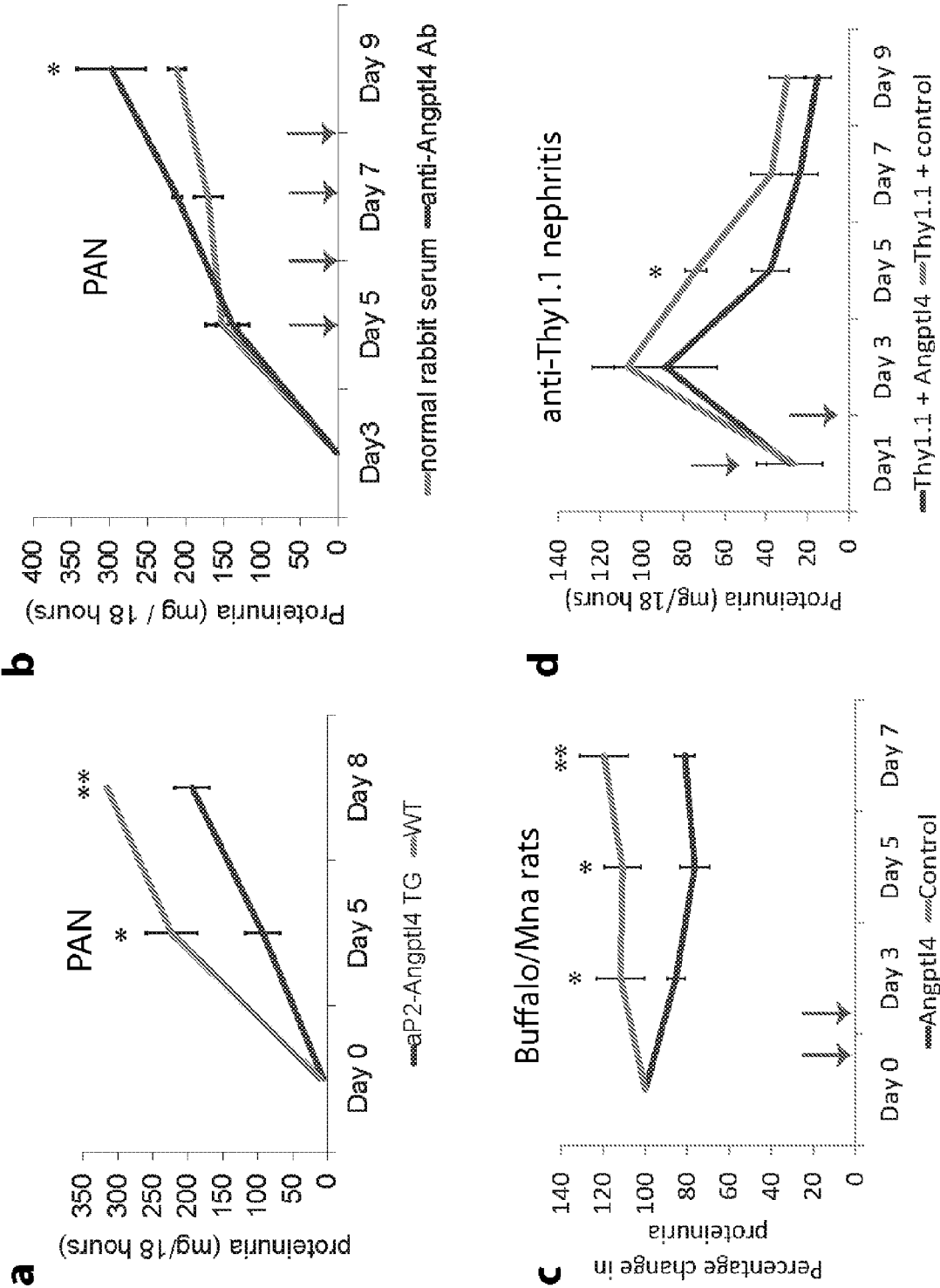


FIG. 9



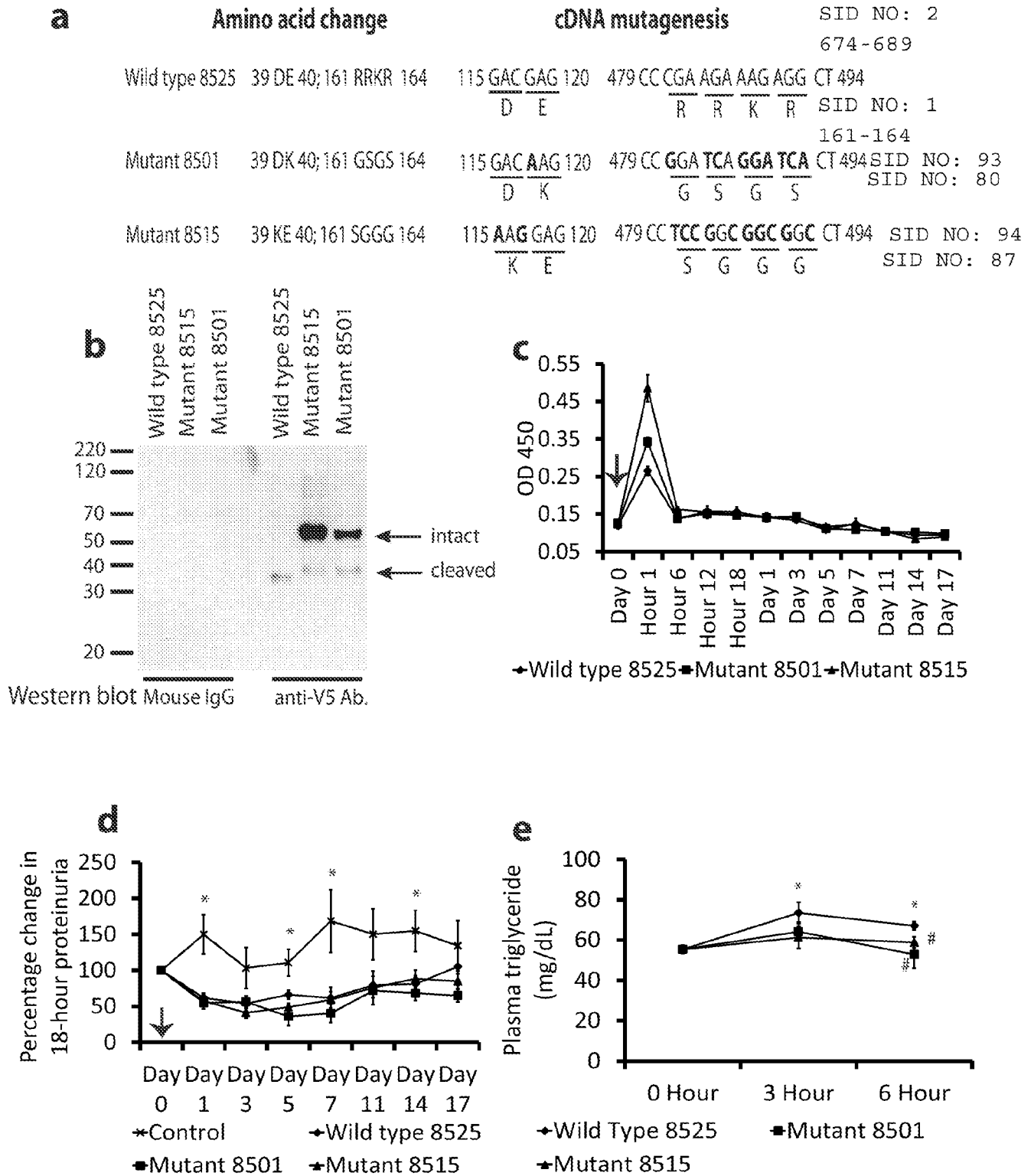


Figure 10

