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

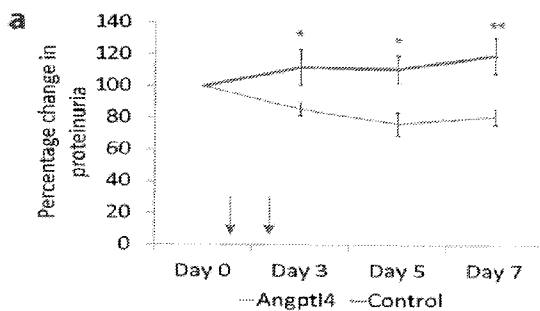
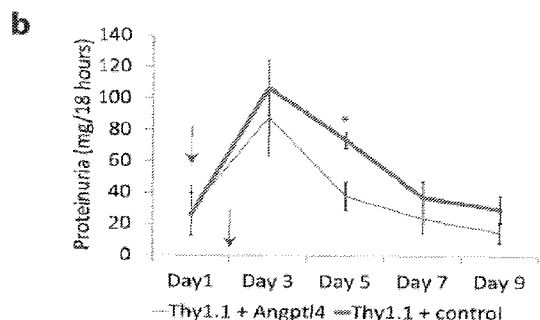


FIG. 4

Buffalo Mna  
rat (FSGS)



Thy1.1 model  
(mesangial injury)

(57) Abstract: The present disclosure provides a method for treating and/or preventing nephrotic syndrome, such as but not limited to MCD and MN, 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

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## 5 STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

Funding for the work described herein was provided by the federal government, which has certain rights in the invention.

## FIELD OF THE DISCLOSURE

10 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

15 Nephrotic syndrome (NS) is a general term that refers to the loss of protein in the urine (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.

20 Podocytes (or visceral epithelial cells) are cells in the outer layer of the glomerular capillary 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 processes, or "foot projections," 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  
25 of the glomerular capillary loop is made of fenestrated endothelial cells. Kidneys affected by nephrotic syndrome have abnormalities in the glomerular capillary loop that cause leakage of blood proteins, resulting in proteinuria.

30 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 membranous glomerulonephritis, MGN), and membranoproliferative glomerulonephritis (MPGN). For years pathologists found no changes in MCD tissue when  
5 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. Diabetic nephropathy is the most common cause of nephrotic syndrome.

10 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 angiopoietin-like 4 (Angptl4), inhibit the activity of LPL.

The molecular basis of nephrotic syndrome is not known. Increased levels of Angptl4  
15 have 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  
20 proteinuria and glucocorticoid sensitivity in 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  
25 levels lags behind the response of proteinuria to glucocorticoids in certain forms 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  
30 preventing nephrotic syndrome, such as but not limited to, MCD, FSGS, MN/MGN, MPGN and diabetic nephropathy as 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  $\mu$ g 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  $\mu$ g 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  $\mu$ g 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  $\mu$ g / 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).

- 5 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

- 10 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

- 15 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

- 20 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 show 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.

- 25 FIG. 4B 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. 4B 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

5 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.

## 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 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, 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, 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 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.

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 Angptl4 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

Angptl4 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 $\gamma$  (8) and PPAR $\alpha$  (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,

5 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  
5 Angptl4 polypeptide derivatives.

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  
10 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  
15 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  
20 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  
25 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  
30 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 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 alleviates one or more symptoms of nephrotic syndrome by providing

Angptl4 function. In an 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 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 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 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.

### 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.

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 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 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.

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 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 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  
5 *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  
10 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  
15 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  
20 mutations conferring reduced triglyceride levels implying a decreased inhibition of LPL (33 and 34, each of which are incorporated by reference for the teaching of Angptl4 variants). 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 (R<sub>161</sub>RKR<sub>164</sub> of SEQ ID NOS: 1 and 3) to  
25 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  
30 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. In one embodiment, amino acid residues of the wild type Angptl 4 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.

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

<b>G</b>	Glycine	<b>P</b>	Proline
<b>A</b>	Alanine	<b>V</b>	Valine
<b>L</b>	Leucine	<b>I</b>	Isoleucine
<b>M</b>	Methionine	<b>C</b>	Cysteine
<b>F</b>	Phenylalanine	<b>Y</b>	Tyrosine
<b>W</b>	Tryptophan	<b>H</b>	Histidine
<b>K</b>	Lysine	<b>R</b>	Arginine
<b>Q</b>	Glutamine	<b>N</b>	Asparagine
<b>E</b>	Glutamic Acid	<b>D</b>	Aspartic Acid
<b>S</b>	Serine	<b>T</b>	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 (see references 33 and 34 which are herein incorporated by reference for such teaching). In one embodiment, such change occurs in residues 1-187 with respect to SEQ ID NO: 1 or residues 1-182 of SEQ ID NO: 3. In an alternate embodiment, such change occurs at position 40 with respect to SEQ ID NOS: 1, 3, 5 or 7 or SEQ ID NOS: 1 or 3. 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 of SEQ ID NOS: 1 and 3 is selected from those shown in Table 1 below. In yet a further embodiment, the amino acid at position 39 (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 of a D39A substitution. In a further particular embodiment, the change at position 39 of SEQ ID NOS: 1 and 3 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.

Table 1

Modifications of E <sub>40</sub> 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 variant 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 R<sub>161</sub>RKR<sub>164</sub> region of Angptl4. The change may be a replacement, deletion and/or substitution of one or more residues in this region. The R<sub>161</sub>RKR<sub>164</sub> 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 R<sub>161</sub>RKR<sub>164</sub> region are changed; in an alternate embodiment, 1, 2 or 3 amino acid residues of the R<sub>161</sub>RKR<sub>164</sub> 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 R<sub>161</sub>RKR<sub>164</sub> amino acid sequence of SEQ ID NOS: 1 or 3 is replaced with G<sub>161</sub>AAG<sub>164</sub>; in a further specific embodiment, the R<sub>161</sub>RKR<sub>164</sub> amino acid sequence of SEQ ID NOS: 1 or 3 is replaced with G<sub>161</sub>SGS<sub>164</sub>. Exemplary amino acid sequences for replacement of the entire R<sub>161</sub>RKR<sub>164</sub> region of SEQ ID NOS: 1 or 3 is provided in Table 2 below.

