RNAi targeting liver Angiopoietin-like protein 3 protects from nephrotic syndrome in a rat model via amelioration of pathologic hypertriglyceridemia

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Running Title Page

a) Running Title:
Angptl3 siRNA Treatment for Nephrotic Syndrome

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d) Abbreviations
Alpha-smooth muscle actin- α-SM actin
Angiopoietin like protein 3- Angptl3
Glyceraldehyde 3-phosphate dehydrogenase- GAPDH
High density lipoprotein- HDL

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Lipoprotein lipase- LPL
Low density lipoprotein- LDL
Intermediate density lipoprotein- IDL
Monocyte Chemoattractant Protein-1- MCP1
NADPH-oxidase 4- NOX4
Nephrotic syndrome- NS
Nuclear Factor kappa-light-chain-enhancer of activated B cells- NF-κB
Proprotein convertase subtilisin/kexin type 9- PCSK9
RNA interference- RNAi
Transforming growth factor-β- TGF-β
Very low density lipoprotein- VLDL

**Recommended section**

Drug Discovery and Translational Medicine
ABSTRACT

Nephrotic syndrome (NS) is associated with metabolic perturbances including profound dyslipidemia characterized by hypercholesterolemia and hypertriglyceridemia. A major underlying mechanism of hypertriglyceridemia in NS is lipoprotein lipase (LPL) deficiency and dysfunction. There is emerging evidence that elevated angiopoietin-like protein 3 (ANGPTL3), a Lipoprotein Lipase (LPL) inhibitor that is primarily expressed and secreted by hepatocytes, may be in part responsible for these findings. Furthermore, there is evidence pointing to the contribution of ANGPTL3 to the pathogenesis of proteinuria in NS. Therefore, we hypothesized that inhibition of hepatic ANGPTL3 by RNA interference (RNAi) will ameliorate dyslipidemia and other symptoms of NS and pave the way for a new therapeutic strategy. To this end, we used a subcutaneously delivered, GalNAc-conjugated small-interfering RNA (siRNA) to selectively target and suppress liver Angptl3 in rats with puromycin-induced NS which exhibit clinical features of NS including proteinuria, hypoalbuminemia, hyperlipidemia and renal histological abnormalities. The study demonstrated that siRNA-mediated knockdown of the liver Angptl3 relieved its inhibitory effect on LPL and significantly reduced hypertriglyceridemia in nephrotic rats. This was accompanied by diminished proteinuria and hypoalbuminemia which are the hallmarks of NS and significant attenuation of renal tissue inflammation and oxidative stress. Taken together, this study confirmed the hypothesis that suppression of Angptl3 is protective in NS and points to the possibility that the use of RNAi to suppress hepatic Angptl3 can serve as a novel therapeutic strategy for NS.
Significance:

Current standard of care for mitigating nephrotic dyslipidemia in nephrotic syndrome is statins therapy. However, the efficacy of statins and its safety in the context of impaired kidney function is not well established. Here we present an alternate therapeutic approach by using siRNA targeting Angptl3 expressed in hepatocytes. As liver is the major source of circulating Angptl3, siRNA treatment reduced the profound hypertriglyceridemia in a rat model of nephrotic syndrome and was also effective in improving kidney and cardiac function.
Introduction

Nephrotic syndrome (NS) is associated with significant hyperlipidemia and substantial alterations in lipid and lipoprotein metabolism (Moradi and Vaziri, 2018; Vaziri, 2016a). Serum cholesterol, triglycerides and the apolipoprotein B-containing lipoproteins including very low density lipoprotein (VLDL), intermediate density lipoprotein cholesterol (IDL-C), and low density lipoprotein cholesterol (LDL-C), are significantly elevated in NS (Joven et al., 1990). While altered biosynthesis of fatty acids and cholesterol play a minor role in the pathogenesis of these abnormalities, their impaired clearance is a key cause of the NS-induced dyslipidemia (Agrawal et al., 2018; Kaysen et al., 1992). The latter results in significant elevations of serum triglycerides (Dewey et al.) and triglyceride-rich lipoproteins including VLDL and IDL (Kaysen and de Sain-van der Velden, 1999; Vaziri, 2016b). The major underlying cause of these abnormalities is lipoprotein lipase (LPL) deficiency and dysfunction (Agrawal et al., 2018; Garber et al., 1984; Vaziri, 2016a). LPL is the rate-limiting enzyme that mediates lipolysis of chylomicrons and VLDL and release of their fatty acids for uptake by adipocytes for storage of energy and the muscle and other tissues for production of energy. NS is associated with significant reduction of LPL activity and marked reductions of heparin-releasable and intracellular LPL protein (Liang and Vaziri, 1997a; b; Sato et al., 2002; Shearer and Kaysen, 2001; Vaziri et al., 2012). These abnormalities are present despite normal LPL mRNA expression in adipose tissue, skeletal muscle and myocardium, therefore indicating presence of post-
transcriptional and translational mechanisms of NS-induced LPL dysfunction (Liang and Vaziri, 1997b; Sato et al., 2002).

