

Chapter 3

Angiotensin-Like 4 (Angptl4) in MCNS

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Abstract Minimal-change nephrotic syndrome (MCNS) affects children and adults, with onset most commonly seen between ages 2 and 10 years. The cardinal characteristics of this disease include massive and selective proteinuria, normal-appearing glomeruli on light microscopy, diffuse effacement of podocyte foot processes on electron microscopy, loss of glomerular basement membrane charge, glucocorticoid sensitivity, and explosive onset of edema. Recent progress in this field suggests that secretion of the glycoprotein angiotensin-like 4 (Angptl4) from podocytes in human and experimental MCNS can explain most of these features. Disease-related podocyte Angptl4 upregulation is highly glucocorticoid sensitive in vivo, and a podocyte-specific Angptl4 overexpressing transgenic rat reproduces most of these features. There are two different forms of podocyte-secreted Angptl4: a high isoelectric point (pI) form exclusively overproduced by podocytes in disease states that lacks attachment of sialic acid residues (hyposialylated Angptl4) and a neutral-pI sialylated form, secreted also by the heart, adipose tissue, and skeletal muscle. Hyposialylated Angptl4 is directly implicated in the pathogenesis of proteinuria in MCNS, and conversion of this form into sialylated Angptl4 using sialic acid precursor in vivo reduces proteinuria. Sialylated circulating Angptl4 has anti-proteinuric effects but causes hypertriglyceridemia in nephrotic states. Conversion of hyposialylated Angptl4 into sialylated protein using sialic acid precursors represents the next-generation treatment of MCNS.

Keywords Angiotensin-like 4 • Minimal-change nephrotic syndrome • Sialylation • Therapy • ManNAc

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3.1 Introduction

Minimal-change nephrotic syndrome (MCNS), also known as *minimal-change disease*, was described for the first time in 1913 by Munk, who named it *lipoid nephrosis* due to the presence of lipid in the tubular epithelial cells and urine. MCNS is the primary cause of nephrotic syndrome in children (75–80 % of nephrotic syndrome) and also causes 10–15 % of primary nephrotic syndrome in adults [1].

Clinically, the cardinal features of MCNS are rapid onset of proteinuria (which is selective), explosive onset of edema (sometimes within a day), and response to glucocorticoid therapy in the majority of patients. Along with these specific symptoms, nephrotic syndrome characteristics are present and include hypoalbuminemia and hyperlipidemia (increased total cholesterol, low-density lipoproteins (LDL), and triglyceride). Light microscopy does not reveal any sign of glomerular lesion, electron microscopy presents extensive effacement of podocyte (visceral epithelial cell) foot processes, and tubulointerstitial fibrosis is absent despite nephrotic-range proteinuria. The glomerular basement membrane (GBM) shows a loss of negative charge. The relationship of this loss of charge to the pathogenesis of proteinuria in MCNS is still unclear [2].

MCNS generally responds well to initial therapy as 90 % of patients will go on remission, but nearly 85 % of children with this disease develop one or more relapse.

3.1.1 *Quest for the Elucidation of Molecular Mechanisms Leading to MCNS*

In the past, MCNS was thought to be initiated by T-cell-secreted factors. This hypothesis, put forth four decades ago by Shalhoub [3], was based on the following assumptions: lack of a humoral antibody response, remission brought about by measles (an infection that alters cell-mediated immunity), the benefits of steroids and cyclophosphamide therapy (which also lessen cell-mediated immunity), and occurrence in Hodgkin disease. Although this hypothesis was appropriate when proposed, it no longer receives substantial support. Glucocorticoids [4, 5] and cyclophosphamide [6] are now known to have direct effects in the kidney independent of their effects on the immune system. Most patients with Hodgkin disease do not develop MCNS, and the exponential growth in our knowledge of cell-mediated immunity and multiple genes co-expressed in immune cells and podocytes makes the measles-induced remission point weak.

Given the fact that none of these assumptions would hold ground in the twenty-first century, it is not surprising that there have not been any major breakthroughs in this area. Emphasis has now shifted on identifying molecular mechanisms causing this kidney disease.

Over a decade ago, studies to identify mechanisms of proteinuria were performed by injecting sheep anti-rat glomerular γ 2-nephrotoxic serum (γ 2-NTS) into rats. This antiserum is reactive to many podocyte proteins and induces complement- and leukocyte-independent glomerular injury by direct antibody binding [7]. In this animal model, the rat develops nephrotic-range proteinuria within 24 h after injection of γ 2-NTS. Kidneys from those proteinuric rats and control rats were harvested, glomeruli were isolated, and differentially expressed glomerular genes identified by suppression subtractive hybridization were studied by TaqMan realtime PCR. Angptl4 was identified as the most highly upregulated gene in glomeruli in γ 2-NTS-injected rats, and in situ hybridization confirmed upregulation in a peripheral capillary loop pattern localizing its expression to podocyte [4]. γ 2-NTS induced significantly lower albuminuria in Angptl4 knockout mice than in wild-type mice, demonstrating that Angptl4 is involved in the pathogenesis of proteinuria.

While searching for Angptl4 expression in animal models of human glomerular diseases, significant Angptl4 upregulation was noted in the puromycin aminonucleoside (PAN) model of human (MCNS), starting just before the onset of proteinuria and increasing thereafter. Upregulation was mild and starting after the onset of proteinuria in the passive Heymann nephritis (PHN) model of membranous nephropathy [4] and absent in two models of focal and segmental glomerulosclerosis [4, 8] and in the Thy 1.1 nephritis animal model of mesangial injury [4]. Studies in human MCNS biopsies showed increased Angptl4 protein expression in podocytes, with substantial staining showing also overlapping with the GBM. Elevated plasma levels and urinary excretion were also noted.