Table 2

Modifications of 161RRKR164 in the human Angptl4 protein								
GAAG	GA AV	GA VV	GV VV	GAAA	AA VV	VVVV	GSGS	GSGG
GAGA	GA VA	GV AV	VG VV	AGAA	AA VA	SSSS	GSSG	SGGG
GGAA	GV AA	GV VA	VV VG	AA AG	AA AV	GGGG	GGSS	GGSG
AGGA	AG VA	AG VV	VV GV	AAGA	AV AA	AAAA	SGSG	GGGS
AGAG	AG AV	AV VG			VAAA		SGGS	
AAGG	AA VG	AV GV			AV VV		SSGG	
	AAGV	VG AV			VAVV			
	AV AG	VG VA			VV VA			
	AV GA	VAGV			VV AV			
	VG AA	VAVG						
	VA AG	VV GA						
	VAG A	VV AG						

In a further embodiment, one or more of the amino acids in the R<sub>161</sub>RKR<sub>164</sub> amino acid sequence of SEQ ID NOS: 1 or 3 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 C<sub>76</sub> and/or C<sub>80</sub> region of Angptl4. The C<sub>76</sub> and/or C<sub>80</sub> region has been shown to be involved in oligomerization of the Angptl4 polypeptide (34, which reference is incorporated herein for such teaching). 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.

In a further embodiment, the variant comprises one or more changes in the R<sub>161</sub>RKR<sub>164</sub> 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 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 X<sub>39</sub>, X<sub>40</sub>, X<sub>76</sub>, X<sub>80</sub>, X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub>, 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 X<sub>39</sub>, X<sub>40</sub>, X<sub>76</sub>, X<sub>80</sub>, X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub>, respectively.

In SEQ ID NOS: 9 and 10, X<sub>39</sub> may be A, G, P, V, L, I, M, C, F, Y, W, H, R, Q, N, S, T or K. In one embodiment, X<sub>39</sub> is a neutral or positively charged amino acid. In a further embodiment, X<sub>39</sub> may be A or K. In still a further embodiment, X<sub>39</sub> may be D.

In SEQ ID NOS: 9 and 10, X<sub>40</sub> may be A, G, P, V, L, I, M, C, F, Y, W, H, R, Q, N, S, T or K. In one embodiment, X<sub>40</sub> is a neutral or positively charged amino acid. In a further embodiment, X<sub>40</sub> may be A or K. In still a further embodiment, X<sub>40</sub> may be E. In yet a further embodiment, X<sub>40</sub> may be E when X<sub>39</sub> is not D and X<sub>39</sub> may be D when X<sub>40</sub> is not E.

In SEQ ID NOS: 9 and 10, at least one of X<sub>76</sub> and X<sub>80</sub> may be substituted. In one embodiment, X<sub>76</sub> and X<sub>80</sub> are independently A or S or C. In one embodiment, one of X<sub>76</sub> and X<sub>80</sub> may be A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C. In a further embodiment, both of X<sub>76</sub> and X<sub>80</sub> may be independently A or S. In still a further embodiment, both of X<sub>76</sub> and X<sub>80</sub> may C.

In SEQ ID NOS: 9 and 10, at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> may be substituted. In one embodiment, all 4 of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are substituted; in an alternate embodiment, 1, 2 or 3 of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are substituted. In a further embodiment, X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> 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 hydropathic index of +/- 1 or less or with residues having a difference in hydrophilicity values of +/- 1 or less.

In a one embodiment, X<sub>39</sub> is D, X<sub>40</sub> is A or K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X<sub>39</sub> is D, X<sub>40</sub> is A or K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are selected from the combinations shown in Table 2. In still another embodiment, X<sub>39</sub> is D, X<sub>40</sub> is A or K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are GSGS or GAAG.

In an additional embodiment, X<sub>39</sub> is D, X<sub>40</sub> is A or K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3. In a further embodiment, X<sub>39</sub> is D, X<sub>40</sub> is A or K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are selected from the combinations shown in Table 2. In still a further embodiment, X<sub>39</sub> is D, X<sub>40</sub> is A or K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are GSGS or GAAG.

In one embodiment, X<sub>39</sub> is A or K, X<sub>40</sub> is E, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X<sub>39</sub> is A or K, X<sub>40</sub> is E, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are selected from the combinations shown in Table 2. In still another embodiment, X<sub>39</sub> is A or K, X<sub>40</sub> is E, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are GSGS or GAAG.

In one embodiment, X<sub>39</sub> is D, X<sub>40</sub> is K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X<sub>39</sub> is D, X<sub>40</sub> is K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are selected from the combinations shown in Table 2. In still another embodiment, X<sub>39</sub> is D, X<sub>40</sub> is K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are GSGS or GAAG.

In one embodiment, X<sub>39</sub> is D, X<sub>40</sub> is K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3. In another embodiment, X<sub>39</sub> is D, X<sub>40</sub> is K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are selected from

the combinations shown in Table 2. In still another embodiment, X<sub>39</sub> is D, X<sub>40</sub> is K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> 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.

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 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 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 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 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.

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  $\pm 2$  may be used; in an alternate embodiment, the hydropathic indices are with  $\pm 1$ ; in yet another alternate embodiment, the hydropathic indices are within  $\pm 0.5$ .

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			
Original Amino Acid	Amino	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 Or 3 or 5 or 7, 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 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. 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 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 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 found in Remington: The Science and Practice of Pharmacy (20<sup>th</sup> 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 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 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 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 (20<sup>th</sup> Ed., 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 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, 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 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 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 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.

## RESULTS

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 (this reference is hereby incorporated by reference for the disclosure contained therein regarding the use of Angptl4 polypeptides).

Patients with Nephrotic Syndrome Have Increased Levels of Circulating Angptl4

Patients with Nephrotic syndrome have increased circulating levels of Angptl4 polypeptide. 200  $\mu$ g 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 pI form and is present as monomers and oligomers.

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 (BAT) and white adipose tissue (WAT) (FIG. 1C).

2D gel electrophoresis of 200  $\mu$ g 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  $\mu$ g plasma, followed by Western blotting using anti-Angptl4 and anti-V5 antibodies show the 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 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), which is hereby incorporated by reference for such teaching).

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.

#### 5 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 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  $\mu$ g / lane, except MCD remission) (densitometry readings are provided under each lane). The intact  
 10 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-Angptl4 TG rats (a rat transgenic model having podocytes specific Angptl4 expression and shown to develop MCD with proteinuria; see US  
 15 Provisional application No. , which is hereby incorporated by reference for such teaching), 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 controls. FIG. 2C shows the same results for aP2-Angptl4 heterozygous male TG rats. FIG. 2D shows  
 20 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 nephrotic syndrome.