Angiopoietin-like protein 3 (ANGPTL3), secreted by the liver (Conklin et al., 1999), is an inhibitor of LPL and endothelial lipase (EL) has a major role in lipid metabolism (Musunuru et al., 2010; Wu et al., 2020). Homozygous loss of ANGPTL3 function causes familial combined hypolipidemia characterized by low plasma levels of triglycerides (Dewey et al.), high-density lipoprotein cholesterol (HDL-C) and LDL-C and a decreased risk of coronary artery disease (CAD) (Minicocci et al., 2012; Musunuru et al., 2010; Robciuc et al., 2013; Romeo et al., 2009; Stitziel et al., 2017). Following an effort to replicate this advantageous human phenotype for treating dyslipidemia, several therapeutic modalities have been developed. Antibodies targeted towards circulating ANGPTL3 lowers plasma TG, LDL-C and HDL-C in mice (Gusarova et al., 2015), monkeys (Gusarova et al., 2015) and humans (Dewey et al., 2017; Gaudet et al., 2017). RNAi mediated suppression of liver Angptl3 transcript has shown to have a similar effect in several mouse models and humans (Graham et al., 2017; Xu et al., 2018). ANGPTL3 inhibition has thus emerged as highly promising and alternative therapeutic option for mitigating hyperlipidemia in various lipoprotein disorders (Arca et al., 2013; Kersten, 2019).

Recent studies have identified ANGPTL3 and ANGPTL4 as proteins that by inhibiting LPL activity play a significant role in the pathogenesis of NS-induced LPL deficiency (Clement et al., 2014; Kersten, 2017; Vaziri and Moradi, 2014). ANGPTL3 is a secreted protein which is mainly expressed in hepatocytes (Conklin
et al., 1999). Recent studies have found that the Angptl3 knockdown via siRNA or use of knock out animal models markedly ameliorated these findings, improved structure and integrity of podocyte and decreased proteinuria (Dai et al., 2015; Dai et al., 2019; Lin et al., 2013; Liu et al., 2015). NS, in addition to heavy proteinuria and glomerular disease (as evidenced by heavy proteinuria, glomerulosclerosis) also results in tubulointerstitial disease marked by inflammation and oxidative stress. The latter abnormalities are worsened and contributed to by the significant dyslipidemia of NS including profound hypertriglyceridemia. It is well known that free fatty acids can cause cellular toxicity and contribute to oxidative stress and inflammation (Han et al., 2013; Hoffman and Levy, 1989). Therefore, any therapy that would ameliorate glomerular damage and tubulointerstitial disease would also reduce oxidative stress and inflammation. Our evaluations were constructed to show that not only was proteinuria reduced, but also this salutary effect is accompanied by reduced markers of inflammation and oxidative stress.

Given these findings and that liver is the major source of circulating ANGPTL3, we hypothesized that Angptl3 mRNA knockdown specifically in the hepatocytes by using GalNAc conjugated siRNA (Rajeev et al., 2015) may not only ameliorate NS-associated hypertriglyceridemia, but it can also have a salutary effect on proteinuria and renal function. Therefore, we evaluated the impact of Angptl3 siRNA treatment in a rat model of puromycin induced nephrotic syndrome.
Material and Methods