3.1.2 What Is Angptl4?

Angptl4 is a secreted glycoprotein discovered around the turn of the century, belonging to the angiopoietin-like protein family (8 members, Angptl1-8), which shares some structural similarities with angiopoietins. Functionally, Angptl4 protein was shown to be implicated in the mechanisms of hypertriglyceridemia [9], proteinuria [4], wound repair [10], and tumor metastasis [11].

The human ANGPTL4 gene is well conserved among different species, sharing ~77 % and 99 % amino acid sequence similarity with mouse and chimpanzee, respectively. It encodes a 406-amino acid glycoprotein with a molecular mass of ~45–65 kDa, has a secretory signal peptide, contains a predicted N-terminal coiled-coil quaternary domain structure and a large C-terminal fibrinogen-like domain, and has two *O*- and two *N*-glycosylation sites where it is likely to be sialylated [4].

The native full-length ANGPTL4 can form higher-order structures via intermolecular disulfide bonds [12]. The N-terminal region of Angptl4 is responsible for its assembly into oligomeric structures [13], and oligomerization of Angptl4 is important for its function as a lipoprotein lipase inhibitor [12–14]. The cleaved C-terminal region circulates as a monomer.

Angptl4 is highly expressed in the liver and adipose tissue and is strongly induced by fasting in these organs [15]. It is a target gene of the nuclear transcription factors peroxisome proliferator-activated receptor- α (PPAR- α) [15] and PPAR- γ [16] and is an apoptosis survival factor for vascular endothelial cell under normoxic conditions [17]. Other studies have shown lower expression in cardiomyocytes and skeletal muscle, and Northern blot analysis has shown low expression in the whole kidney [16]. Population-based studies of ANGPTL4 have identified variants that affect triglyceride levels in humans [18, 19]. Both the full-length and truncated forms of Angptl4 are found in blood plasma, and most normal circulating Angptl4 in rodents is secreted from the liver as a cleaved protein that binds high-density lipoprotein particles [20].

To study the biological role of Angptl4 upregulation in nephrotic syndrome, a previously established Angptl4 transgenic mouse model was first studied [20]. Angptl4 is expressed in the podocyte, and its glomerular expression is increased in 3-month-old transgenic mice. Glomeruli appeared normal by light microscopy, and electron microscopy revealed 50 % effacement or broadening of foot processes. But these mice had only mild proteinuria compared to heavy proteinuria noted in patients, so another transgenic rat model closer to human MCNS was developed.

3.1.3 Study of Angptl4 in Generated Transgenic Rat Models

To independently study the effects of increased podocyte-expressed and circulating Angptl4, both of which are observed in human MCNS, on glomerular function, two different rat lines overexpressing Angptl4 were generated. First, rats overexpressing Angptl4 from the podocyte, using the podocin promoter (NPHS2-Angptl4 rat), were developed in order to study the effects of increased podocyte expression of Angptl4 on glomerular function. Next, rats overexpressing Angptl4 from the adipose tissue, using the adipose tissue marker adipocyte protein 2 (aP2) promoter (aP2-Angptl4 rat), were developed in order to study the effects of increased levels of circulating Angptl4 on glomerular function [4, 21].

3.1.3.1 NPHS2-Angptl4 Transgenic Rats

Rats that specifically overexpress Angptl4 from the podocyte (NPHS2-Angptl4 transgenic rats) develop many features of human MCNS but in slow motion [4]. Albuminuria develops as early as age 1 month, when foot processes are still well preserved, and immunogold electron microscopy pictures show the transit of Angptl4 protein from the podocyte into the GBM. 3-month-old NPHS2-Angptl4 transgenic rats present patchy foot process effacement, with clustering of Angptl4 protein in the GBM across the area of foot process effacement. Albuminuria increases with age and often exceeds 500–800 times the level in age- and sex-matched controls in 5-month-old NPHS2-Angptl4 transgenic rats. By this age, extensive foot process effacement is noted in these transgenic rats. This

model represents an opportunity to study the pathogenesis of proteinuria in slow motion as the onset of proteinuria is gradual in these transgenic rats, whereas the onset of proteinuria in human MCNS is explosive.

Proteinuria in NPHS2-Angptl4 transgenic rats is highly selective, with 92 % of urinary proteins being albumin. This is comparable with 86 % in human MCNS. The NPHS2-Angptl4 transgenic rat is the only known animal model of massive and selective proteinuria and thus is a better animal model for human MCNS than the more commonly used PAN rat model.

Loss of GBM charge in MCNS was reported more than four decades ago [22]. This is another cardinal feature of human MCNS that is present in NPHS2-Angptl4 transgenic rats. Loss of GBM charge was studied in these transgenic rats by two different techniques: Alcian Blue staining (light microscopy) and polyethyleneimine staining (electron microscopy). Both techniques showed reduction of GBM charge in NPHS2-Angptl4 transgenic rats. The decrease in Alcian Blue staining is very similar between NPHS2-Angptl4 transgenic rats and generalized Angptl4 overexpression transgenic mice, even though the mice are only slightly proteinuric compared to the transgenic rats [4]. This would argue against a strong relationship between charge and proteinuria. However, unlike NPHS2-Angptl4 transgenic rats, the transgenic mice also have high circulating Angptl4 levels. As discussed later, circulating Angptl4 has anti-proteinuric effects. Therefore, the relationship of charge and proteinuria cannot be resolved using these animal models. Regardless of this controversy, Angptl4 is the only known substance produced by glomeruli that has been demonstrated to cause loss of GBM charge. Angptl4 likely does this by binding to the glycosaminoglycan (GAG) chains of GBM heparan sulfate proteoglycans, since a heparan sulfate proteoglycans-Angptl4 interaction was previously described at other sites in the body [23]. It is possible that the loss of GBM charge is an epiphenomenon related to the transit of Angptl4 across the GBM against the direction of the fluid flow. aP2-Angptl4 transgenic rats do not develop loss of GBM charge, thereby suggesting that this phenomenon requires the presence of hyposialylated Angptl4.