25 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 were subject to oxidative injury by addition of hydrogen peroxide and into  
 30 the culture media and incubated with concentrated supernatant (600  $\mu$ 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 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  $\mu$ g 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 theThy1.1 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

### 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.

#### Injection of NTS into Angptl4 <sup>-/-</sup> mice

Angptl4 <sup>-/-</sup> 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 <sup>-/-</sup> or <sup>+/+</sup> mice (n = 4 mice/group) were injected intravenously with 1.5 mg  $\gamma$ 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

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  $\mu$ M H<sub>2</sub>O<sub>2</sub>, along with 600  $\mu$ 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 H<sub>2</sub>O<sub>2</sub> or stable cell line supernatant was added.

#### Statistical analysis

5 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.

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## CLAIMS

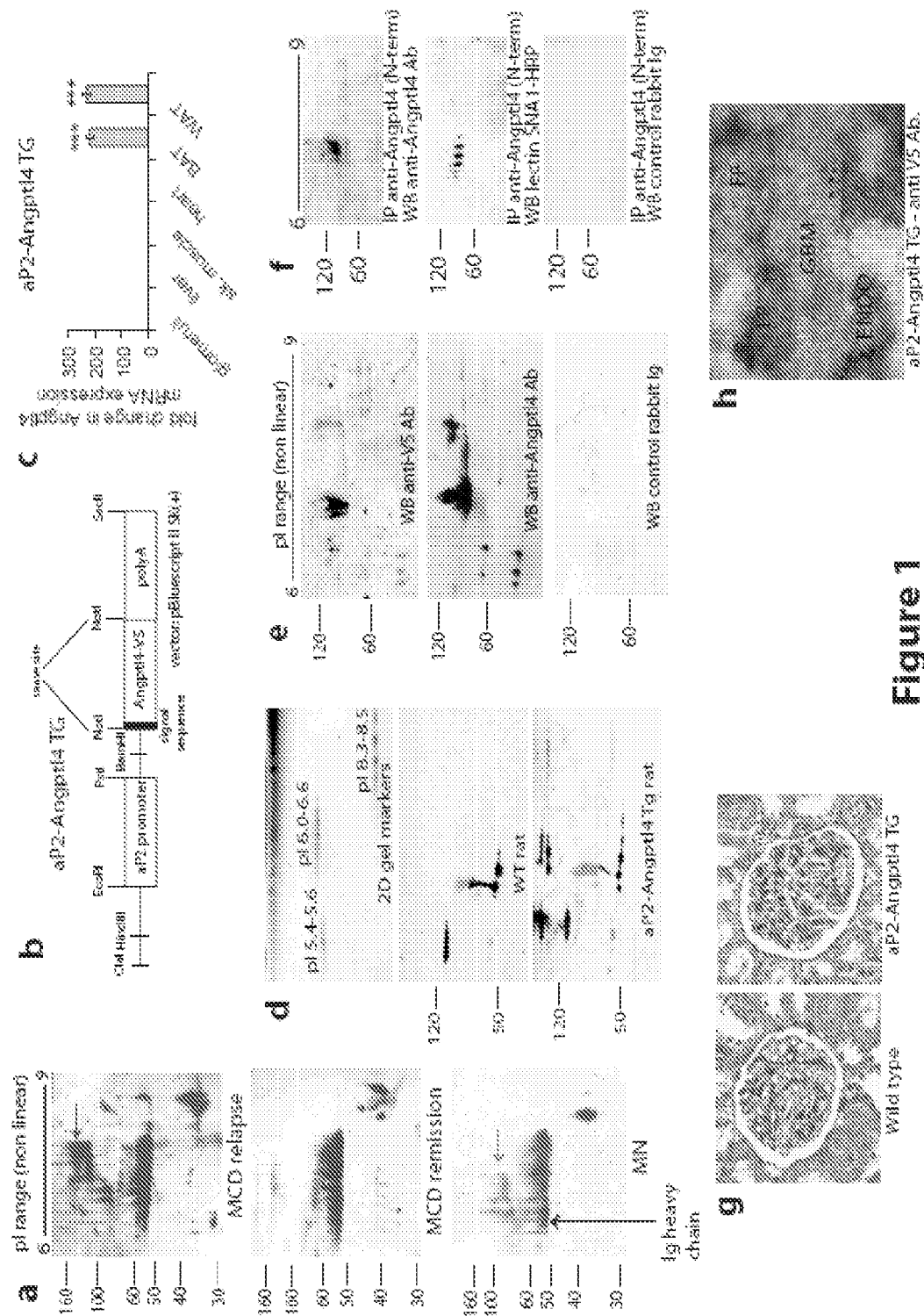
What is claimed:

