ANGPTL3 Impacts Not Only Proteinuria But Also Hyperlipidemia in Nephrotic Syndrome

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Research

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Abstract

**Background:** Why primary nephrotic syndrome (PNS) patients often accompany with dyslipidemia is unknown. Recent studies discovered that angiopoietin-like protein 3 (ANGPTL3) is an important regulator in lipid metabolism. In this study, we explored how ANGPTL3 impact dyslipidemia in PNS development.

**Methods:** We detected the expression serum level of ANGPTL3 in PNS patients. Further, degree of proteinuria and lipid metabolism were tested in angptl3 overexpression-transgenic (angptl3-tg) mice at different weeks of age. Meanwhile, this study used CRISP/Cas 9 system to build angptl3-knockout (angptl3/-) mice to observe LPS-treated nephrotic mice.

**Results:** There was a significant correlation between the expression level of serum ANGPTL3 and the level of cholesterol, triglyceride and low density lipoprotein in PNS patients. Along with the age growing, the angptl3-tg mice emerged more and more severe hypertriglyceridemia and proteinuria. The pathological features showed rich lipid droplets deposition of hepatocytes and diffuse podocytes effacement with these angptl3-tg mice. Compared to wild type mice, angptl3/- mice showed significant less degree of lipid dysfunction and proteinuria after treated with LPS. The ANGPTL3' effects on nephrotic dyslipidemia was confirmed in the cultured hepatocyte with knock-down or overexpressed angptl3. Finally, the significant alters of lipoprotein lipase (LPL) were tested in liver tissues between Angptl3-/- and wild type mice with LPS treatment.

**Conclusion:** ANGPTL3 could be involved in development of dyslipidemia, besides proteinuria in PNS pathogenesis. Inhibiting LPL expression is a reason why ANGPTL3 induce hyperlipidemia in PNS.

Introduction

Proteinuria and hyperlipidemia are considered to be the most important clinical features of primary nephrotic syndrome (PNS). Among them, proteinuria is the core performance of PNS. Podocyte injury in the outermost layer of the glomerular filtration barrier plays a key role in the development of PNS proteinuria\(^1\). Hyperlipidemia is another important feature of PNS, which often appears during the acute phase and disappears in the remission phase of the disease. It is considered that abnormal lipoprotein lipase (LPL) activity is one of the mechanisms leading to PNS hyperlipidemia\(^2\). But, why does PNS produce massive proteinuria and hyperlipidemia at the same time, whether these two different pathophysiological phenomena have a common pathogenesis is a question that we have been interested in.

Angiopoietin-like protein 3 (ANGPTL3) belongs to the angiopoietin-like protein family. ANGPTL3 is mainly synthesized by liver cells and is especially expressed in the kidney podocyte\(^3\). Recently, a series of experiments discovered that ANGPTL3 could induce cytoskeleton rearrangement of podocytes, leading to increased podocyte motility\(^4\). And, some study revealed ANGPTL3 is involved in the development of
nephrotic proteinuria via attenuating podocyte foot effaced, even podocyte detachment from glomerular[5].

As a lipid regulating reagent, ANGPTL3 has been extensively studied with respect to lipid metabolism[6]. It is known that ANGPTL3 can significantly inhibit the activity of LPL, leading to reduce triglycerides and cholesterol decomposition, and increase blood lipid, which is a key molecule in regulating lipid metabolism[7].

In this study, we emphasized on whether ANGPTL3 could regulate lipid metabolism either in vivo or in vitro PNS model, and using angptl3-tg mice tested the multiple effects of ANGPTL3. Finally, we explored LPL’s role for mechanism of ANGPTL3 on PNS’s hyperlipidemia.

### Methods

#### Antibodies and reagents

The antibodies and reagents used in this study are listed with their sources in parentheses as follows:
- monoclonal antibody against glyceraldehyde-phosphate dehydrogenase (GAPDH) (ImmunoWay Biotechnology, Texas, USA);
- polyclonal antibody against ANGPTL3 (R&D Systems, Minneapolis, USA);
- polyclonal antibody against LPL (Santa Cruz Biotechnology, Santa Cruz, CA).