Finally, NPHS2-Angptl4 transgenic rats do not develop tubulointerstitial fibrosis despite the nephrotic-range proteinuria. This is another hallmark of MCNS encountered in this rat model.

Two features found in human MCNS, but not in these NPHS2-Angptl4 transgenic rats, limit the use of these animals as a perfect model for this pathology. A major difference of this model compared to human MCNS is that young NPHS2-Angptl4 transgenic rats do not develop hypertriglyceridemia, which requires the entry of podocyte-secreted Angptl4 into the circulation. Further study of these rats showed that podocyte-specific overexpressed Angptl4 remains within the glomerulus or is lost in urine but does not enter the circulation. This would suggest that entry into the circulation requires other GBM permeability factors. To simulate this, NPHS2-Angptl4 transgenic rats were injected with a small dose of puromycin aminonucleoside. The production of other as yet unknown factors by puromycin aminonucleoside increases the permeability of the glomerular filter to allow entry of podocyte-secreted Angptl4 into the circulation and induces hypertriglyceridemia (Fig. 3.1). A second factor representing a limitation of that rat model is the fact that

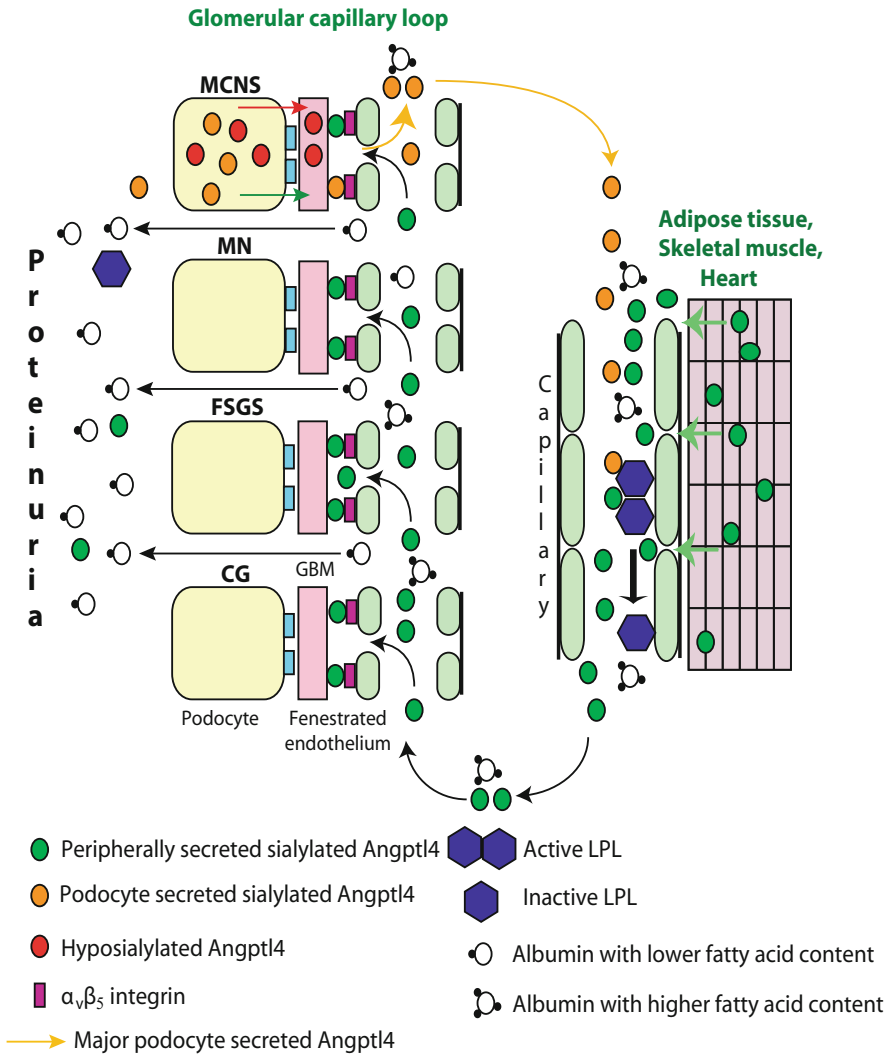


Fig. 3.1 Pathobiology of circulating Angptl4 in nephrotic syndrome (From [21]). Diagram representing the production of circulating Angptl4 protein and its biological effects. The circulating, sialylated form of Angptl4 is secreted from peripheral organs (mostly the skeletal muscle, heart, and adipose tissue) in minimal-change nephrotic syndrome (MCNS), membranous nephropathy (MN), focal and segmental glomerulosclerosis (FSGS), and non-HIV collapsing glomerulopathy (CG). In addition, podocytes in MCNS secrete a hyposialylated form of the protein that remains restricted to the kidney and induces proteinuria [4] and a normosialylated form that enters the circulation. Circulating Angptl4 binds to glomerular endothelial $\alpha_v\beta_5$ integrin to reduce proteinuria or inactivates endothelium-bound lipoprotein lipase (LPL) in the skeletal muscle, heart, and adipose tissue to reduce the hydrolysis of plasma triglycerides to free fatty acids (FFA), resulting in hypertriglyceridemia. Some Angptl4 and LPL are lost in the urine

the onset of proteinuria in these transgenic rats is gradual. In human MCNS, the onset of proteinuria is more rapid. This suggests that additional genes/proteins may be involved in the rapid development of proteinuria in human MCNS.