In vivo

All animal experiments were approved by the University of California Irvine Institutional Committee for the Use and Care of Experimental Animals. Male Sprague-Dawley rats with an average body weight of 180-200 g (Charles River Labs, Raleigh, NC, USA) were used in this study. Animals were housed in a climate-controlled vivarium with 12h day/night cycles and with access to food and water ad libitum. At the beginning and the final week of the study, the animals were placed in metabolic cages for a 24h urine collection and systolic blood pressure (SBP) was measured by tail plethysmography as described previously (Vaziri et al., 2002). NS was induced in a randomly selected group of animals via sequential intraperitoneal injections of puromycin aminonucleoside on day 1 (130 mg/kg) and day 14 (60 mg/kg) (Han et al., 2013; Hoffman and Levy, 1989). The rats assigned to the control group received saline injections. Subsequently, the NS animals were randomized to either weekly subcutaneous injection of 1X PBS (Vehicle control) or 10 mg/kg of Angptl3 siRNA and followed for 4 weeks. The siRNA was designed and synthesized as published elsewhere (Nair et al., 2014) to target rat Angptl3 mRNA NM_001025065.1. It targets the sequence at position 265-287, 5'-AAAAAGACTGATCAAATATGTTG -3', with a single mismatch at position 6.

At the end of the study, body weight, tail arterial blood pressure and 24-hour urinary protein excretion were measured. The animals were then fasted overnight and between the hours of 9 and 11 AM were sacrificed under general anesthesia.
Blood was collected using cardiac puncture and subsequently, relevant tissues including kidney and heart were immediately removed and processed for histological evaluation including electron microscopy and frozen and stored at -70 °C for biochemical analysis.

For euthanasia, the animals were placed into a sealed anesthesia induction chamber under 5% isoflurane (Piramal Clinical Care, Bethlehem, PA, USA)/oxygen gaseous mixture to induce sedation and maintained at 2-4%.

Urinary protein was measured using Rat Urinary Protein Assay Kit, Cat # 9040 (Chondrex, Inc., Redmond, WA, USA). Serum urea was measured by QuantiChrom™ Urea Assay Kit, Cat # DIUR-500 (BioAssay Systems, Hayward, CA, USA). Urinary creatinine was measured by QuantiChrom™ Creatinine Assay Kit, Cat # DICT-500 (BioAssay Systems, Hayward, CA, USA). Serum cholesterol and triglyceride were measured by Cholesterol Fluorometric Assay Kit, Item # 10007640 and Triglyceride Colorimetric Assay Kit, Item # 10010303 (Cayman Chemical, Ann Arbor, MI, USA). HDL (High density lipoprotein) cholesterol was measured by HDL-Cholesterol assay kit Cat # STA-394 (Cell Biolabs, INC., San Diego, CA, USA). Serum LDL was measured by Rat LDL-Cholesterol Assay Kit, Cat # 79960 (Crystal Chem, Elk Grove Village, IL, USA). Serum urea was measured using Urea Assay Kit, Cat # DIUR-500 and serum albumin was measured using QuantiChrom™ BCP Albumin Assay Kit, Cat # SIAP-250 (BioAssay Systems, Hayward, CA, USA). All kits were used according to manufactures instructions. Serum creatinine was measured by capillary electrophoresis using PA800 Plus Pharmaceutical Analysis System (Beckman...
Coulter) from George M O'Brien Kidney Research Core at University of Texas Southwestern Medical Center.

**Histologic Analysis**

The kidneys were removed after sacrifice and fixed with 10% formalin solution. Histologic sections were stained with hematoxylin and eosin, and Masson trichrome. Briefly, 30 glomeruli from each trichrome-stained section were evaluated for glomerulosclerosis. Any degree of glomerulosclerosis was counted as positive and the total number of affected glomeruli were resulted for each animal. The mean number of sclerotic glomeruli was calculated for each experimental group. Tubulointerstitial damage marked by inflammatory cells infiltration, fibrosis, tubular dilatation, and atrophy was evaluated semi-quantitatively according to the extension of the damaged area in the renal cortex: 0, normal; grade 1, <10%; grade 2, 10% to 25%; grade 3, 25% to 50%; grade 4, 50% to 75%; and grade 5, 75% to 100%.

**Electron Microscopy**

Samples were fixed in 4% glutaraldehyde for 3-4 hours; tissue were then cut into 1.2-2.0 mm$^3$ sections and fixed in Osmium tetroxide for one hour. After rinsing with distilled water, the tissues were dehydrated using 50%, 75%, 95% and 100% ethanol, then placed in propylene oxide followed by 1:1 of propylene and embedded in Epon media. Sections measuring approximately 1000 microns were stained with methylene blue to identify the appropriate segments to be used and
subsequently 100-micron sections were stained with uranyl acetate and lead acetate and examined using a Tecnai Spirit electron microscope at 80 KV.