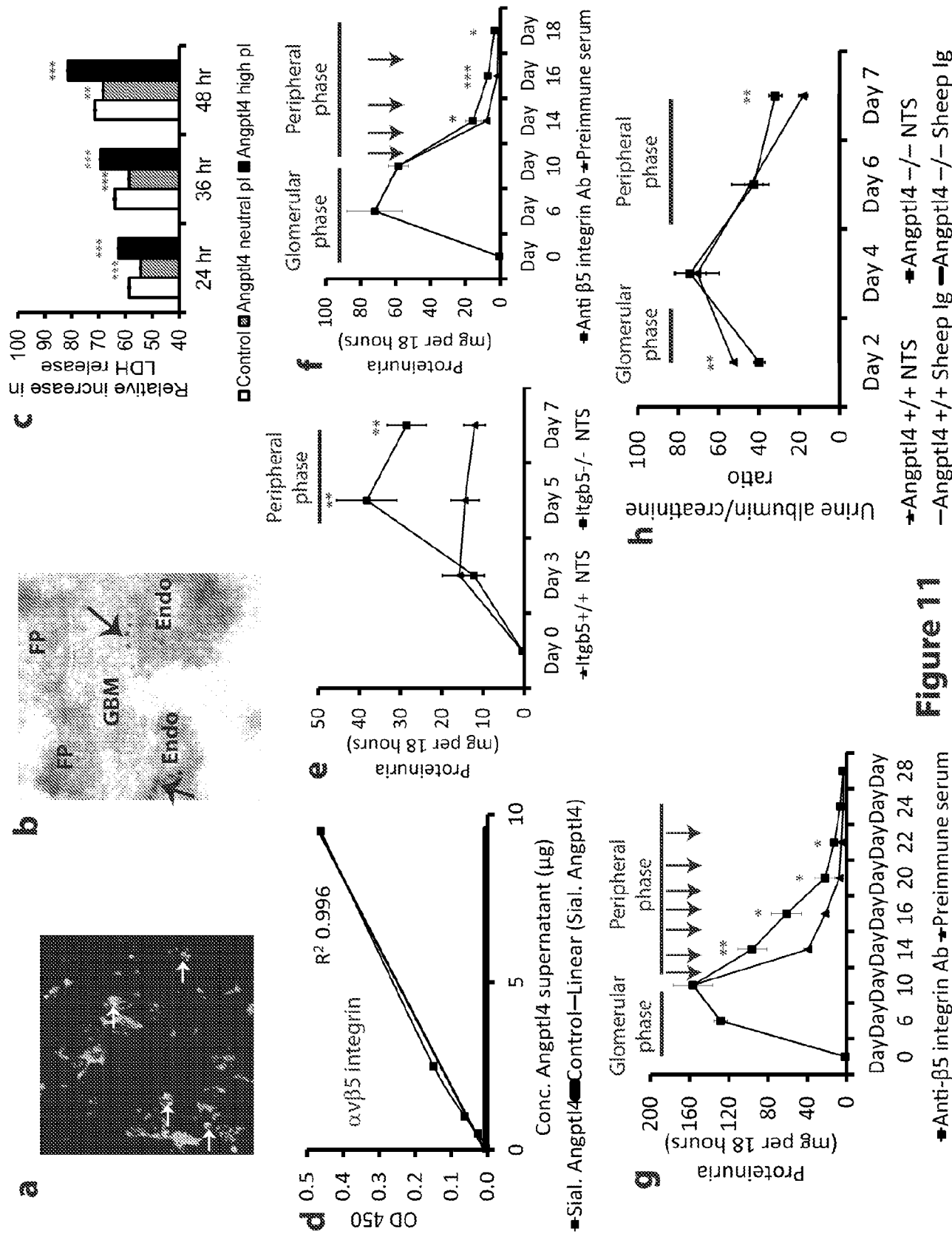


FIG 12A

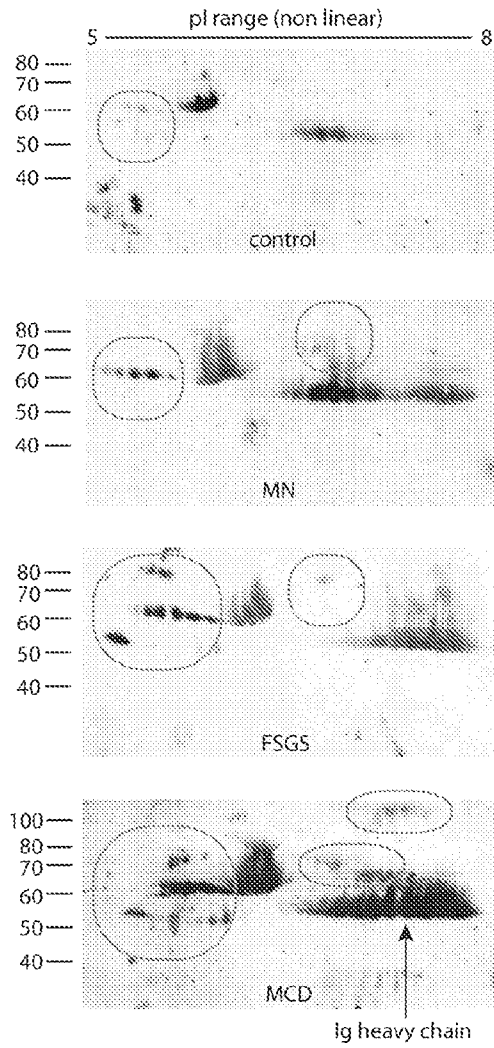
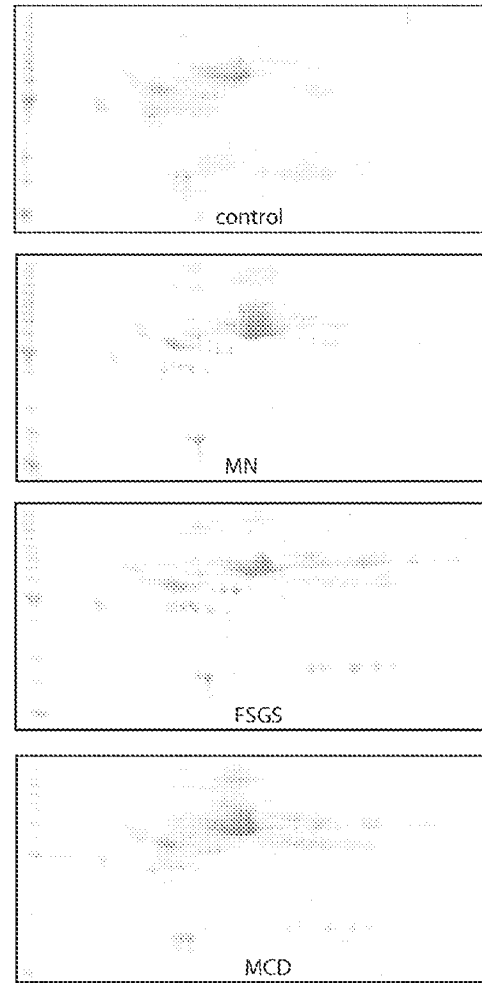