#### Production of Cas9 mRNA and sgRNA

T7 promoter was added to Cas9 coding sequence by PCR amplification using PX330 vector (Addgene) and the primer Cas9 (F and R) (Table S1). T7-Cas9 PCR product was gel purified by QIAquick Gel Extraction Kit (Qiagen, USA) and used as the template (500 ng) for in vitro transcription (IVT) using mMESSAGEmMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Scientific, USA). Both T7 promoter and targeting sequence was added to sgRNA by PCR amplification using the primer sgAngptl3 (F and R) (Table S1). The T7-sgRNA PCR product was also purified on gels using QIAquick Gel Extraction Kit (Qiagen, USA) and then used as templates (250 ng) for IVT using MEGA short script T7 kit (Thermo Fisher Scientific, USA). Both the Cas9 mRNA and the sgRNA were purified according to the standard protocol by phenol: chloroform extraction and ethanol precipitation, and then dissolved in DNase/RNase-free water (Thermo Fisher Scientific, USA).

#### Generation of Angptl3 knockout mice

C57BL/6 female mice (6–8 weeks old) were used as embryo donors. C57BL/6 female mice were superovulated by intraperitoneally injecting with PMSG and hCG, and then mated to C57BL/6 male mice. Fertilized embryos (zygotes) were collected from their oviducts. Cas9 mRNA (100 ng/μL) and sgRNA (angptl3) (50 ng/μL) were mixed and injected into the cytoplasm of fertilized eggs with both pronuclei visible in CZB (Chatot–Ziomek–Bavister) medium. The injected zygotes were then cultured in Quinn's
Advantage cleavage medium (In-Vitro Fertilization, Inc.) at 37 °C under 5% CO2 in air for about 24 h, and 18–20 2-cell stage embryos were transferred into the oviduct of a pseudo-pregnant ICR female mouse at 0.5 dpc. This work was taken in Shanghai Gemple Biotech Co.Ltd.

Mouse identification and maintenance

Angptl3-/- mice were generated by CRISPR/Cas9 system. All mice had access to food and water. All experiments were performed in accordance with the Health Guide for the Care and Use of Laboratory Animals and were approved by the Biological Research Ethics Committee of Gansu province People's hospital (No. syll20130331). Genotyping of angptl3-/- mice was performed by PCR of mice tail-tip genomic DNA using primer angptl3 (F and R) (Table S2) and then analyzed by Sanger sequencing. This work was done in the animal center of Gansu University of Traditional Chinese medicine. All mice were housed in an air-conditioned room and were provided free access to food and water (22 ± 2°C; 12:12-hour light: dark cycle). After the 10% chloral hydrate(400 mg/kg) anesthesia, the mice were euthanized by cervical dislocation, and all efforts were undertaken to minimize pain and discomfort. The mice did not exhibited signs of peritonitis after the administration of 10% chloral hydrate(400 mg/kg).

Generation of angptl3 gene knock-out mice

The double-strand breaks (DSBs) induced by the CRISPR/Cas9 system stimulate DNA repair by at least two distinct mechanisms, non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is error-prone and introduces unpredictable patterns of insertions and deletions (Indel), which can lead to disruption of the protein-coding capacity of a defined locus. To generate angptl3 knockout mice, we injected into fertilized eggs with Cas9 mRNAs and sgRNA targeting the angptl3 exon 1, which contains the start codon (Fig S1a). Subsequent sub-cloning of flanking regions surrounding the sgRNA targeting site identified four founder mice with frame shift mutations (Fig S1b).

Generation of angptl3 transgenic mice

The murine angptl3 cDNA, synthesized by Shanghai Gemple Biotechnology, was cloned into the pCDNA3.1 vector (Fig S2a). This plasmid, designated pCDNA3.1- Angptl3, was linearized by MluI/DraIII, and fragment of interest was then purified for oocyte injection using C57Bl/6 mouse-derived fertilized eggs[19-20]. Transgenic mice were identified by PCR using oligonucleotide primers specific for the construct was inserted (CMV-F, 5’-CGCGTTGACATTGATTATTGAC-3’ and angptl3-R, 5’-CAGGAGGCCATTCGCTAAAA-3’; PCR fragment =892bp) (Fig S2b).