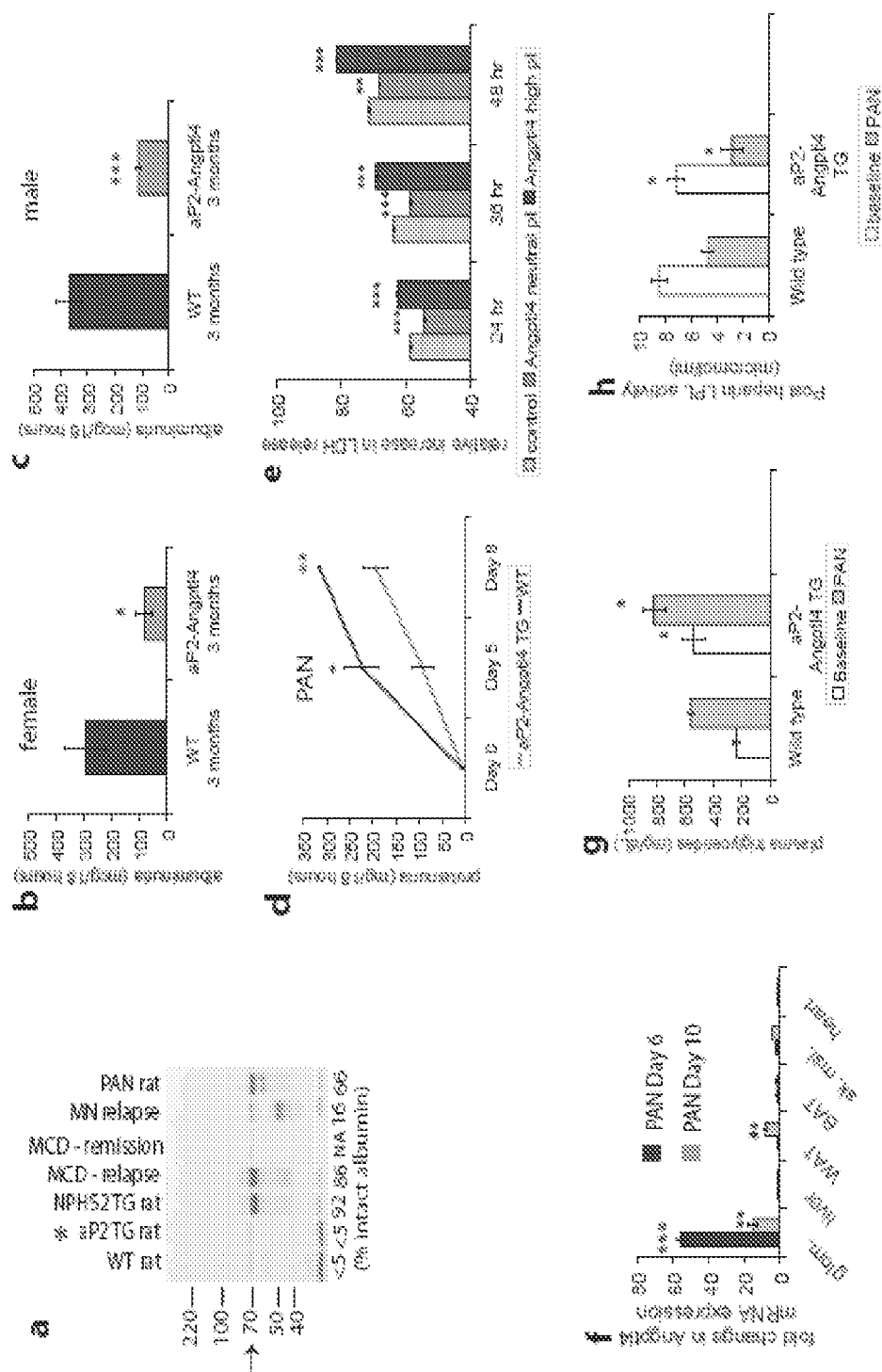
1. A method for the treatment or prevention of nephrotic syndrome in a subject, said method comprising the step of administering an Angptl4 polypeptide, an Angptl4 polypeptide derivative or a combination of the foregoing to the subject.
2. The method of claim 1, wherein the nephrotic syndrome is characterized as minimal change disease, focal segmental glomerulosclerosis, membranous nephropathy/membranous glomerulonephritis, membranoproliferative glomerulonephritis or a diabetic condition.
3. The method of claim 1, wherein said method comprises the step of administering an Angptl4 polypeptide.
4. The method of claim 1, wherein the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 1 or 3.
5. The method of claim 1, wherein the Angptl4 polypeptide comprises the sequence of SEQ ID NOS: 5 or 7.
6. The method of claim 1, wherein said method comprises the step of administering an Angptl4 polypeptide derivative.
7. The method of claim 6, wherein the Angptl 4 polypeptide derivative has decreased lipoprotein lipase inhibitory activity, is resistant to cleavage, or a combination of the foregoing.
8. The method of claim 6, wherein the Angptl 4 polypeptide derivative has decreased lipoprotein lipase inhibitory activity.
9. The method of claim 8, wherein the Angptl 4 polypeptide derivative contains an amino acid substitution at position 40 with respect to the wild-type Angptl4 polypeptide.
10. The method of claim 8, wherein the Angptl 4 polypeptide derivative contains and E40K substitution.
11. The method of claim 8, wherein the Angptl 4 polypeptide derivative contains and E40A substitution.
12. The methods of claims 10 or 11, wherein the Angptl 4 polypeptide derivative further contains an D39K or a D39A substitution.

13. The method of claim 6, wherein the Angptl 4 polypeptide derivative is resistant to cleavage.
14. The method of claim 13, wherein the Angptl 4 polypeptide derivative contains an amino acid substitution at one or more of positions 161, 162, 163 or 164.
15. The method of claim 13, wherein the Angptl 4 polypeptide derivative contains at least one amino acid substitution, wherein the arginine residue at one or more of positions 161, 162 and 164 substituted with a neutral amino acid and the lysine residue at position 163 is substituted with a neutral amino acid.
16. The method of claim 13, wherein the Angptl 4 polypeptide derivative contains at least one amino acid substitution, wherein the arginine residue at one or more of positions 161, 162 and 164 substituted with D, R, K, G, A, V or S and the lysine residue at position 163 is substituted with D, R, K, G, A, V or S.
17. The method of claim 13, wherein the Angptl 4 polypeptide derivative contains an R<sub>161</sub>RKR<sub>164</sub> to a G<sub>161</sub>SGS<sub>164</sub> substitution.
18. The methods of claim 6, wherein the Angptl 4 polypeptide derivative has the sequence of SEQ ID NOS: 9 or 10, wherein X<sub>39</sub> is D, X<sub>40</sub> is A or K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3.
19. The methods of claim 6, wherein the Angptl 4 polypeptide derivative has the sequence of SEQ ID NOS: 9 or 10, wherein X<sub>39</sub> is D, X<sub>40</sub> is A or K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3.
20. The methods of claim 6, wherein the Angptl 4 polypeptide derivative has the sequence of SEQ ID NOS: 9 or 10, wherein X<sub>39</sub> is A or K, X<sub>40</sub> is E, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3.
21. The methods of claim 6, wherein the Angptl 4 polypeptide derivative has the sequence of SEQ ID NOS: 9 or 10, wherein X<sub>39</sub> is D, X<sub>40</sub> is K, X<sub>76</sub> and X<sub>80</sub> are C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S,

optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3.