FIG. 12B



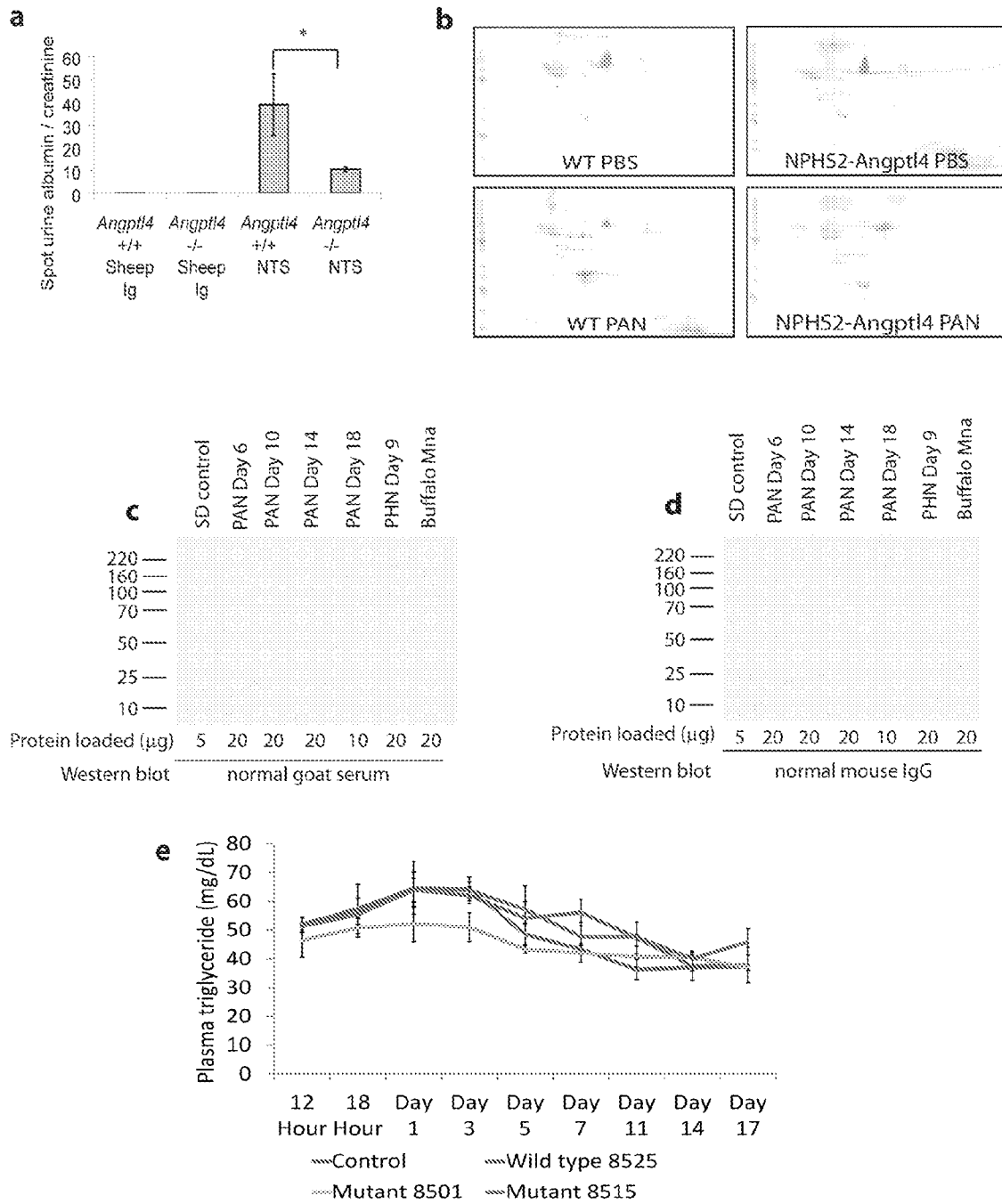


FIG. 13

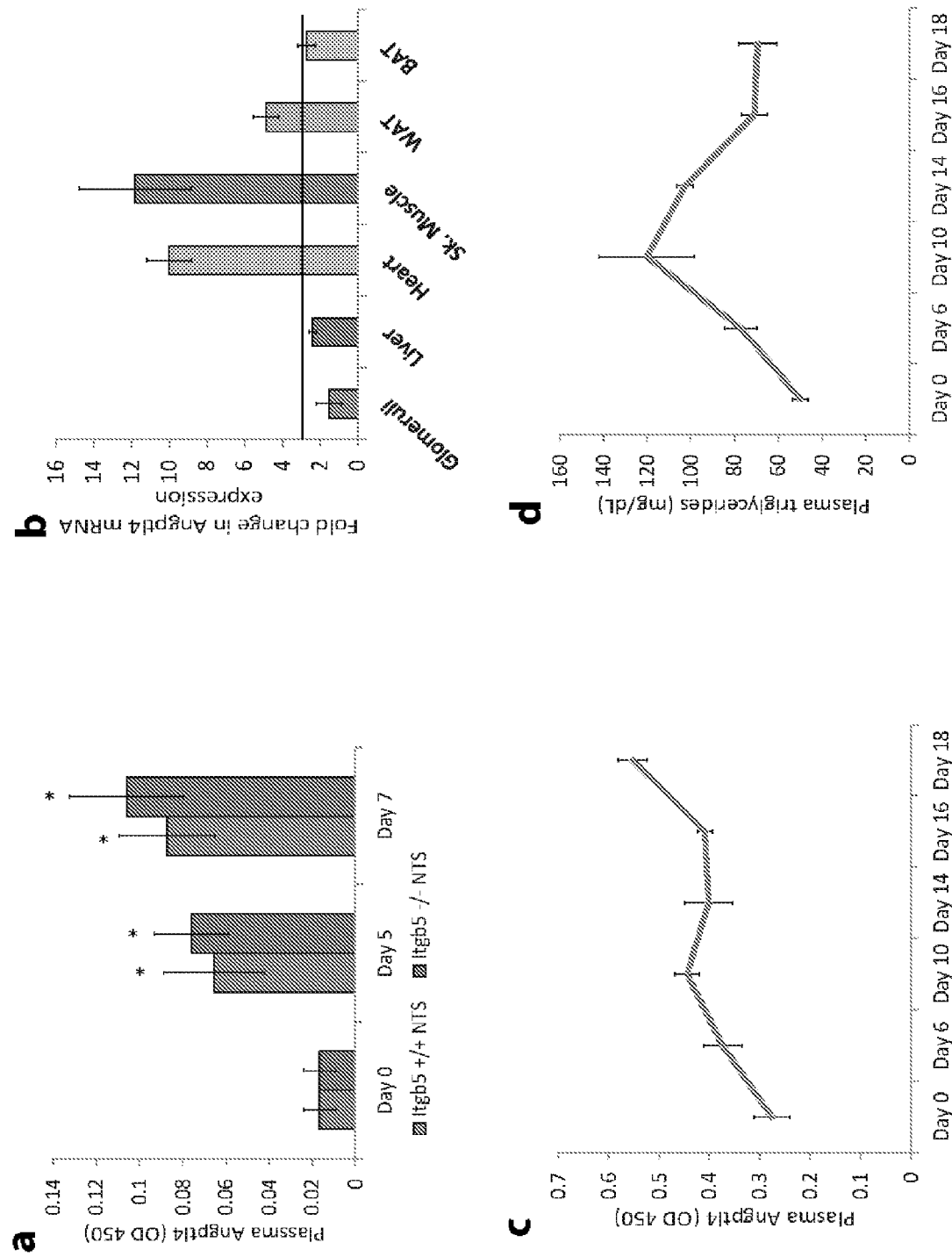


FIG. 14

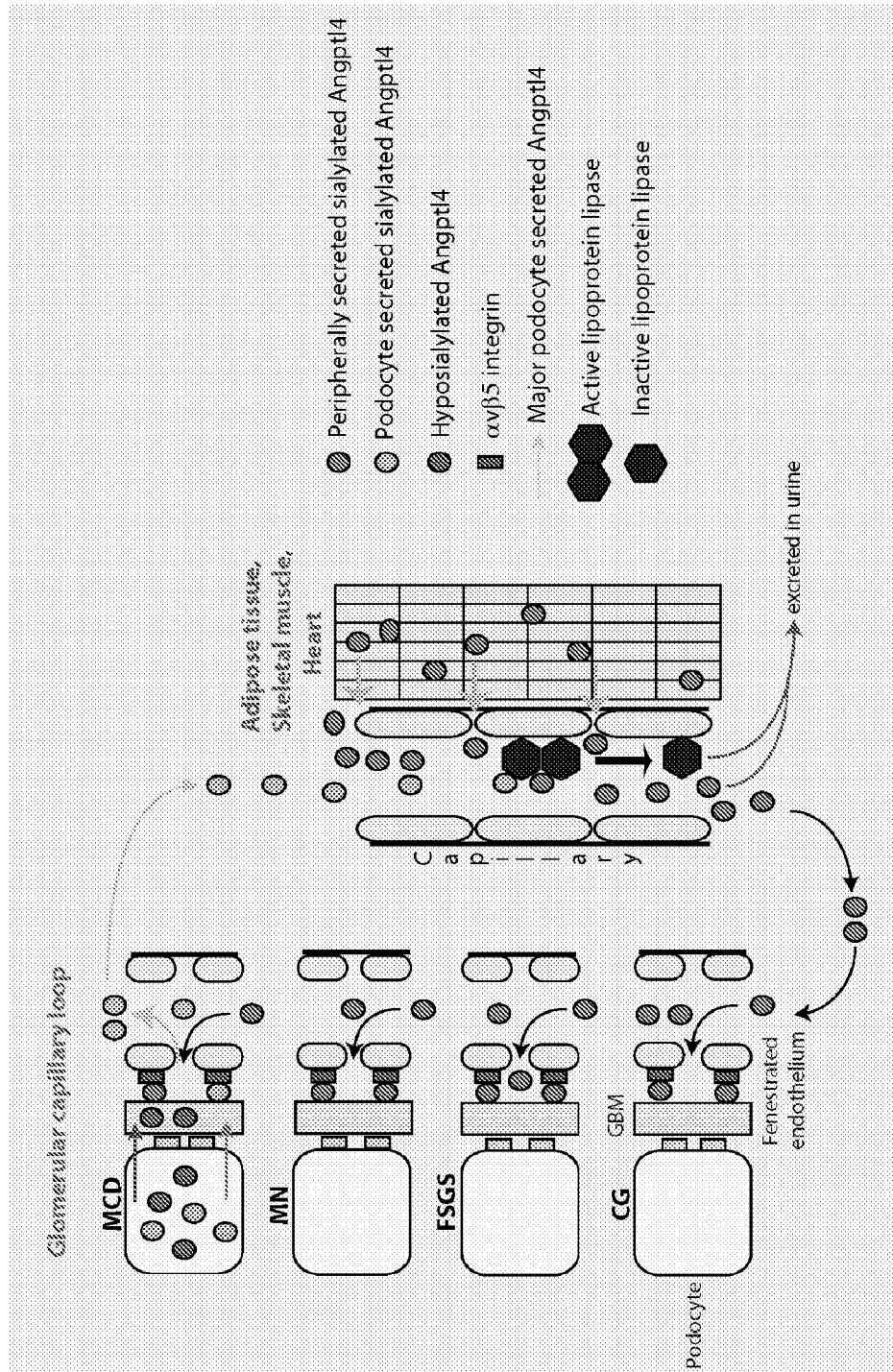


FIG. 15

FIG. 16

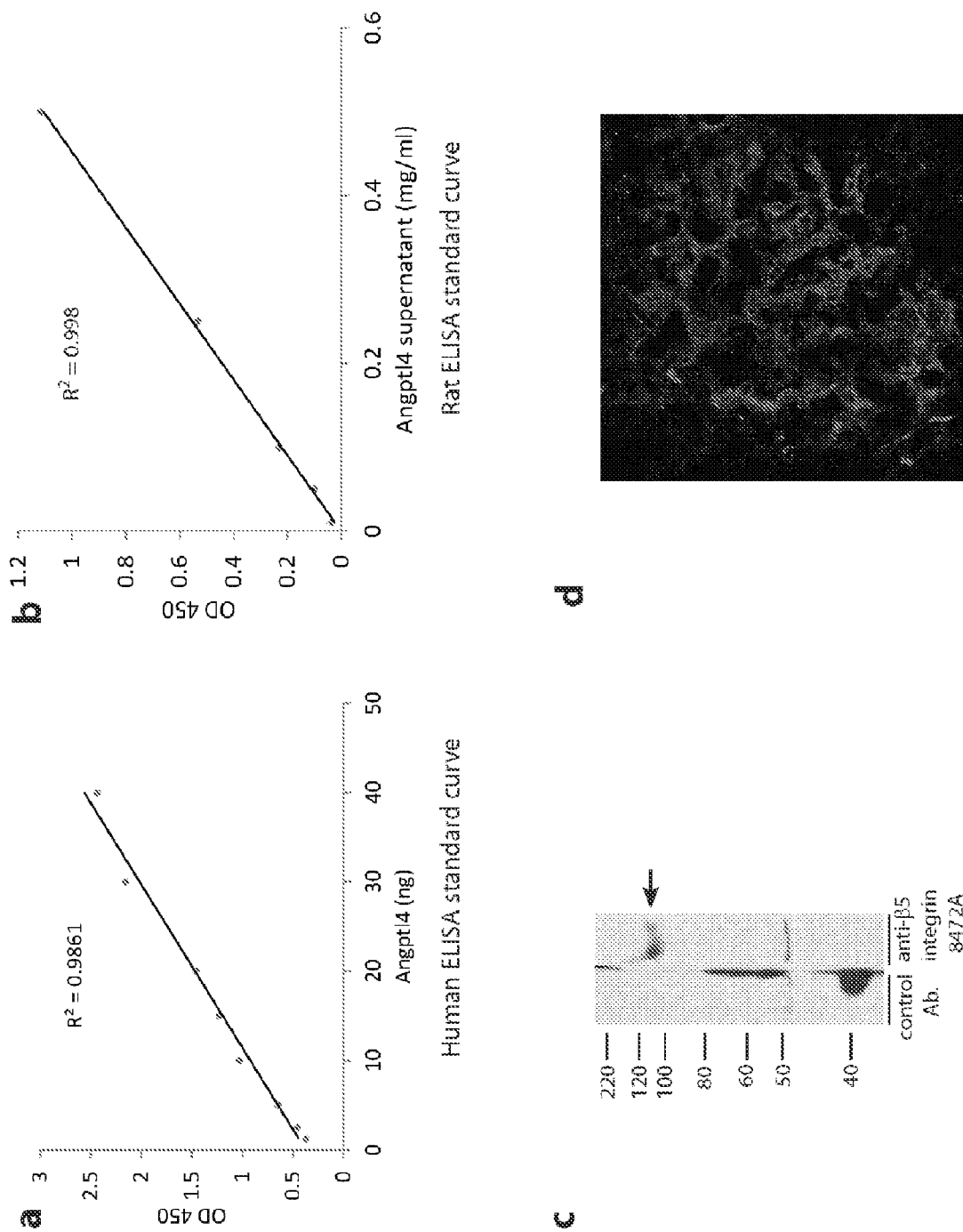


FIG. 17

Amino acid change		cDNA mutagenesis	
Wild type 8525	39 DE 40; 161 RRKR 164	115 <u>GAC</u> <u>GAG</u> 120	479 CC <u>CGA</u> <u>AGA</u> <u>AAG</u> <u>AGG</u> CT 494 D E R R K R