Generation of LPS nephrosis in mice

All animal studies were approved by the Subcommittee on Research Animal Care of the Gansu province People's Hospital (No. syll20130331) and performed in the animal center of Gansu University of Traditional Chinese medicine. Either of thirty-six wild type and Angptl3-/- male 6-8week C57BL/6 mice were given free access to standard laboratory food and water. The both mice were injected
intraperitoneally with either 200 μg LPS (1 mg/ml in sterile LPS-free PBS) in a total volume of 200 μl. As the control group, the both mice (n=5) received an identical volume of intravenous saline. After these four group were injected at 24th hour, 48th hour, 72th hour, the 24h urinary protein excretion was measured, and kidney and liver tissues were harvested and processed for H&E. FP effacement was assessed by transmission electron microscopy according to our published protocols\[8\].After LPS injection, the mice were killed at 24h, 48h, 72h, during which no death was found. The humane endpoint is defined as weight loss of 20%, dyspnea, or difficulty in feeding after LPS injection within 72 hours. Death was confirmed by the absence of a pulse, breathing, corneal reflex, response to toe pinch as well as a lack of respiratory sounds and heartbeat.

**Objectives**

In this study, 196 patients with PNS admitted to Gansu Province People's Hospital from Jan 2016 to Jan 2018 were collected, including 124 males and 72 females. The health control was 60 cases. The study protocol conforms to the ethical guidelines of the 2013 Declaration of Helsinki. We insured that all patients and healthy controls provided informed and a written consent for the study, and the ethical approval was obtained from the Gansu province People's Hospital Research Ethics Committee hsyll20160037. PNS met the inclusion criteria of nephrotic syndrome, with urinary protein > 3.5 g/d and plasma albumin < 30 g/L, accompanied by edema of varying degrees and/or hyperlipidemia\[9-10\]. Exclusion criteria: secondary NS; other acute and chronic kidney diseases; tumors, infectious diseases; liver dysfunction, thyroid dysfunction, systemic sclerosis and dermatomyositis, heart failure, etc.

**Experimental methods**

(1) Sample collection: All fasting subjects' elbow venous blood 3 ml in the morning was collected and placed in anticoagulant test tube. Serum was separated after centrifugation for 5 minutes (800 x g) and separated into EP tube. Urine 5 ml was collected and placed in a test tube without any additives. After centrifugation for 5 minutes (800 x g), the supernatant was taken and separated into EP tubes. Serum and urine are frozen in - 80 C refrigerator for use.

(2) Detection of serum and urine biochemical indicators: Biochemical indicators such as triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), serum creatinine (Scr), urea nitrogen (BUN), 24-hour urea protein (24 hUP) were detected by automatic biochemical analyzer(ABBOTT ARCHITECT c1600, USA).

(3) Determination of ANGPTL3 level in serum: the concentration of ANGPTL3 in serum was detected by ELISA kit of human ANGPTL3, and the specific operation was carried out strictly according to the instructions.

**Hepatocyte cell line culture and treatment**
A nontumorigenic mouse hepatocyte cell line, AML12, was obtained from American type culture collection (ATCC), which was cultured in DMEM/F12 medium (Gibco) containing 5 μg/ml ITS premix (Sigma-Aldrich, USA), 40 ng/ml dexamethasone (Sigma-Aldrich), and 10% fetal bovine serum (FBS, Gibco) at 37 °C in a humidified atmosphere of 5% CO2. Lipopolysaccharide (LPS; working concentration: 25μg/ml) were purchased from Sigma-Aldrich. AML12 was collected after LPS treatment for 24h.

**Lentiviral infection**

For production of ANGPTL3 overexpression or knockdown lentivirus, the lentivirus with mouse angptl3 coding sequence or angptl3 shRNA and blank control were purchased from Gemple Biotechnology (Shanghai, China). The target sequences of angptl3 shRNA are as follows: sh angptl3#1, 5’-GCTGGGTCATGGACTTTAAAG-3’; and sh angptl3#2, 5’-GCAGCTAACCAACTTATTT-3’. AML12 cells were infected with recombinant lentivirus units plus 8 ug/ml polybrene (Sigma-Aldrich) with a multiplicity of infection (MOI) of 20. The stable lentivirus infection cells were selected and enriched by flow cytometry (BD).