3.1.3.2 aP2-Angptl4 Transgenic Rats

Under normal conditions, major Angptl4-secreting tissues in the body are the adipose tissue, skeletal muscle, heart, and liver. These organs secrete a sialylated form of Angptl4 that enters circulation and affects plasma triglyceride levels.

Rats that specifically overexpress Angptl4 from adipose tissue (aP2-Angptl4 transgenic rats) have high levels of circulating sialylated Angptl4 protein. High circulating sialylated Angptl4 derived from these organs is increased in many forms of human nephrotic syndrome, including MCNS, membranous nephropathy, focal and segmental glomerulosclerosis, and collapsing glomerulopathy [21]. The only difference between the other conditions and MCNS is that the podocyte is an additional source of circulating sialylated Angptl4 in MCNS. As discussed later, podocytes produce a combination of hyposialylated and sialylated Angptl4.

aP2-Angptl4 transgenic rats do not develop proteinuria, since circulating sialylated Angptl4 has an anti-proteinuric effect. These transgenic rats have normal glomerular morphology on light and electron microscopy, and they do not have any modification in the GBM charge. They have significant hypertriglyceridemia, since circulating Angptl4 is a potent inhibitor of lipoprotein lipase (LPL), the endothelium-bound enzyme that hydrolyzes triglycerides to release free fatty acids (FFA).

3.1.4 Study of Angptl4 in the PAN Model of Human MCNS

The PAN model is the most commonly used model of MCNS, induced by a single intravenous injection of puromycin aminonucleoside into rats [24]. These rats present some features of the human MCNS: explosive onset of proteinuria, no visible glomerular lesions by light microscopy, foot process effacement by electron microscopy, hypertriglyceridemia and hypercholesterolemia, and loss of GBM charge. However, proteinuria is not selective and is only partially glucocorticoid sensitive [5].

Confocal microscopy shows co-localization of Angptl4 with nephrin, indicating its expression in podocyte. There is low constitutive Angptl4 protein expression in the normal rat and human podocyte. In both human MCNS and PAN model, Angptl4 secreted from podocytes enters the GBM (co-localizes with proteoglycans) and the blood circulation and is also lost in urine.

In the PAN model, upregulation of Angptl4 gene expression and amount of proteinuria induced are dependent on the dose of puromycin aminonucleoside injected into rats. The proteinuria induced in the model is relatively nonselective,

since only 66 % of urinary proteins comprise for albumin, compared to about 86 % in human MCNS relapse patient and 92 % in NPHS-Angptl4 transgenic rats. Regarding selectivity of proteinuria, this is not such a good model to study MCNS.

3.1.5 Molecular Mechanisms of Angptl4 in Proteinuria in MCNS: The Role of Sialylation

Two-dimensional electrophoresis gel studies showed that glomeruli from PAN rats express two forms of the Angptl4 protein: a positively charged form migrating at a high isoelectric point (pI) (8–8.5) and a neutral form migrating at pI between 6 and 7. The lack of adequate sialylation in the high-pI form is an important difference between these two forms of Angptl4. NPHS2-Angptl4 transgenic rats similarly express both forms of Angptl4 in glomeruli, making them suitable to further study the role of Angptl4 in MCNS.

In addition to the transgenic rats, two types of stable cell lines overexpressing Angptl4 were developed in mouse glomerular epithelial cells (GEC) and human embryonic kidney cells (HEK293). Cells secrete high-pI form of Angptl4 in the medium from where it can be harvested. Incubation of both cell lines with the naturally occurring sialic acid precursor *N-acetyl-D-mannosamine* (ManNAc) allows the conversion of the high-pI hyposialylated Angptl4 into the neutral-pI sialylated form within the cells and secretion of sialylated Angptl4 in the supernatant.

The choice of using ManNAc for sialylation-based therapeutics was determined by several factors [25]. First, ManNAc has a neutral charge allowing it to cross cell membranes easily. Once in the cell, it is converted into sialic acid and then incorporated into glycoproteins as Angptl4 [4]. Furthermore, ManNAc enters the sialic acid biosynthesis pathway after the rate-limiting enzymatic step catalyzed by GNE (UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase) [26] (Fig. 3.2). This surmounts the intrinsic limitations of the pathway during high-demand states. Second, podocytes are nondividing cells, and therefore, cellular ManNAc content is not divided by cell division. Lastly, orally administrated ManNAc is rapidly lost in the urine (90 % within 4 h). During this process, the podocyte, being the outermost layer of the glomerular filter, gets normal exposure during transit across this filter.