**Western Blot Analyses**

Cytoplasmic extracts of the renal tissue were prepared as described previously (Sakurai et al., 1996). The proteins of interest in the cytoplasmic fractions of the kidney tissue were measured by Western blot analysis as previously described (Aminzadeh et al., 2012; Kim and Vaziri, 2010) using the following antibodies. Rabbit against rat NADPH-oxidase 4 (NOX4, Cat # ab133303) and transforming growth factor-β (TGF-β, Cat # ab215715) were purchased from Abcam (Cambridge, MA). Rabbit antibody against Monocyte Chemoattractant Protein-1 (MCP1, Cat # NBP1-07035) were purchased from Novus Biologicals. Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB, Cat # ab16502) was obtained from Cell Signaling. Mouse antibody against alpha-smooth muscle actin (α-SM actin) (Cat # 701457) was obtained from Invitrogen. Mouse antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat # TH270379) was purchased from Invitrogen and used for measurement of loading control reference.

**Lipoprotein Lipase Activity**

Lipoprotein lipase activity in hepatic, muscle and white adipose tissues was determined using an assay kit purchased from Abcam (ab204721) following the manufacturer’s protocol. Briefly, 50 mg of tissue samples were homogenized with
200 µl ice cold PBS. Samples were then centrifuged at 4 °C at 10,000 * g for 10 minutes and supernatant was collected in a new tube. 50 µl of samples were added to 96-well plate followed by 50 µl of Substrate (in DMSO, included in the kit). The plate was incubated at 37 °C for 10 minutes protected from light and measured using a microplate reader at Ex/Em = 482/515 nm every 10 minutes for 1 hour.

**Real-time PCR analysis**

Total RNA was isolated from liver tissue using the miRNeasy kit (Qiagen) following manufacturer’s protocols. 1ug of RNA from each sample was reverse transcribed using the High capacity Reverse transcription kit (Invitrogen). Quantitative real time PCR was performed on the cDNA using Roche light cycler and the Light cycler 480 master mix (Roche). All experimental samples were analyzed using TaqMan assays (Invitrogen) to detect Angptl3 (Rn01433283_m1) and normalized with the expression level of a reference gene, Gapdh (4352339E)

**Statistical analysis**

Data are presented as mean ± SD. One-way ANOVA and multiple comparisons were performed using GraphPad Prism 8.4 (GraphPad Software, San Diego, CA). Tukey’s post-test was used to determine differences between the groups. Grubbs test was used to analyze potential outliers. P values less than 0.05 were considered significant.
Results

*Angptl3* siRNA treatment reduced liver transcript *Angptl3* levels and correspondingly increased LPL activity

To study the effects of *Angptl3* siRNA on NS, we utilized a previously established rat model of puromycin induced NS. The rats were subcutaneously treated with siRNA targeting *Angptl3*. Compared to the control group, the placebo-treated nephrotic group exhibited a trend for increased hepatic tissue *Angptl3* gene expression. *Angptl3* siRNA administration significantly decreased hepatic tissue *Angptl3* mRNA expression by 86.2 ± 7.4% (Fig. 1A). This level of *Angptl3* transcript knockdown, using this particular siRNA duplex has routinely shown significant decrease in circulating ANGPTL3 in various rodent models (Wu et al., 2020). As expected, this led to a disinhibition of LPL observed by an increase in LPL activity in the serum (Fig. 1B). In addition, *Angptl3* siRNA treatment significantly increased LPL activity in tissues including hepatic tissue, muscle and adipose tissue (Fig. 1C-E) when compared with the vehicle-treated controls.