Mutant 8496	39 DK 40; 161 GAAG 164	115 <u>GAC</u> <u>AAG</u> 120	479 CC <u>GGA</u> <u>GCA</u> <u>GCA</u> <u>GGA</u> CT 494 D K G A A G
Mutant 8501	39 DK 40; 161 GSGS 164	115 <u>GAC</u> <u>AAG</u> 120	479 CC <u>GGA</u> <u>TCA</u> <u>GGA</u> <u>TCA</u> CT 494 D K G S G S
Mutant 8506	39 DK 40; 161 AAVV 164	115 <u>GAC</u> <u>AAG</u> 120	479 CC <u>GCC</u> <u>GCC</u> <u>GTG</u> <u>GTG</u> CT 494 D K A A V V
Mutant 8511	39 DA 40; 161 GVVA 164	115 <u>GAC</u> <u>GCA</u> 120	479 CC <u>GGC</u> <u>GTC</u> <u>GTC</u> <u>GCG</u> CT 494 D A G V V A
Mutant 8515	39 KE 40; 161 SGGG 164	115 <u>AAG</u> <u>GAG</u> 120	479 CC <u>TCC</u> <u>GGC</u> <u>GGC</u> <u>GGC</u> CT 494 K E S G G G
Mutant 8520	39 AE 40; 161 VAVA 164	115 <u>GCA</u> <u>GAG</u> 120	479 CC <u>GTC</u> <u>GCC</u> <u>GTG</u> <u>GCG</u> CT 494 A E V A V A