To answer the question of the biological significance of Angptl4 sialylation, *in vivo* studies were conducted to verify its effects on albuminuria. Treatment of NPHS2-Angptl4 transgenic rats with daily oral ManNAc supplementation results in an over 40 % decrease in albuminuria from baseline over 12 days. Concomitantly, conversion of significant amounts of high-pI to neutral-pI Angptl4 occurs in the glomeruli of these ManNAc-treated rats compared to untreated rats. The neutral fraction reacts with sialic acid-binding lectin from *Sambucus nigra* (SNA I), confirming the increase in sialylation of Angptl4 in ManNAc-treated rats. This

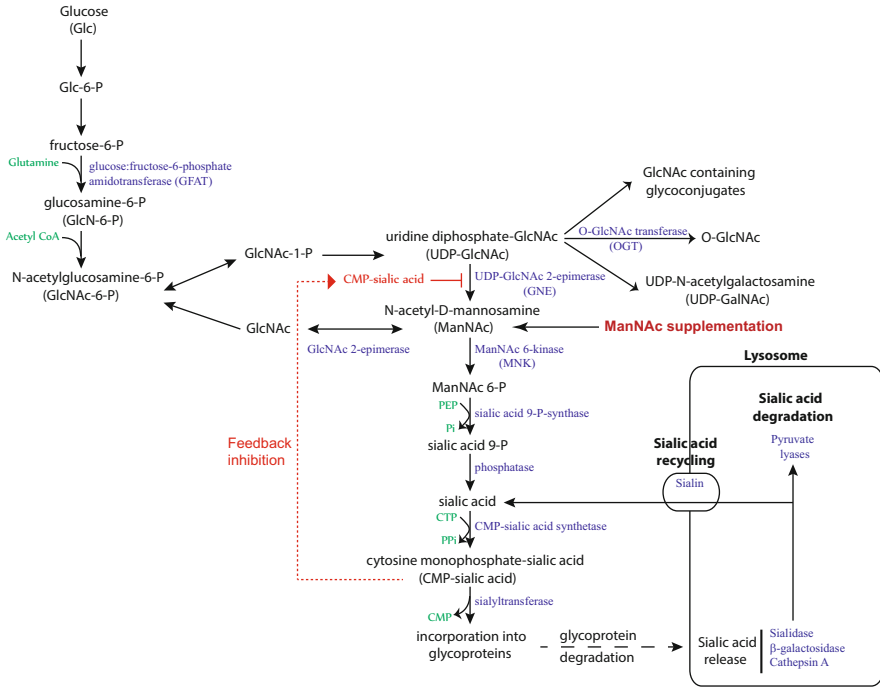


Fig. 3.2 Sialic acid biosynthesis and recycling pathway (From [27]). In humans, sialic acid is synthesized from glucose. The rate-limiting step, catalyzed by UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), is subject to feedback inhibition. ManNAc is the product of this rate-limiting step, so exogenous ManNAc supplementation enters the pathway after this step. A substantial amount of sialic acid is recycled via the anion transporter sialin following lysosomal degradation of glycoproteins and glycolipids

form of therapy has a significant memory effect, since it took 24 days for the proteinuria to rise back to the level of control-treated NPHS2-Angptl4 rats. To verify the biological effects of ManNAc on sialylation in the glomeruli, the two-dimensional electrophoresis gel patterns of another important protein in the podocyte were studied. Podocalyxin is a structural sialoglycoprotein expressed in podocytes. The non-modification of the overall charge of podocalyxin suggests that podocalyxin was not affected by ManNAc therapy [4].

The lack of sialylation of Angptl4 in MCNS is still under investigation and could have several origins. A simple possible explanation is based on the low constitutive expression of Angptl4 in normal podocytes. Therefore, only small amounts of sialic acid are required by this pathway at baseline. Due to severe and rapid upregulation of Angptl4 expression in experimental MCNS disease (70-fold increase in messenger RNA expression), demand exceeds supply, resulting in the secretion of hyposialylated high-pI protein. Another possible explanation for the lack of sialylation of Angptl4 is that the activity of a class of enzymes called sialyltransferases, which add sialic acid residues to proteins, is decreased in podocytes in MCNS.

3.1.6 Link Between Proteinuria and Hypertriglyceridemia in Nephrotic Syndrome

There are large gaps of knowledge in our understanding of the molecular relationship between proteinuria, the primary driver in nephrotic syndrome, and most of the other components. Only the link between proteinuria and hypertriglyceridemia has been clearly elucidated [21, 27], revealing the intrinsic role of circulating Angptl4 in nephrotic syndrome. This relationship is strongly influenced by the link between FFA and albumin. Albumin is the most abundant plasma protein that circulates as a 69 kDa monomer and serves as a vehicle to transport cations, hormones, and FFA. FFA are a critical energy source for the body and also serve as an important molecular mediator in nephrotic syndrome. FFA can be used as a source of energy by organs like the skeletal muscle and heart [28] or can be recycled by adipose tissue, since it both releases and takes up FFA for storage as triglycerides. These organs also have high expression for LPL, Angptl4, and peroxisome proliferator-activated receptor (PPAR) family members, which regulate Angptl4 expression in response to FFA uptake.

Normal sources of fatty acids include diet, mobilization from adipose tissue, and conversion of excess carbohydrates into fat by the liver. Some fatty acids are coupled with glycerol to form triglycerides (or triacylglycerols) for transport or storage and can be converted back into FFA by lipases. FFA that are not part of triglycerides circulate in the blood, mostly coupled non-covalently with albumin. Each albumin molecule has six high-affinity FFA-binding sites and many low-affinity-binding sites, and up to ten FFA molecules can be bound to an albumin molecule at any given time [29]. Adipose tissue releases FFA into circulation after serial conversion of triglycerides to diglycerides by adipose triglyceride lipase and diglycerides to monoglycerides by hormone-sensitive lipase. After digestion of dietary fat, medium-chain fatty acids (8–12 carbon chain) are transported coupled with albumin, whereas long-chain fatty acids (14 or more carbon chains) are converted back to triglycerides, incorporated into chylomicrons, and transferred to the circulation via the thoracic duct.