**RNA extraction and quantitative RT-PCR**

Total RNA was extracted using Direct-zol RNA MiniPrep (Zymo Research, USA) according to the manufacturer's instructions. Total mRNA (1 μg) was reverse transcribed using 5X All-In-One RT MasterMix (Abm, Canada) according to the manufacturer's instructions. Real-time PCR was performed using SYBR FAST qPCR Kit Master Mix (2X) Universal (KAPA, USA) on an Applied Biosystems 7,500 Fast Real-Time PCR System (Foster City, USA). The RT-PCR system involved in cDNA 1.0 μl, 2X SYBR-Green Mix 10 μl, Forward Primer (10 μM) 0.5 μl, Reverse Primer (10 μM) 0.5 μl, and made up to 20 μl with RNase-free water. The reaction conditions were: 2 min of denaturation at 94 °C, 40 cycles of 1 min at 94 °C, 30 sec at 56 °C, 2 min at 72 °C, and a final extension step at 72 °C for 10 min. The cycle threshold (Ct) values were analyzed using the comparative Ct (ΔΔCt) method following MIQE Guidelines. The amount of target was normalized to an endogenous reference (GAPDH) and expressed relative to a control (non-treated cells). Used primers are as follows: ANGPTL3: (forward, 5’- GCGAACATACAAGTGGCGTG-3’; reverse, 5’-CTGTGAGCCATCT TTCCGGT-3’); LPL: (forward, 5’- GAAAACCCCAGC AAGGCATAC -3’; reverse, 5’-CATCTTGCTGCTTCTTTGGC -3’).

**Oil Red O Staining**

Oil Red O Staining was performed with Oil Red O Stain Kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Results were examined by a light microscope and OD560 was measured for quantification.

**Western blot**

We performed immunoblotting experiments as described[4].

**Statistical analysis**
Quantitative data consistent with normal distribution is represented by x±s, using independent sample t test; non-normal distribution quantitative data is expressed by M(1/4, 3/4), using Mann-Whitney U test and Kruskal-Wallis test; the difference of sex ratio was tested by $X^2$ test; relationship between indicators was determined by Spearman correlation analysis. The values from animal or cells were subjected to one-way ANOVA tests, and Pearson correlations among the groups were calculated. $P$-values of <0.05 were considered statistically significant. The data was statistically processed using SPSS 20.0 software.

**Results**

The expression level of serum ANGPTL3 was correlated with blood lipid in PNS patients.

There were 196 patients with PNS included in this study, including 72 females (36.54%) and 124 males (63.46%), with a male to female ratio of 1:0.58. The average age of the PNS group was 35.32±14.35, and that of the healthy control group was 36.10±14.38 which showed no significant difference between the two groups ($P > 0.05$), as shown in Table 1. Compared with the healthy group, as shown in Table 1, the serum ANGPTL3 level in PNS group significantly increased (33.0±14.260 ng/ml vs 70.23± 37.45 ng/ml, $T = 8.23$, $P < 0.001$).

ANGPTL3 is known to be involved in triglyceride and cholesterol metabolism by inhibiting lipoprotein lipase activity[11]. The mechanism of PNS hyperlipidemia is still unclear. In this study, we analyzed the correlation between serum ANGPTL3 level and major indicators of blood lipid and found that ANGPTL3 was positively correlated with CHO in a low degree ($r=0.34$, $P < 0.001$), with TG in a low degree ($r = 0.25$, $P = 0.001$), and with LDL in a moderate degree ($r = 0.50$, $P < 0.001$), but no correlation with HDL (seen in Table 2, $r = 0.15$, $P = 0.07$).

In physical condition, lipid level and proteinuria with knocked-out angptl3 gene C57 mice shows nearly normal

Although it was reported that mice on B6;129S5 gene background was knocked out angptl3[8], there is unknown about how about kidney and liver characteristic with C57 mice knocked out this gene via CRISPR/Cas 9 system. Here, we observed lab indication and pathological features with the angptl3-/- mice in physiology condition. And, there was no significant difference in the serum TG and TC between wild-type mice and angptl3-/- mice (Fig. 1a, b $P > 0.05$). 24h urine protein results showed that there was no difference between angptl3-/- mice and wild-type mice (Fig. 1c, $P > 0.05$). The liver structure of angptl3-/- mice was observed under light microscope, and there was no difference between the wild-type and the angptl3-/- mice. No inflammatory cell infiltration was observed (Fig. 1d). The glomerular structure of the knockout mice was also normal under light microscope and electron microscope (Fig. 1e, f).