22. The methods of claim 6, wherein the Angptl 4 polypeptide derivative has the sequence of SEQ ID NOS: 9 or 10, wherein X<sub>39</sub> is D, X<sub>40</sub> is K, one of X<sub>76</sub> and X<sub>80</sub> is A or S and the other of X<sub>76</sub> and X<sub>80</sub> is C and X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> are independently substituted with D, R, K, G, A, V or S, optionally provided that at least one of X<sub>161</sub>, X<sub>162</sub>, X<sub>163</sub> and X<sub>164</sub> is an amino acid not found in SEQ ID NOS: 1 or 3.
23. The method of claim 1, wherein the Angptl4 polypeptide or the Angptl4 polypeptide derivative is administered in a therapeutically effective amount.
24. The method of claim 1, wherein the administering reduces edema, reduces proteinuria, increases plasma albumin levels, reduces hypercholesterolemia, reduces hypertriglyceridemia or a combination of the foregoing.
25. The method of claim 1, wherein the Angptl4 polypeptide or the Angptl4 polypeptide derivative is sialylated.
26. The methods of claim 1 wherein the nephrotic condition is minimal change disease.
27. The methods of claim 2, wherein the diabetic condition is diabetic nephropathy, diabetes mellitus, lupus nephritis or primary glomerular disease.





Gene / transgene	Species	Forward primer	Reverse primer	Taqman probe
Angptl4	rat	tcctggatctccaccattttg	tcaccgcccagctccat	caacigtgagatgacac
Angptl4	rat	cgccaccgcgttaccaca	cagaggctggatciggaaaagt	tgcagggaactcttt
aP2-Angptl4 construct	rat	tgttgatccagcccattgga	aggatagggttaccttcgaag	caggagctccc
Prolactin (genomic)	rat	ctgaagggaattgaaaagataattagc	ccatgagtcagaaaagcattgaac	aggtagcattttcttg

**Figure 3**

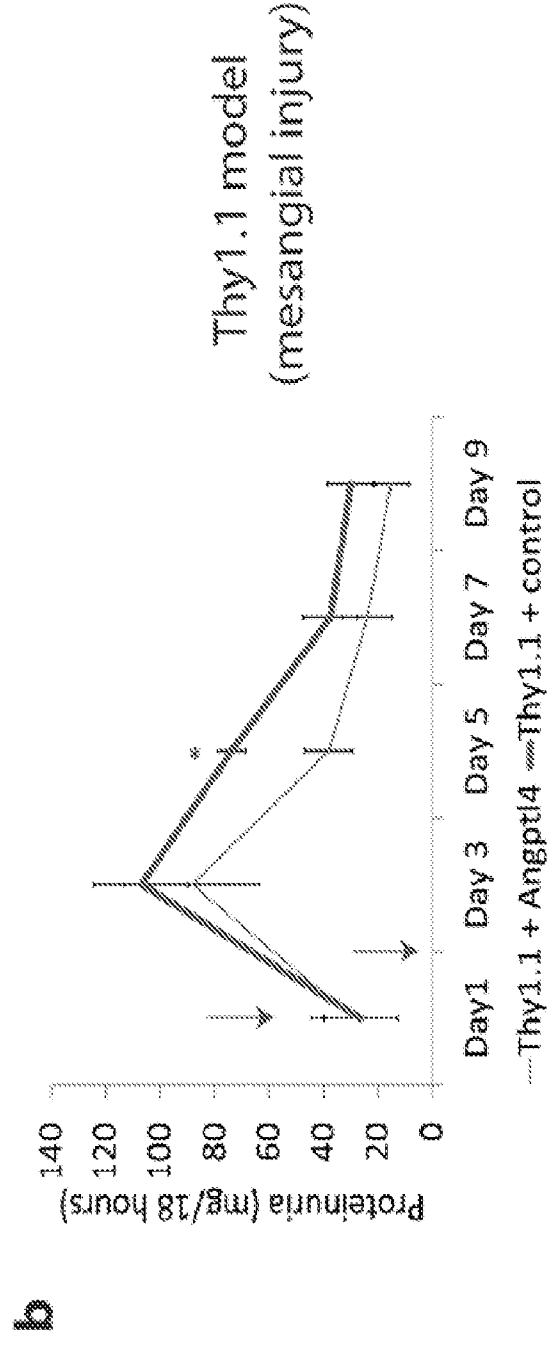
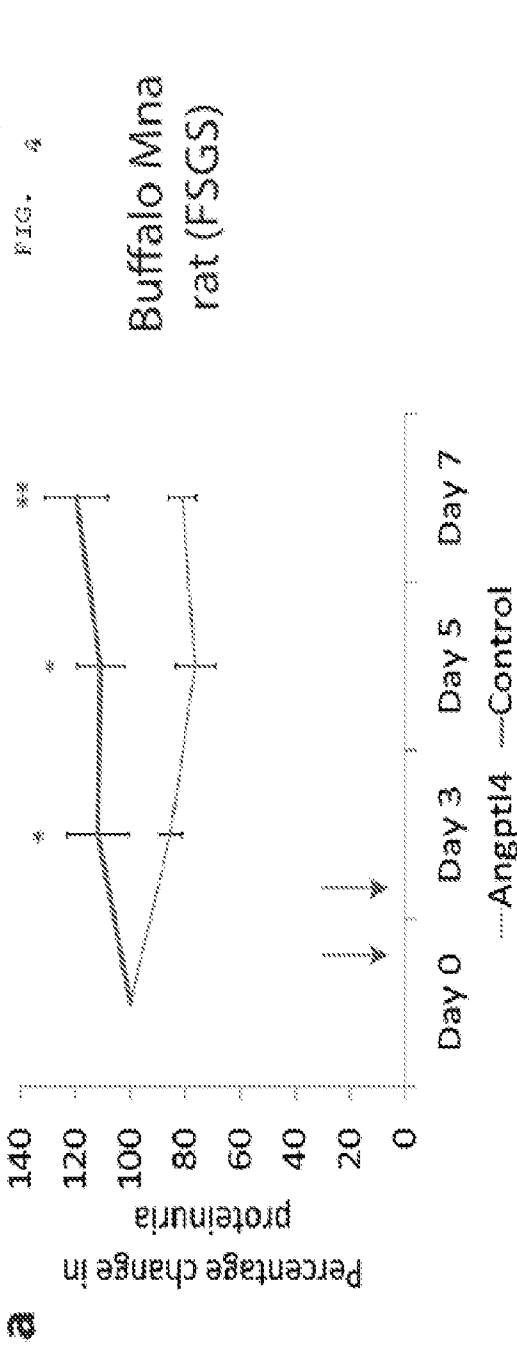