There are two sources of FFA for uptake by organs: albumin-bound FFA and conversion of circulating triglycerides into FFA by the endothelium-anchored enzyme LPL (Fig. 3.3). The balance between these two sources of FFA uptake is significantly altered in nephrotic syndrome. For reasons that are unknown and need to be explored in the future, albumin with a low FFA content is preferentially lost in urine by proteinuric kidneys, but albumin with high FFA content is not, so the result is a progressive retention of albumin with high FFA content [29, 21]. As proteinuria reaches nephrotic range, hypoalbuminemia develops, and a combination of high FFA containing albumin and hypoalbuminemia raises the plasma ratio of FFA to albumin [21]. This elevated plasma FFA-to-albumin ratio induces increased FFA uptake in the skeletal muscle, heart, and adipose tissue, which in turn increases Angptl4 expression and secretion from these tissues. Since Angptl4 is a known PPAR target gene [30–32], and PPAR expression is increased during the nephrotic

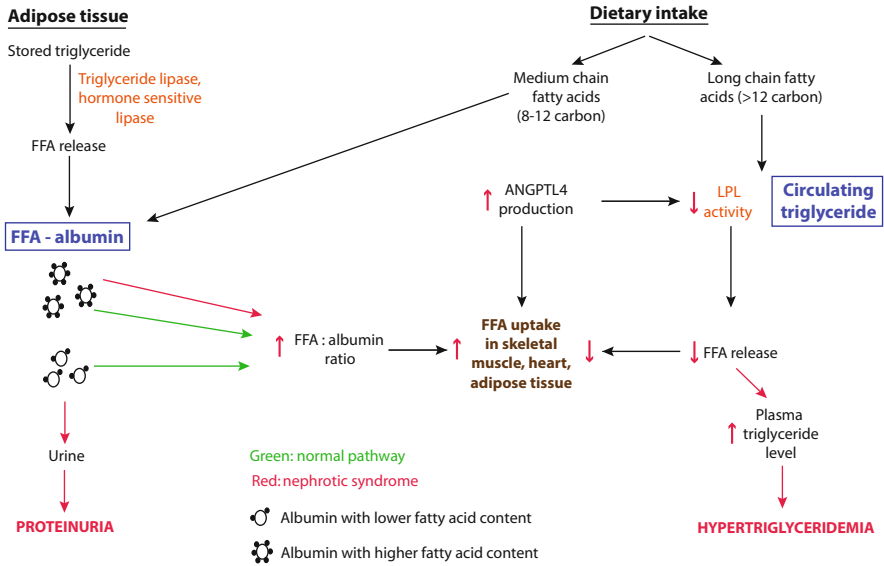


Fig. 3.3 Schematic illustration of the two sources of free fatty acids (FFA) available for uptake by the skeletal muscle, heart, and adipose tissue in the normal and nephrotic state (From [41]). *Green* shows normal conditions, and *red* illustrates changes in nephrotic syndrome. The balance shifts significantly to albumin-bound free fatty acids (FFA) because of retention of albumin with high FFA content in nephrotic syndrome. Angptl4 secreted from these organs reduces the conversion of triglycerides to FFA by inactivating lipoprotein lipase (LPL), thereby reducing use of triglycerides and resulting in hypertriglyceridemia

phase in these tissues [21], at least part of this Angptl4 upregulation is likely to be PPAR mediated. Angptl4 secreted from these organs into the circulation has two effects presented in a local and a systemic feedback loops (Fig. 3.4). First, it binds to the $\alpha\beta 5$ integrin in glomerular endothelium and reduces proteinuria (Fig. 3.4, systemic feedback loop). The precise mechanism by which the Angptl4- $\alpha\beta 5$ integrin interaction reduces proteinuria is not known, but it is possible that additional feedback loops within the glomerulus are involved. Second, Angptl4 inactivates LPL activity in these organs, thereby reducing the conversion of triglycerides into FFA, which reduces FFA uptake by this pathway (Fig. 3.4, local feedback loop), and also results in hypertriglyceridemia. Overall, it looks like the local feedback loop, in which Angptl4 decreases LPL activity and then reduces the availability of FFA generated from triglycerides, reduces the effectiveness of the systemic feedback loop by limiting the extent of Angptl4 upregulation [21]. This attempt to reduce proteinuria by Angptl4 represents a systemic response against rising proteinuria.