In LPS nephrotic mice model, C57 mice with knocked out angptl3 relieved either proteinuria or hyperlipidemia
To explored whether ANGPLT3 impact on dyslipidemia in PNS condition, we compared the changes of main indexes of blood lipid in wild type and angptl3/-/- mice after LPS modeling respectively. As shown in the Fig. 2a, b compared with the WT group, mice in the WT+LPS group developed significantly hypertriglyceridemia and hypercholesterolemia after modeling 48 hours ($P < 0.05$). The TG and TC levels in the angptl3/-/-+LPS group were lower than those in the WT+LPS group at 24h and 48h and the significant difference was observed at 72 h ($P < 0.05$).

Then we observed the lipid droplet deposition in each group liver tissues of mice at 72 h. The results showed that liver cell vacuolar degeneration was found in the WT+LPS group, compared with the WT group, which indicating that lipid droplets accumulated in large amounts in the hepatocytes (Fig. 2d). Different from wild-type mice, the lipid droplet deposition in liver tissues of angptl3/-/- mice showed a significant less trend after LPS treatment.

In this study, we also compared the proteinuria changes of angptl3/-/- mice and wild-type mice in LPS nephropathy model. As shown in the Fig. 2c, at 24, 48 and 72 hours after LPS modeling, the proteinuria level of angptl3/-/- mice at different time points was significantly lower than that of wild-type mice ($P < 0.05$).

Observation of the structure of glomeruli and podocytes showed that it was consistent with the pathological type of MCD, and the structure of glomeruli in wild-type mice was basically normal under light microscope after LPS modeling. LPS nephropathy mouse podocytes were extensively fused under electron microscope. However, the degree of podocyte fusion in angptl3/-/- mice was significantly lower than that in wild-type mice after LPS modeling (Fig. 2e).

**Transgenic angptl3 mice could develop hyperlipidemia accompany with proteinuria**

To verify whether ANGPTL3 is involved in both lipid metabolism and proteinuria, the transgenic mice of angptl3 were tested. As shown in the Fig 3a, b, the triglycerides and total cholesterol of angptl3-tg mice were significantly higher than those of wild-type mice at the three time points of observation ($P < 0.01$), the serum lipid index of angptl3-tg mice gradually increased since 6 weeks after birth. Further, we compared possible difference in dyslipidemia degree between LPS nephrotic model and angptl3-tg mice. The wild type mice at 6, 24 and 36 weeks of age were treated with LPS. The triglyceride and total cholesterol with LPS mice were tested after LPS treatment 72 hours. At the same age, the triglycerides of angptl3-tg mice was significantly lower than that of LPS mice (Fig 3a, $P<0.01$). Different to triglycerides level, the total cholesterol level was not observed obviously different between the angptl3-tg mice and the LPS model mice at each age.

In addition, compared with wild-type mice, the 24-hour proteinuria quantification results suggested that the angptl3-tg mice proteinuria levels increased significantly at week 6 (Fig 3c, $P < 0.01$). Then, after of week 24, the trend of increase was decreased, but the level of proteinuria was still significantly higher than that of wild type mice of the same age ($P < 0.05$). The 24-hour proteinuria level of angptl3-tg group was always significantly lower than that of wild-type mice after modeling ($P < 0.01$) at each time point.
And the podocyte injury with angptl3-tg mice under electronic microscopy showed that the podocyte foot effacement became more and more diffuse accompanying with the mice age week (Fig 3d).

**ANGPTL3 regulates lipid metabolism on nephrotic hepatocytes in vitro.**

In this study, the lipid metabolism of nephrotic hepatocytes was observed by oil red staining. The results showed that the staining area of wild-type hepatocytes treated with LPS was significantly increased compared with the untreated group (Fig. 4a, b). The oil red staining area in hepatocytes overexpressed ANGPTL3 was enlarged (Fig. 4a, b). While the area of lipid droplets in the hepatocytes with knocked-down ANGPTL3 gene was significantly smaller than that of the wild-type hepatocytes, that the both cells were treated respectively with LPS (Fig. 4a, b).