FIG. 18

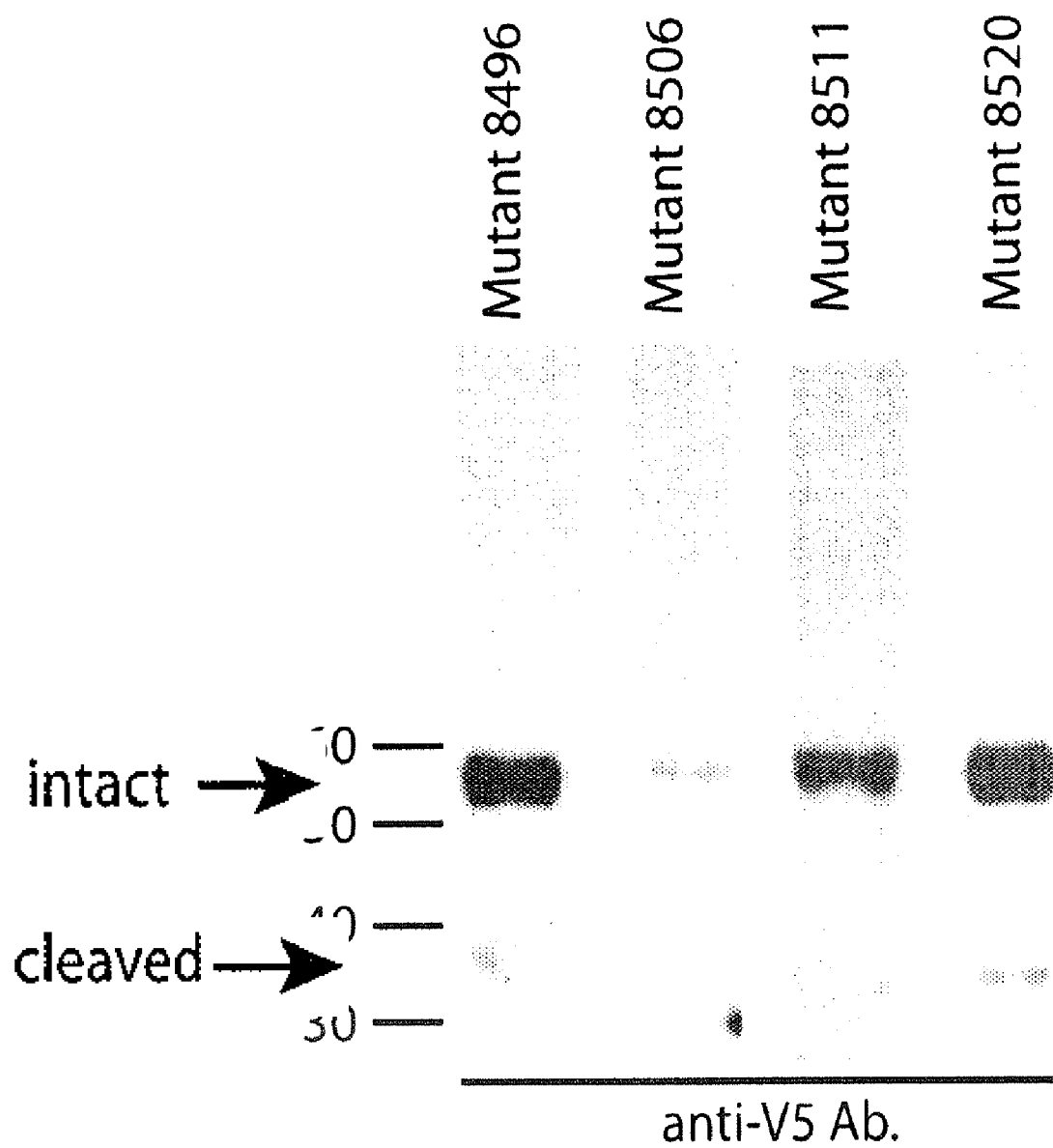


FIG. 19

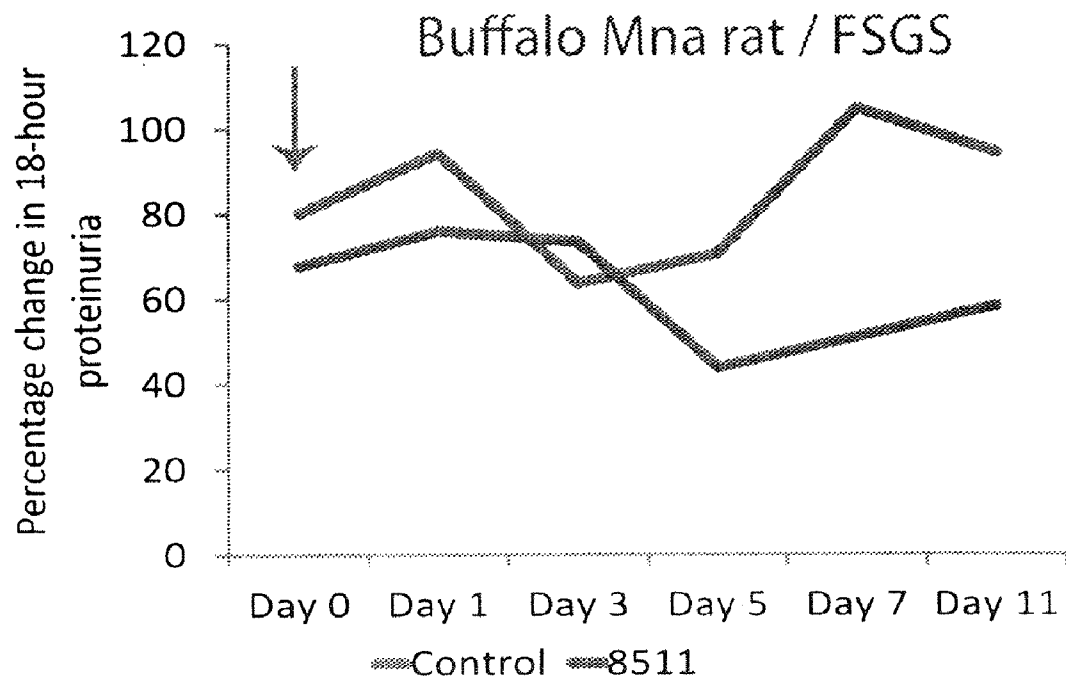