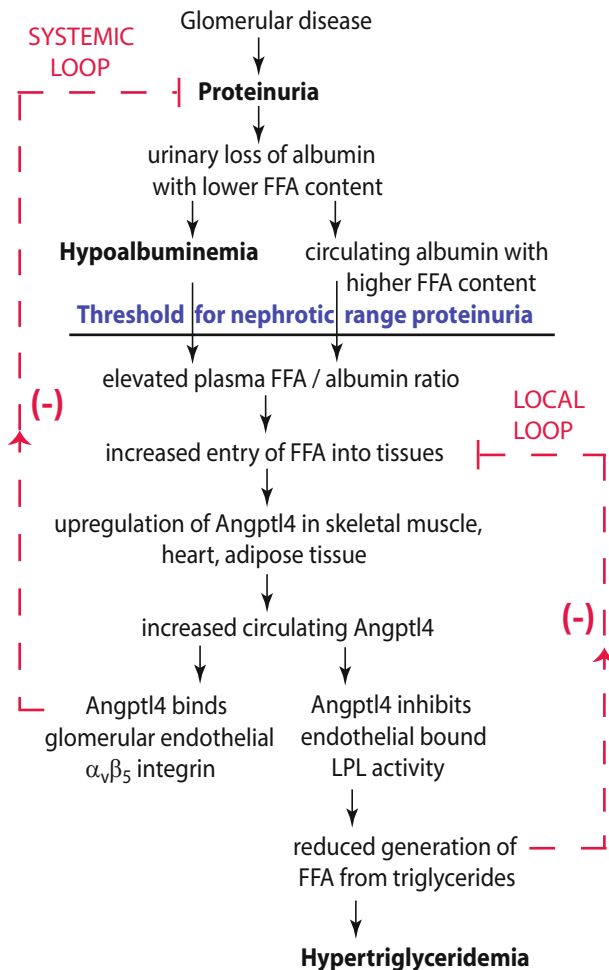


Fig. 3.4 Circulating Angptl4: link between proteinuria and hypertriglyceridemia in nephrotic syndrome (From [21]). Schematic illustration of negative feedback loops in the link between proteinuria, hypoalbuminemia, and hypertriglyceridemia that are mediated by Angptl4 and free fatty acids (FFA) (unesterified fatty acids with a free carboxylate group). Plasma FFAs are non-covalently bound to albumin, and because of the preferential loss of albumin with low FFA content during proteinuria, albumin with higher FFA content is retained in circulation. As glomerular disease progresses and proteinuria increases, hypoalbuminemia develops, and the combination of high albumin-FFA content and lower plasma albumin levels increases the plasma ratio of FFAs to albumin. This increased available FFA enters the skeletal muscle, heart, and adipose tissue to induce upregulation of Angptl4, mediated at least in part by peroxisome proliferator-activated receptor (PPAR) transcription factors. Angptl4 secreted from these organs participates in two feedback loops. In the systemic loop, it binds to glomerular endothelial $\alpha_v\beta_5$ integrin and reduces proteinuria. In a local loop, it inhibits lipoprotein lipase (LPL) activity in the same organs from which it is secreted to reduce the uptake of FFAs, thereby curtailing the stimulus for its own upregulation

3.1.7 Importance of the Nephrotic-Range Proteinuria Threshold

In patients, manifestation of the other components of nephrotic syndrome starts only after proteinuria crosses the nephrotic-range proteinuria threshold, which is usually defined as about 3.5 g/d in adults, but it is likely to be quite variable between different individuals and within the same individual among different components. Until recently, the molecular basis for this threshold was not known. It is, at present, only possible to explain the nephrotic threshold in the context of hypertriglyceridemia [21]. Hypertriglyceridemia is dependent on the retention of albumin with high FFA content, resulting in a plasma FFA-to-albumin ratio that is high enough to induce upregulation of Angptl4 expression in the skeletal muscle, heart, and adipose tissue. Studies in experimental animals reveal that, during mild proteinuria, plasma FFA-to-albumin ratio, plasma Angptl4 levels, peripheral organ Angptl4, and PPAR mRNA expression are similar to control non-proteinuric animals. During severe proteinuria, all these parameters are significantly elevated, suggesting that the threshold for nephrotic-range proteinuria, in the context of hypertriglyceridemia, correlates with the downstream effects of an increased plasma FFA-to-albumin ratio.

3.1.8 Current and Future Therapies for MCNS

3.1.8.1 Current Therapies

Current therapies for kidney disease related to proteinuric disorders rely on agents used in other fields than nephrology. In one category, glucocorticoids, cyclophosphamide, azathioprine, chlorambucil, mycophenolate mofetil, cyclosporine, tacrolimus, and the anti-CD20 antibody have been used to treat glomerular disease because of their immunosuppressive properties. Over the past decade, it became clear that several of these drugs have direct effects on glomerular cells [33, 4]. In another category, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, spironolactone, and renin inhibitors such as aliskiren block the renin-angiotensin system and are used for supportive therapy.

Among these drugs, glucocorticoids are usually the first line of therapy for MCNS. Indeed, up to 95 % of patients with MCNS are sensitive to glucocorticoid therapy [1], which suggests that the key mediators of this disease are either encoded by glucocorticoid-sensitive genes or controlled by glucocorticoid-sensitive pathways. Glomerular ANGPTL4 upregulation noted in the PAN model is glucocorticoid sensitive, since administration of glucocorticoids in PAN reduces glomerular ANGPTL4 expression and proteinuria [4]. There is limited value in treating the NPHS2-ANGPTL4 transgenic rat with glucocorticoids to study glucocorticoid

sensitivity in the context of MCNS because transgenic expression is not driven by the native ANGPTL4 promoter.

However, prolonged and repeated exposure to glucocorticoids has numerous serious side effects. Since MCNS patients relapse frequently and often become glucocorticoid dependent, it is more appropriate to explore additional ways of treating this disease.

3.1.8.2 ManNAc: A New Therapeutic Agent for MCNS

The upregulation of podocyte-secreted Angptl4 is present in human and experimental MCNS and induces heavy proteinuria. Podocyte-secreted hyposialylated Angptl4 induces proteinuria, and conversion of hyposialylated Angptl4 to its sialylated form reduces proteinuria in NPHS2-Angptl4 transgenic rats. Therefore, sialylation of Angptl4 using ManNAc is a reasonable approach toward proteinuria.