FIG. 5

## SEQ ID NO: 1

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWDEMNVLAHGLLQLGQGLREHAERTRSQLSALER  
 RLSACGSACQGTEGSTDLPLAPESRVDPEVLHSLQTQLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLQSQFGL  
 LDHKHLDHEVAKPARRKRLPEMAQPVDPAHNVSRLLHRLPRDCQELFQVGERQSGLFQIQQGSPFFLVNCKMTSD  
 GGWTVIQRRHDGSVDFNRPWEAYKAGFGDPHGEFWLGLEKVHSITGDRNSRLAVQLRDWDGNAELLQFSVHLGGE  
 DTAYSLQLTAPVAGQLGATTVPFSGLSVPFSTWDQDHDLLRRDKNCAKSLSGGWFWGTCSHNSNLNGQYFRSIPQQR  
 QKLKKGIFWKTWRGRYYPLQATTMLIQPMAAEAAAS

406 amino acids

## SEQ ID NO: 2

1	ataaaaaccg	tcctcggggc	cggcggggag	aagccgagct	gagcggatcc	tcacacgact
61	gtgatccgat	tctttccagc	ggcttctgca	accaagcggg	tcttaccccc	ggtcctccgc
121	gtctccagtc	ctcgcacctg	gaaccccaac	gtccccgaga	gtccccgaat	ccccgcctcc
181	aggctaccta	agaggatgag	cggtgtctcg	acggccgggg	cagccctgat	gctctgcgcc
241	gccaccgcgc	tgctactgag	cgcctcaggg	ggaccctgct	agtcctgaag	gcccgccttt
301	gcgtcctggg	acgagatgaa	tgctcctggc	cacggactcc	tgagcgtcgc	ccaggggcgtg
361	cgcgaacacg	cggagcgcac	ccgcagtcag	ctgagcgcgc	tgagcggcgc	cctgagcgcg
421	tgccgggtcg	cctgtcaggg	aaccgagggg	tccaccgacc	tcccgttagc	ccctgagagc
481	cggtgtggac	ctgaggtcct	tcacagcctg	cagacacaa	tcaaggtc	gaacagcagg
541	atccagcaac	tcttccacaa	ggtggccag	cagcagcggc	acctggagaa	gcagcacctg
601	cgaattcagc	atctgcaaag	ccagtttggc	ctcctggacc	acaagcacct	agaccatgag
661	gtggccaagc	ctgcccgaag	aaagaggctg	cccagagatg	cccagccagt	tgaccgcggc
721	cacaatgtca	gccgcctgca	ccggctgccc	agggattgcc	aggagctgtt	ccaggttggg
781	gagaggcaga	gtggactatt	tgaatccag	cctcaggggt	ctccgccatt	tttgggtgaa
841	tgcaagatga	cctcagatgg	aggctggaca	gtaattcaga	ggcgccacga	tggtcagtg
901	gacttcaacc	ggccctggga	agcctacaag	gcggggtttg	gggatcccca	cggcgagttc
961	tggtctgggt	tggagaaggt	gcatagcata	acgggggacc	gcaacagccg	cctggccgtg
1021	cagctgcggg	actgggatgg	caacgccgag	ttgctgcagt	tctccgtgca	cctgggtggc
1081	gaggacacgg	cctatagcct	gcagctcact	gcaccctggg	ccggccagct	gggcgccacc
1141	accgtcccac	ccagcggcct	ctccgtaccc	ttctccactt	gggaccagga	tcacgacctc
1201	cgcagggaca	agaactgcgc	caagagcctc	tctggaggct	ggtgggtttg	cacctgcagc
1261	cattccaacc	tcaacggcca	gtacttccgc	tccatccac	agcagcggca	gaagcttaag
1321	aagggaatct	tctggaagac	ctggcggggc	cgctactacc	cgctgcaggc	caccaccatg
1381	ttgatccagc	ccatggcagc	agaggcagcc	tcctagcgtc	ctggctgggc	ctggtcccag
1441	gcccacgaaa	gacggtgact	cttggtctct	cccagggatg	tgcccggttc	ctgcctgggc
1501	aggggctcca	aggagggg	atctggaaac	ttgtggacag	agaagaagac	cacgactgga
1561	gaagccccct	ttctgagtgc	aggggggctg	catgcgttgc	ctcctgagat	cgaggctgca
1621	ggatatgctc	agactctaga	ggcgtggacc	aaggggcatg	gagcttccct	ccttgctggc
1681	caggaggttg	gggactcaga	gggaccactt	ggggccagcc	agactggcct	caatggcgga
1741	ctcagtcaca	ttgactgacg	gggaccaggg	cttgtgtggg	tcgagagcgc	cctcatggtg
1801	ctgggtgctg	tgtgtgtagg	ttccctgggg	acacaagcag	gcgccaatgg	tatctggggc
1861	gagctcacag	agttcttggg	ataaaagcaa	cctcagaaca	cttaaaaaaa	aaaaaaaaaa
1921	aaaaaaaaaa	aaaaaaaaaa	aaaaaaaaaa	aaaaaaaaaa	aaaaaa	

## SEQ ID NO: 3

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWDEMNVLAHGLLQLGQGLREHAERTRSQLSALERRLSA  
 CGSACQGTEGSTDLPLAPESRVDPEVLHSLQTQLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLQSQFGLLDHK  
 HLDHEVAKPARRKRLPEMAQPVDPAHNVSRLLHGGWTVIQRRHDGSVDFNRPWEAYKAGFGDPHGEFWLGLEKVH  
 SITGDRNSRLAVQLRDWDGNAELLQFSVHLGGEDTAYSLQLTAPVAGQLGATTVPFSGLSVPFSTWDQDHDLLRRD  
 KNCASLSGGWWFGTCSHNSNLNGQYFRSIPQQRQKLKKGIFWKTWRGRYYPLQATTMLIQPMAAEAAAS

368 amino acids

## SEQ ID NO: 4

1	ataaaaaccg	tcctcggggc	cggcggggag	aagccgagct	gagcggatcc	tcacacgact
61	gtgatccgat	tctttccagc	ggcttctgca	accaagcggg	tcttaccccc	ggtcctccgc