FIG. 20

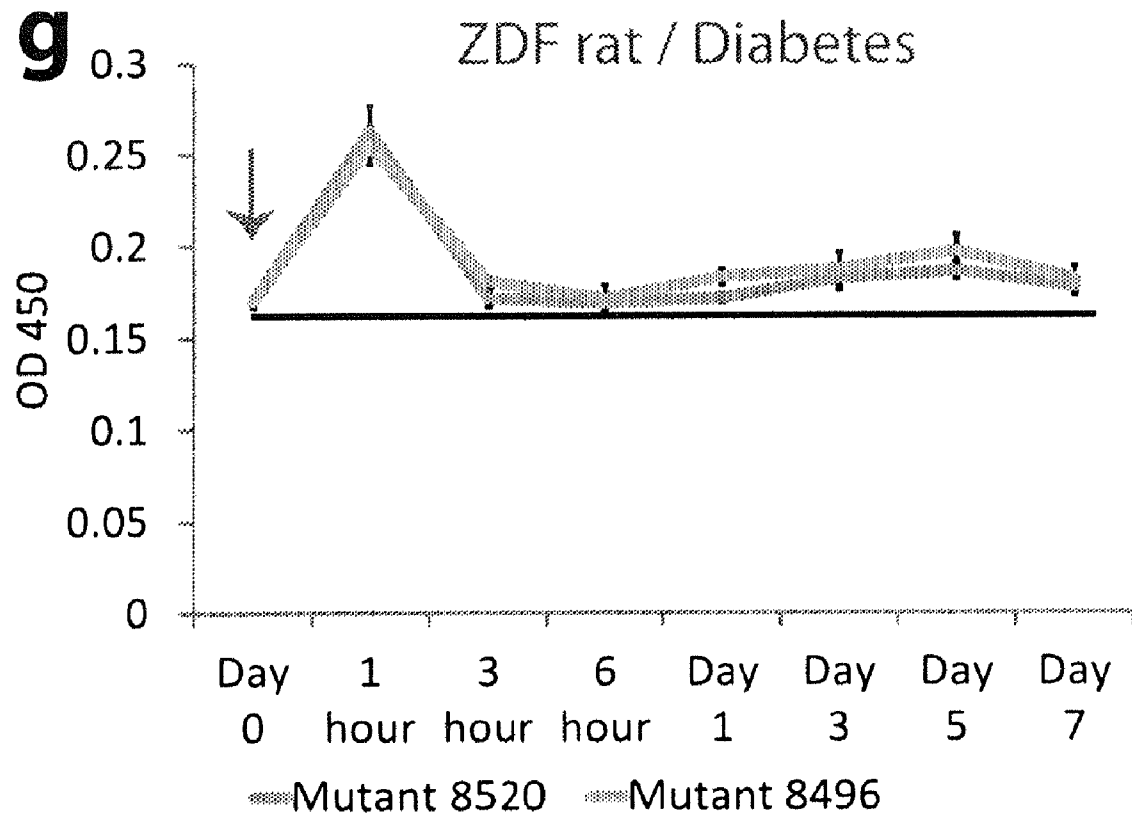


FIG. 21

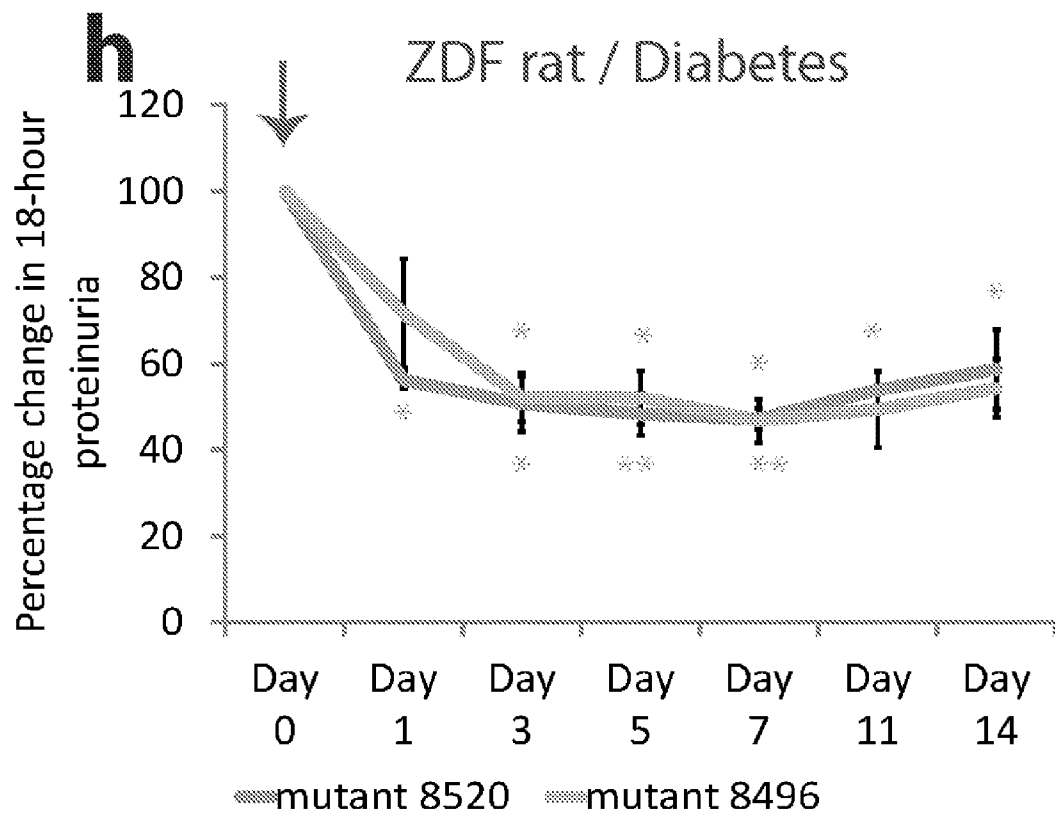