Sialic acid is commonly present as terminal carbohydrate on glycoproteins. ManNAc is the precursor of all physiological sialic acids. In biological systems it is the precursor compound for the biosynthesis of the sialic acids especially N-acetylneuraminic acid (Neu5Ac/NANA). Sialic acid is essential for a variety of cellular functions. In humans, mutations of the GNE gene result in a severe neuromuscular disorder called hereditary inclusion body myopathy (HIBM) [34]. These patients never develop kidney disease. Mice with mutations at the same site in the mouse GNE gene develop kidney disease, which suggests that mice are very different from humans in this aspect, and data from such mutant mice bears no relevance to human disease. This is further strengthened by the observation of the GNE knockout mice, with a knock-in of the human gene with the common HIBM mutation, that develop muscle disease only and have no sign of kidney disease [34].

Sialylation of proteins is a natural event in human cells. A substantial amount of sialic acid in cells is recycled, which reduces tremendously the requirement for de novo sialic acid synthesis [35]. Sialic acid is most commonly incorporated at *O*- and *N*-glycosylation sites of glycoproteins and in glycosphingolipids (gangliosides). The predominant form of sialic acid present in humans is NANA/Neu5Ac [36]. Humans synthesize sialic acid from glucose since there is no major nutritional source of NANA/Neu5Ac. Animals convert NANA into *N*-glycolylneuraminic acid (NGNA/Neu5Gc) using the enzyme NANA hydroxylase. This enzyme is nonfunctional in humans due to an exon deletion/frameshift mutation [37]. Therefore, under most circumstances, NGNA present in food is not incorporated into human proteins, excluding diet as a source of sialic acid in humans. During normal condition and during times of high demand (e.g., fasting, nephrotic syndrome), sialylated Angptl4 is secreted into the circulation from organs having very active sialic acid biosynthesis pathway like the liver and adipose tissue. Podocytes likely have a less active sialic acid biosynthesis pathway, and in condition of severe upregulation of Angptl4, like in MCNS, this pathway is unable to sialylate newly produced Angptl4, despite a slight increase in the expression of the rate-limiting

enzyme GNE. This results in the secretion of a combination of hyposialylated and sialylated Angptl4 from podocytes. The fact that exogenously administered ManNAc enters the sialic acid biosynthesis pathway after the rate-limiting step catalyzed by UDP-GNE allows the cells to synthesize sialic acid when it is needed.

ManNAc could be used as a therapy in diseases involving the podocyte for two reasons. First, unlike most other cells in the body, podocyte division is very rare. This cellular specificity would allow the podocyte to accumulate ManNAc, and consequently, ManNAc therapy would require lower oral dose compared to therapy-targeting dividing cells. Finally, 90 % of the ManNAc oral dose is secreted into urine within 4 h [34]. This rapid urinary excretion is an advantage, since podocytes are exposed to most of the oral dose of ManNAc while it is filtered through the glomerulus. Overall chances of toxicity from ManNAc are dramatically decreased by the combination of low-dose requirement for podocyte disease and rapid urinary excretion.

In MCNS, ManNAc could be used as a complementary therapy to glucocorticoids. Indeed, glucocorticoids reduce podocyte Angptl4 gene expression, but ManNAc improves sialylation of the protein itself. ManNAc therapy would be very useful in patients who have frequent relapses, those developing resistance to glucocorticoids, or patients in whom complete remission is not achieved or who require very prolonged glucocorticoid therapy. ManNAc therapy could be considered for maintenance therapy, either as a daily very low-dose regimen or intermittent low-dose therapy. Knowing that long-term glucocorticoid therapy is associated with multi-organ complications, the first episode of MCNS would still be treated with glucocorticoids, especially in children, at least in part to prove that the disease is indeed glucocorticoid sensitive. Another mechanism by which glucocorticoids are effective in MCNS is by increasing Angptl4 secretion from the skeletal muscle, heart, and adipose tissue [38], which has anti-proteinuric effects. Knowing that 80–90 % of children relapse after the first remission [39, 40], ManNAc is perfectly suited as a maintenance drug to reduce the frequency and intensity of relapse.

3.1.9 Can Angptl4 Be Used as a Novel Biomarker to Diagnose the Type of INS?

The development of novel biomarkers for MCNS is important. Hyposialylated Angptl4 is lost in the urine in MCNS patients. Detailed studies would need to be conducted to see if high-pI hyposialylated Angptl4 in the urine can be used as a biomarker for MCNS.

3.2 Conclusion

Even if the podocyte Angptl4 upregulation explains most of the cardinal manifestations of MCNS, it clearly does not explain the acute onset of symptoms. For this phenomenon, additional mechanisms need to be explored.

Angptl4 has been conclusively implicated in the pathogenesis of proteinuria in human and experimental MCNS. When it is overexpressed in the podocyte, it is secreted within two forms, a hyposialylated and a sialylated form, and the hyposialylated induces proteinuria. This lack of sialylation can be corrected by supplementation of animal drinking water with the sialic acid precursor ManNAc. Furthermore, it is implicated in other hallmarks of MCNS like hyperlipidemia, effacement of foot process, and loss of GBM charge.

In the near future, Angptl4 could be used as a biomarker of MCNS depending on the ability to measure hyposialylated Angptl4 in urine from patients. Sialylation-based therapeutic strategy holds significant promise in the treatment of common forms of proteinuric chronic kidney disease. This is a novel area, innovative and mechanism based, and has been extensively studied in vivo in appropriate human disease models. Manipulating Angptl4-related pathways in the context of therapeutics seems to have a high chance of success. This new therapy could be used to treat relapse patients and could prevent prolonged and repeated exposure to corticosteroids and their drug-related adverse events.

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