The results of TG and LDL in each group were further detected by ELISA, as shown in Fig. 4c, d, the wild-type and the ANGPTL3 knockdown hepatocytes were compared and treated with LPS respectively. We found that the levels of TG and LDL in the miRNA+LPS group were significantly lower than those in the WT+LPS group. TG and LDL of the Teg podocytes with over-expression ANGPTL3 were both higher than those of WT group.

**ANGPTL3 may affect the occurrence of PNS hyperlipidemia by affecting LPL**

In this study, we tested the expression of ANGPLT3 in liver tissue, and found that the mRNA and protein expression levels of ANGPTL3 in liver with wild type mice significantly enhanced since LPS treatment 24 h (Fig. 5a, b, c, \( P < 0.01 \)). It is known that ANGPTL3 powerfully inhibits the activity of LPL which is an important factor of metabolism power for triglycerides and cholesterol. Real time-PCR and Western blot were used to test the mRNA and protein expression levels of LPL in the liver tissues after the mice were took LPS treats 24 h, 48 h and 72 h. The data showed that the mRNA and protein expression levels of LPL in the WT+LPS mice were significantly lower than those in the WT mice at each time point (Fig.5d, e, f, \( P < 0.05 \)). But, the expression level of LPL in the Angptl3-/-+LPS group was significantly higher than that in the WT+LPS group (Fig. 5e, f, \( P < 0.05 \)).

**Discussion**

The mechanism of massive proteinuria and hyperlipidemia in primary nephrotic syndrome has not been clearly explained. Over the past 10 years, there were a large number of important signaling molecules and mechanisms related to proteinuria have been found in the study of podocytes injury\[^{12-14}\], but the mechanism of PNS complicated with hyperlipidemia is still rarely reported.

As a member of the angiopoietin-like protein family, ANGPTL3 is well known as a powerful regulator for lipid metabolism via inhibiting LPL function. There has been very little study about this factor in PNS dyslipidemia. Recently, more and more evidences suggest that ANGPTL3 has been involved in the occurrence of nephropathy proteinuria\[^{4,8,15,16}\]. In our study, we explored the dual role of ANGPTL3, that means, it is not only involved in the occurrence of PNS proteinuria but also hyperlipidemia. Firstly, the
data from the PNS patients suggested that serum ANGPTL3 level was correlated with the degree of hyperlipidemia, such as TG, CHO and LDL.

It's known that the B6;129S5 gene back mice is natural resistance to nephropathy modeling drugs\textsuperscript{[17]}, while C57 BL/6 mice are known to be sensitive to the modeling drugs commonly used in nephropathy\textsuperscript{[17–19]}. Therefore, before establishing of the animal model of nephropathy in this study, we firstly used CRISP/Cas9 technology to knock out the \textit{angptl3} gene of C57 BL/6 mice and established the \textit{angptl3}-/- mice\textsuperscript{[20,21]}. Then, we observed the liver and kidney function and structural characteristics of \textit{angptl3}-/- mice in physiological state. We found that the deletion of \textit{angptl3} gene did not affect the kidney morphology, glomerular and tubular structures of the mice, and no obvious structural abnormalities were found in the podocytes under electron microscope. No obvious abnormalities in liver cells and bile duct structures were observed by light microscopy of liver tissue. At the same time, there were no significant changes in the serum lipid markers and urine protein level of \textit{angptl3}-/- mice compared with the wild-type mice.

The further experimental results of exploring the characteristics of \textit{angptl3} in the nephrotic model confirmed that the proteinuria level of \textit{angptl3}-/- mice after LPS modeling were significantly lower than those of wild-type mice. In accordance with our previous studies in B6;129S5 gene back mice, the podocytes in the mice with \textit{angptl3} gene knockout showed slight podocyte fusion under electron microscope, and the degree of podocyte fusion was significantly reduced compared with wild-type nephropathy mice\textsuperscript{[8]}.