*Angptl3* siRNA treatment attenuated hyperlipidemia and proteinuria

To assess the effects of the *Angptl3* siRNA treatment, we looked at the well-established markers of NS. Puromycin treated nephrotic animals exhibited all the hallmarks of NS and dyslipidemia as observed by heavy proteinuria, significantly increased serum cholesterol, low density lipoprotein (LDL), and triglycerides levels and decreased serum albumin concentrations. In addition, the nephrotic group had modest increases in systolic blood pressure and serum urea concentration and
reduced creatinine clearance (Table 1). Treatment with *Angptl3* siRNA in NS animals resulted in a remarkable and statistically significant lowering of serum triglyceride levels (307.5±88.9 vs 89.7±78.4 mg/dL). This was accompanied by partial improvement of hypoalbuminemia and increased creatinine clearance. In accordance with these markers of improved kidney function, the *Angptl3* siRNA treated animals also showed decreased proteinuria at week 4 post puromycin treatment when the maximum effect of the puromycin is observed in this model (Fig. 2). In addition, there was a modest but significant improvement in arterial blood pressure and decreased left ventricular hypertrophy in the *Angptl3* siRNA treated group indicating potential for a modest betterment of cardiac function probably as a direct effect of improved kidney function.

**Angptl3 siRNA treatment partially protected against the pathological renal injury in NS rats**

We next observed the effect of *Angptl3* siRNA treatment on kidney injury. Puromycin treatment induced sclerosis of the glomeruli. *Angptl3* siRNA treatment partially attenuated the glomerulosclerosis score (Fig. 3A and B). Further evaluation by electron microscopy confirmed moderate podocyte swelling and effacement in the NS group (Fig. 3C) when compared to controls (Fig. 3D). When treated with *Angptl3* siRNA, the podocyte swelling and effacement where reduced (Fig. 3E). Puromycin treatment also induced tubulointerstitial injury seen with tubular atrophy, tubular dilation and interstitial fibrosis. However, there was no resolution of the tubulointerstitial abnormalities between the vehicle and siRNA-treated NS animals (Fig. 3F and G). This is consistent with mild renal injury.
observed in this animal model and possible renal recovery after week 4 when the effects of puromycin are waning.

**Angptl3 siRNA treatment improved the inflammatory, oxidative stress and fibrotic pathways dysregulation seen in NS kidneys.**

Molecular pathways of inflammation, oxidative stress and fibrosis are upregulated in NS and contributes to the kidney dysfunction. We next sought to examine the effect of Angptl3 siRNA on some of these molecular components of pathogenesis. Compared to the control group, the NS rats exhibited increased NF-κB protein abundance in the renal tissue ($p < 0.01$) pointing to activation of the inflammatory pathway. Angptl3 siRNA treatment reversed upregulation of NF-κB in the kidney tissues of NS rats (Fig. 4A). Compared with the control group the NS group had significant increases in protein abundance of MCP-1 and NOX4 in their renal tissue. Upregulations of the renal tissue MCP-1 and NOX4 were ameliorated with Angptl3 siRNA treatment (Fig. 4B and C). Furthermore, renal tissue from the NS animals exhibited upregulations of the profibrotic proteins, TGF-β ($p < 0.01$) and α-SM actin ($p < 0.01$), which were markedly attenuated with siRNA treatment (Fig. 4D and E).
Discussion

Heavy proteinuria, which is a hallmark of Nephrotic syndrome (NS), is also associated with various metabolic complications including hypoalbuminemia and dyslipidemia (Vaziri, 2016a; Vaziri et al., 2003). One of the defining characteristic of NS-associated dyslipidemia is hypertriglyceridemia and significantly elevated serum levels of triglyceride-rich lipoproteins (Agrawal et al., 2018). This hyperlipidemia contributes to an increase in cardiovascular disease and adults with NS have an increased risk for myocardial infarction (Agrawal et al., 2018; Ordonez et al., 1993). Despite their sub-par efficacy on triglyceride lowering, Statins are used as treatment for hyperlipidemia in NS (Wheeler, 2001). A major cause of the hypertriglyceridemia has been shown to be LPL deficiency and dysfunction which has been attributed to many different factors, chief among them elevated levels of ANGPTL3 and ANGPTL4 proteins (Jiang et al., 2019; Mace and Chugh, 2014; Vaziri and Moradi, 2014). These proteins have an inhibitory effect on LPL thereby their elevated level leads to impairment of fatty acid and TG clearance and significant hypertriglyceridemia. The latter point was demonstrated in study by Chugh et. al. who showed that increased circulating levels of ANGPTL4 caused significant LPL inhibition leading to hypertriglyceridemia while elevated renal levels of this protein caused decreased urinary protein excretion (Clement et al., 2014). These observations have led to the intriguing possibility of utilizing Angptl3 targeting therapies to prevent dyslipidemia of NS and thus ameliorate symptoms of NS (Jiang et al., 2019; Mace and Chugh, 2014). In accordance with this idea, other investigators have shown that deletion or decreased expression of Angptl3
ameliorates proteinuria and protects renal structure and function in animal models of nephrotic syndrome (Dai et al., 2015; Dai et al., 2019; Liu et al., 2015). In addition, the feasibility of a siRNA mediated approach to mitigate NS is highly supported by other successful and safe siRNA treatments for dyslipidemia such as the PCSK9 inhibitor Inclisiran, developed using a similar siRNA technology (Fitzgerald et al., 2017; Ray et al., 2020).