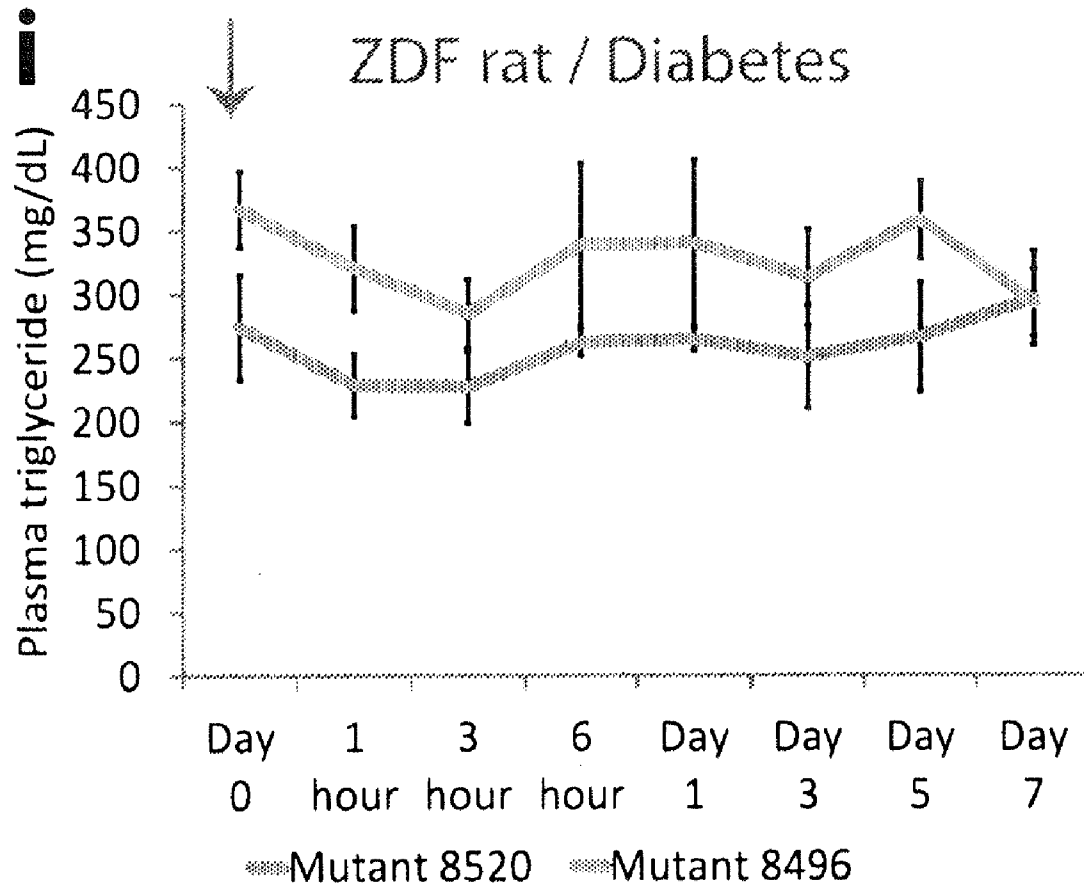


FIG. 22



Buffalo Mna rat / FSGS