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121      gtctccagtc  ctgcacactg  gaaccccaac  gtccccgaga  gtccccgaat  ccccgctccc
181      aggctaccta  agaggatgag  cgggtgctccg  acggccgggg  cagccctgat  gctctgcgcc
241      gccaccgccg  tgctactgag  cgctcagggc  ggaccctgctc  agtccaagtc  gccgcgcttt
301      gcgtcctggg  acgagatgaa  tgtcctggcg  caccgactcc  tgcagctcgg  ccaggggctg
361      cgcgaacacg  cggagcgcac  ccgcagtcag  ctgagcgcgc  tggagcggcg  cctgagcgcg
421      tgcgggtccg  cctgtcaggg  aaccgagggg  tccaccgacc  tcccgttagc  ccctgagagc
481      cgggtggacc  ctgaggctct  tcacagcctg  cagacacaac  tcaaggctca  gaacagcagg
541      atccagcaac  tcttccacaa  ggtggcccg  cagcagcggc  acctggagaa  gcagcacctg
601      cgaattcagc  atctgcaaag  ccagtttggc  ctccctggacc  acaagcacct  agaccatgag
661      gtggccaagc  ctgcccgaag  aaagaggctg  cccgagatgg  cccagccagt  tgacccgctt
721      cacaatgtca  gccgcctgca  ccatggaggc  tggacagtaa  ttcagaggcg  ccacgatggc
781      tcagtggact  tcaaccggcc  ctgggaagcc  tacaaggcgg  ggtttgggga  tccccacggc
841      gagttctggc  tgggtctgga  gaaggtgcat  agcatcacgg  gggaccgcaa  cagccgcctg
901      gccgtgcagc  tgccgggactg  ggatggcaac  gccgagttgc  tgcagttctc  cgtgcacctg
961      ggtggcgagg  acacggccta  tagcctgcag  ctactgcac  ccgtggccgg  ccagctgggc
1021     gccaccaccg  tcccaccag  cggcctctcc  gtacccttct  ccacttggga  ccaggatcac
1081     gacctccgca  gggacaagaa  ctgcgccaa  agcctctctg  gaggtcgggtg  gtttggcacc
1141     tgcagccatt  ccaacctcaa  cggccagtac  ttccgctcca  tcccacagca  gcggcagaag
1201     cttaagaagg  gaatcttctg  gaagacctgg  cggggccgct  actaccgct  gcaggccacc
1261     accatgttga  tccagcccat  ggcagcagag  gcagcctcct  agcgtcctgg  ctgggcctgg
1321     tcccaggccc  acgaaagacg  gtgactcttg  gctctgccc  aggatgtggc  cgttccctgc
1381     ctgggcaggg  gctccaagga  ggggccatct  ggaaacttgt  ggacagagaa  gaagaccacg
1441     actggagaag  ccccccttct  gagtgcaggg  gggctgcatg  cgttgccctc  tgagatcgag
1501     gctgcaggat  atgctcagac  tctagaggcg  tggaccaagg  ggcattggagc  ttcactcctt
1561     gctggccagg  gagttgggga  ctgagaggga  ccacttgggg  ccagccagac  tggcctcaat
1621     ggcgactca  gtcacattga  ctgacgggga  ccagggcttg  tgtgggtcga  gacgcctc
1681     atggtgctgg  tgctgttggtg  tgtaggctcc  ctggggacac  aagcaggcgc  caatgggtatc
1741     tgggcggagc  tcacagagtt  cttggaataa  aagcaacctc  agaacttta  aaaaaaaaaa
1801     aaaaaaaaaa  aaaaaaaaaa  aaaaaaaaaa  aaaaaaaaaa  aaaaaaaaaa  aaa

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## SEQ ID NO: 5

MRCAPTAGAALVLC AATAGLLSAQGRPAQPEPPRFASWDEMNL LAHGLLQLGHGLREHVERTRGQLGALERRMAA  
CGNACQGPKGTDPKDRVPEGQAPETLQSLQTQLKAQNSKIQQLFQKVAQQQRYLSKQNLRIQNLSQSIDLLTPTH  
LDNGVDKTSRGKRLPKMAQLIGLTPNATRLHRPPRDCQELFQEGERHSGLFQIQPLGSPFLVNCMTSDGGWTV  
IQRRNLNGSVDFNQSW EAYKDGFGDPQGEFWLGLEKMHSITGDRGSQLAVQLQDWDGNAKLLQFPFIHLGGEDTAYS  
LQLTEPTANELGATNVSPNGLSLPFSTWDQDHLRGDLNCAKSLSGGWVFGTCSHSNLNGQYFHSIPRQRQRKK  
GIFWKTWKGRYYPLQATTLLIQPMEATAAS  
405 amino acids

## SEQ ID NO: 6

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1      atgcgctgcg  ctccgaccgc  aggcgctgct  ctagtgtctat  gcgcagctactgcggggctg
61      ctgagcgcgc  aagggcgccc  tgcacagccg  gagccgcgcg  gcttcgcacccctgggatgaa
121     atgaacttgc  tggctcacgg  gctgctgcag  ctccggtcacg  ggctgcgggaacacgtggag
181     cgcacccgtg  gacagctggg  cgcgctggaa  cgccgcatgg  ctgcctgcggtaacgcttgt
241     caggggcccc  aggggacaga  cccgaaggat  agagtccccg  aaggccaggtccttgagact
301     ctgcagagtt  tacagactca  actcaaggct  cagaacagca  agatccagcaactgttccag
361     aaggtagccc  agcagcagag  atacctatca  aagcagaatc  tgagaatacagaatcttcag
421     agccagattg  acctcttgac  cccacacac  ctagacaatg  gggtagacaagacttcgagg
481     ggaaagaggc  ttcccaagat  ggcccagctc  attggcttga  ctcccaacgccaccgcctta
541     cacaggctc  cccgggactg  ccaggaactc  tttcaagaag  gggagcggcacagtggaactt
601     ttccagatcc  agcctctggg  atctccacca  tttttggtca  actgtgagatgacttcagat
661     ggaggctgga  cggtgattca  gagcgcctg  aacggctctg  tggacttcaatcagtccttgg
721     gaagcctaca  aagatggctt  cggagatccc  caaggcgagt  tctggctggcctagagaag
781     atgcacagca  tcacagggga  ccgaggaagc  cagttggctg  tgcagctccaggactgggat
841     ggcaatgcc  aattgctcca  atttctctac  catttggggg  gtgaggacacagcctacagc
901     ctgcagctca  ccgagcccac  ggccaatgag  ctgggtgcca  ccaatgtttccccaatggc
961     ctttccctgc  ctttctctac  ctgggaccaa  gaccacgacc  tccgaggggaccttaactgt