In the current study, we found that administration of a hepatocyte targeted Angptl3 specific siRNA formulation in rats with Puromycin-induced NS was associated with a significant decrease in serum TGs, but no change in serum cholesterol or LDL levels. This is consistent with the fact that LPL does not play a major role in elevated cholesterol levels in NS as the latter is mainly driven by LDL-receptor deficiency and elevated serum PCSK9 levels (Jin et al., 2014; Liu and Vaziri, 2014; Vaziri, 2016a). We also found a significant improvement in LPL activity in the fat, muscle and liver tissues of animals with NS treated with Angptl3 siRNA, confirming the mechanism through which Angptl3 inhibition improves TG metabolism. Interestingly, treatment with Angptl3 siRNA was also associated with an improvement in serum albumin concentration and significant decrease in urine protein excretion supporting the amelioration of proteinuria in these animals. Given the evidence that elevated fatty acid and triglyceride levels may cause renal damage and in light of the significant lowering of serum TGs after Angptl3 siRNA treatment the improvement in proteinuria could be the result of reduced lipotoxicity (Escasany et al., 2019; Martinez-Garcia et al., 2015; Nosadini and Tonolo, 2011).
Interestingly, it has been shown that renal expression of Angptl3 is significantly upregulated in the setting of podocyte injury and nephrotic syndrome. Furthermore, altered expression of ANGPTL3 in the glomeruli of animals with nephrotic syndrome have been found to be associated with increased proteinuria and foot process effacement (Liu et al., 2015). Lin et. al. further reported that exposure of podocytes to ANGPTL3 results in accelerated Puromycin-induced loss of podocyte via their detachment and apoptosis which was most likely mediated by the rearrangement of the F-actin (Lin et al., 2013). In fact, there was also a small improvement is serum markers of renal function (creatinine clearance), although the mild nature of renal injury in the early stages of this animal model limits the interpretation of these findings. Histologic analysis of renal tissue showed that animals treated with Angptl3 siRNA had decreased glomerulosclerosis on light microscopy and reduced podocyte effacement on electron microscopy. We also found decreased abundance of the markers of inflammation and fibrosis in the renal tissue of nephrotic animals treated with Angptl3 siRNA when compared to vehicle-treated controls.

However, this current study does not delineate additional mechanisms that could contribute to the protection of renal injury. These findings will need to be further delineated in future mechanistic studies. In summary, the data presented here support the hypothesis that inhibition of liver Angptl3 expression by a siRNA could ameliorate symptoms of NS and highlight the potential of RNAi as a therapeutic alternative for NS.
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Authorship contributions

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Foot note

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Figure Legends:

Figure 1. Impact of Angptl3 siRNA treatment on Angptl3 transcript levels and LPL activity. Liver Angptl3 transcript levels and LPL activity in the indicated tissues were measured to assess the efficacy of Angptl3 siRNA treatment in vehicle treated controls-CTL, nephrotic syndrome-NS and Angptl3 siRNA treated NS+siRNA animals. (A) Angptl3 transcript levels were decreased in Angptl3 siRNA treated group, n ≥ 8. Lipoprotein lipase activity was reduced in nephrotic animals (NS) and recovered in Angptl3 siRNA treatment group (NS+siRNA) in (B) Serum, n ≥ 8, (C) Hepatic n ≥ 6, (D) muscle, n ≥ 7, and (E) adipose tissues, n ≥ 7. Data are presented as mean ± SD, * P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2. Impact of Angptl3 siRNA treatment on urinary protein excretion

Kidney dysfunction in NS causes increased protein excretion and is a hallmark of the disease. Urinary protein was measured in vehicle treated controls-CTL, nephrotic syndrome-NS and Angptl3 siRNA treated NS+siRNA animals. (A) Time course of 24h urinary protein excretion post puromycin treatment showing increased urinary protein excretion which was mitigated by the treatment with Angptl3 siRNA. (B) Individual animal data of 24h urinary protein at week 4 post puromycin treatment. Data are presented as mean ± SD, n ≥ 8. * P < 0.05 vs. CTL, † P < 0.05 NS+siRNA vs. NS.

Figure 3. Impact of Angptl3 siRNA treatment on glomerulosclerosis and podocyte morphology. NS causes pathological injury to kidney tissue. Tissue architecture was assessed by histological staining and electron microscopy methods in vehicle treated controls-CTL, nephrotic syndrome-NS and Angptl3
siRNA treated NS+siRNA animals. (A) Photomicrographs show representative glomeruli in the three groups. (B) NS was associated with increased glomerulosclerosis and treatment with Angptl3 siRNA improved this finding. Data are presented as mean ± SD, n ≥ 7, **P < 0.01. (C) Electron microscopy revealed moderate podocyte swelling and effacement which was reduced upon Angptl3 siRNA treatment. a. Normal podocytes in control group (arrow), b. Moderate podocytes swelling in NS group (arrow), c. Mild podocytes swelling in NS+siRNA group (arrow), d. Normal foot processes in control group (arrow), e. Foot process effacement in approximately 20% of foot processes in NS group (arrow), f. Foot process effacement in 5% of foot processes in NS+siRNA group (arrow). n=3 animals analyzed per group. (D) Tubulointerstitial score was increased in NS animals, however there was no improvement with Angptl3 siRNA treatment at the time point tested. Data are presented as mean ± SD. * P < 0.05, **P < 0.01, ****P < 0.0001.

Figure 4. Impact of Angptl3 siRNA treatment on renal injury pathways. NS triggers kidney injury by activating pathological cellular pathways. Markers of inflammation, oxidative stress and fibrosis were assessed using Western blotting in vehicle treated controls-CTL, nephrotic syndrome-NS and Angptl3 siRNA treated NS+siRNA animals. Protein abundance of (A) NF-κB, n = 6, (B) MCP-1, n = 10, and (C) NOX4, n = 6, (D) TGF-β, n = 8, and (E) alpha-smooth muscle actin, n = 10, showed resolution of injury pathways upon siRNA treatment. Multiple proteins were assessed on the same blot and hence normalized to same loading control.
densities. Data are presented as mean ± SD. * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. 
Table 1. Impact of *Angptl3* siRNA treatment on NS markers. *Angptl3* siRNA treatment attenuated hyper triglyceridemia and improved pathological kidney markers in puromycin treated NS animals. Data are presented as mean ± SD, *P* < 0.05 vs. Control-CTL, † *P* < 0.05 *Angptl3* siRNA treatment group-NS+siRNA vs. NS.

<table>
<thead>
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<th>CTL (n = 9)</th>
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<td>0.30 ± 0.1</td>
</tr>
<tr>
<td>Creatinine Clearance (ml/min)</td>
<td>2.6 ± 0.58</td>
<td>1.7 ± 0.4 *</td>
<td>2.2 ± 0.5 †</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.6 ± 0.6</td>
<td>2.5 ± 0.5 *</td>
<td>2.9 ± 0.5 *</td>
</tr>
<tr>
<td>Serum TC (mg/dl)</td>
<td>93.0 ± 27.0</td>
<td>411.2 ± 147.2 *</td>
<td>372.3 ± 103.3 *</td>
</tr>
<tr>
<td>Serum TG (mg/dl)</td>
<td>52.2 ± 13.0</td>
<td>307.5 ± 88.9 *</td>
<td>89.7 ± 78.4 †</td>
</tr>
<tr>
<td>Serum LDL</td>
<td>42.6 ± 6.3</td>
<td>205.3 ± 89.6 *</td>
<td>192.4 ± 72.1 *</td>
</tr>
</tbody>
</table>
REFERENCES:


Figure 2

A

ANGPTL3 siRNA injected

24-h urine protein (mg/dl)

0 1 2 3 4 5 6

Week

B

24-h urine protein Week 4 (mg)

0 200 400 600 800

Week

CTL NS NS+siRNA

**

**** **

CTL NS NS+siRNA
Figure 3
Figure 4