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1021   gccaaagagcc 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  
 CGNACQGPKGKDA PFKDS EDRVPEGQTPETLQSLQTQLKAQNSKIQQLFQKVAQQQRYLSKQNLRIQNLSQIDL  
 LAPTHLDNGVDKTSR GKRLPKMTQLIGLTPNATHLHRPPRDCQELFQGERHSGFLFQIQPLGSPFLVNC EMTSD  
 GGWTVIQRR LNSVDFNQSW EAYKDGFDPQGEFWLGLEKMH SITGNRGSQ LAVQLQDWDGN AKLLQFPIHLGGE  
 DTAYSLQLTEPTANELGATNVSPNGLSLPFSTWDQDHLRDLNCAKSLSGGWWFGTCSHNSNLNGQYFHSIPRQR  
 QERKKGIFWKTWKGRYYPLQATTLLIQPMEATAAS  
 410 amino acids

## SEQ ID NO: 8

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1      acgggctcca gatcttcttc tgcaccagag caagtctaag tctgagccggctccccaga
61     actccagctg ctgggtcttg aactcctgcg ttccggagtc cttagcgttgctgcacccaag
121    gccaccccca gaatcatgcg ctgcgctccg acagcaggcg ctgccctgggtgctatgcgcg
181    gctactgcgg ggcttttgag cgcgcaaggg cgcctgcac agccagagccaccgcgcttt
241    gcatcctggg acgagatgaa cttgctggct cacgggctgc tacagctcggccatgggctg
301    cgcgaacacg tggagcgcac ccgtgggcag ctgggcgcgc tggagcgccgcatggctgcc
361    tgtggtaacg cttgtcaggg gcccaaggga aaagatgcac cttcaaagactccgaggat
421    agagtccctg aaggccagac tctgagact ctgcagagtt tgcagactcagctcaaggct
481    caaaacagca agatccagca attgttccag aagggtggccc agcagcagagatacctatca
541    aagcagaatc tgagaataca gaatcttcag agccagatag acctcttggtccccacgcac
601    ctagacaatg gagtagacaa gacttcgagg ggaaagaggc ttccaagatgaccagctc
661    attggcttga ctccaacgc caccactta cacaggccgc cccgggactgccaggaactc
721    ttccaagaag gggagaggca cagtggactt tccagatcc agcctctggggtctccacca
781    tttttggtca actgtgagat gacttcagat ggaggctgga cagtgattcagagacgcctg
841    aacggctctg tggacttcaa ccagtcctgg gaagcctaca aggatggcttcggagatccc
901    caaggcgagt tctggctggg cctggaaaag atgcacagca tcacagggaaccgaggaagc
961    caattggctg tgcagctcca ggactgggat ggcaatgcca aattgctccaatttcccac
1021   catttggggg gtgaggacac agcctacagc ctgcagctca ctgagcccacggccaatgag
1081   ctgggtgcca ccaatgtttc cccaatggc ctttccctgc cttctctacttgggaccaa
1141   gaccatgacc tccgtgggga ccttaactgt gccaaagacc tctctgggtggctgggtggtt
1201   ggtacctgta gccattccaa tctcaatgga caatactcc actctatcccacggcaacgg
1261   caggagcgta aaaaggggat cttctggaaa acatggaagg gccgctactatcctctgcag
1321   gctaccaccc tgctgatcca gcccatggag gctacagcag cctcttagcctcctcactgg
1381   agcctggttc caggcctaag aagacagtga ctttggttgt ggccctgagatttggccatt
1441   ctctgctggg ggcaggagct ctaagtaggg ctatctgcgt cttgtggacaaagaagaagc
1501   ccgtaactgg agagactgga ggacccttt tccgtgttg ggtctgcaagcattgttgtc
1561   tgaaacagtc agagcaacag gaaacaaatg gccagatcc agaaaacatgggctcgaggg
1621   gcaactgaata tcacttctcg cctaccagag aagttgggga tgcagagggaccactacagt
1681   ccaactagct gggcccttaa tggcggactc agtcatttg actgactggagacaggggtgc
1741   caggagccct ggatacactc atggtgctgt tgtagggtgt gtggatgcacaggtgctaac
1801   tgtggttccc aggcacaact cacagcattc ttacaataaa aacaacctcagaacaaaaaa
1861   aaaaaaaaaa

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## SEQ ID NO: 9

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWX<sub>39</sub>X<sub>40</sub>MNVLAHGLLQLGQGLREHAERTRSQLSALERR  
 LSAX<sub>76</sub>GSAX<sub>80</sub>QGTGEGSTDLPLAPESRVDPEVLHSLQTQLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLQSQF  
 GLLDHKHLDEHAKPAX<sub>161</sub>X<sub>162</sub>X<sub>163</sub>X<sub>164</sub>LPMAQPVDPAHNVSRHLRPRDCQELFQVGERQSGLF EIQPGSPP  
 FLVNC KMTSX<sub>221</sub>GGWTVIQRRHDGSVDFNRPWEAYKAGFGDPHGEFWLGLEKVHSITGDRNSRLAVQLRDWDGNA  
 ELLQFSVHLGGEDTAYSLQLTAPVAGQLGATTVPSPGLSVFPSTWDQDHLRRDKNCAKSLSGGWWFGTCSHNSN  
 LNGQYFRSIPQQRQKLKKGIFWKTWRGRYYPLQATTMLIQPMAAEAAAS  
 406 amino acids

SEQ ID NO: 10

MSGAPTAGAALMLCAATAVLLSAQGGPVQSKSPRFASWX<sub>39</sub>X<sub>40</sub>MNVLAHGLLQLGQGLREHAERTRSQLSALERR  
LSAX<sub>76</sub>GSAX<sub>80</sub>QGTEGSTDLPLAPESRVDPEVLHSLQTQLKAQNSRIQQLFHKVAQQQRHLEKQHLRIQHLSQF  
GLLDHKHLDHEVAKPAX<sub>161</sub>X<sub>162</sub>X<sub>163</sub>X<sub>164</sub>LPEMAQPVDPAHNVSRLLHHGGWTVIQRHDGSVDFNRPWEAYKAGFG  
DPHGEFWLGLKVVHSITGDRNSRLAVQLRDWDGNAELLQFSVHLGGEDTAYSLQLTAPVAGQLGATTVPSPGLSV  
PFSTWDQDHDLRDKNCAKSLSGGWWFGTCSHNLNGQYFRSIPQQRQKLKKGIFWKTWRGRYYPLQATTMLIQP  
MAAEAAS

368 amino acids