Importantly, the hyperlipidemia levels of \textit{angptl3}-/- mice after LPS modeling were significantly lower than those of wild-type mice. And, the liver tissue analysis showed that the liver cell vacuolization of \textit{angptl3}-/- mice after LPS treatment was significantly less than compared with wild-type nephropathy mice. As well as, \textit{in vitro} data showed that the hepatocytes transfected with \textit{angptl3} also showed significantly enhanced oil red staining. Compared with wild-type hepatocytes, the cells with knocked-down \textit{angptl3} gene expression were less stained with oil red after LPS treatment.

To demonstrate ANGPTL3’s role on PNS hyperlipidemia, we also transfected C57 mice with \textit{angptl3}, and continued to observe the changes of blood lipid and proteinuria in teg-angptl3 mice at different weeks of age. Our results demonstrated that highly expressed ANGPTL3 could lead to nephropathic manifestations with the prolongation of week-old, i.e., and proteinuria, which suggest that ANGPTL3 take part in the both development of hyperlipidemia and proteinuria in mice. But it is necessary to get a longer observation to explore possible pathologic changes in the whole life of teg-angptl3 mice.

In this study, the alterations of LPL in liver tissue in wild type or \textit{angptl3}-/- mice with or without LPS treatment, suggested that ANGPTL3 markedly inhibit LPL expression in PNS model. Through our experiment, it was demonstrated that a large number of ANGPTL3 was synthesized by hepatocytes in nephropathy, thus participating in the occurrence of hyperlipidemia. In one word, the role of ANGPTL3 in regulating of lipid metabolism which is tested in this study give a new opinion for the reason of
dyslipidemia in PNS. Based on the reported data, we think some molecular, such as ANGPTL3 play multiple acts not only in proteinuria but also in hyperlipidemia. Our results may provide a new idea also a for further study of the pathogenesis of PNS in future.

**Conclusion**

ANGPTL3 could be involved in development of dyslipidemia, besides proteinuria in PNS pathogenesis. Inhibiting LPL expression is a reason why ANGPTL3 induce hyperlipidemia in PNS.

**Abbreviations**


**Declarations**

**Ethics approval and consent to participate**

All patients and healthy controls agreed to participate in the study and signed informed consent forms. All experiments were performed in accordance with the Health Guide for the Care and Use of Laboratory Animals and were approved by the Biological Research Ethics Committee of Gansu province People's hospital (No. syll20130331).

**Consent for publication**

All authors read and approved to publication.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests.

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Author's contributions

FZ, Xin Gao and ZCX performed the majority of experiments, analyzed data. And, Xia Gao designed and directed the study, and wrote the manuscript. FZ and ZCX took animal experiments and analyzed data. ZCX and GYL collected clinical objects and related data. BQ and WJC took part in hepatocytes study \textit{in vitro}. YHS tested the expressions of LPL and ANGPTL3 in different mice group. HYD reviewed mouse kidney histopathology and some Western blot.

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References


Tables

Table 1. Comparison of basic characteristics between the both groups [x ± s/n%]
<table>
<thead>
<tr>
<th></th>
<th>60n</th>
<th>196PNS</th>
<th>t/x²</th>
<th>P</th>
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<tbody>
<tr>
<td>Male/female</td>
<td>30/30</td>
<td>124/72</td>
<td>1.79</td>
<td>0.18</td>
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<tr>
<td>Ages (years)</td>
<td>36.10±14.378</td>
<td>35.32±14.348</td>
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<td>CH0 (mmol/L)</td>
<td>3.70±0.932</td>
<td>7.01±2.862</td>
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<td>TG (mmol/L)</td>
<td>1.04±0.455</td>
<td>2.44±1.519</td>
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<td>LDL (mmol/L)</td>
<td>1.92±0.494</td>
<td>4.47±2.246</td>
<td>11.48</td>
<td>0.001</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.18±0.314</td>
<td>1.92±0.853</td>
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<td>24hUP (g/d)</td>
<td>0.09±0.019</td>
<td>3.12±5.063</td>
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<td>ALB (g/L)</td>
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<td>31.60±9.162</td>
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<td>Scr (umol/L)</td>
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<td>84.30±57.858</td>
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<td>BUN (mmol/L)</td>
<td>5.35±1.567</td>
<td>9.44±28.193</td>
<td>0.65</td>
<td>0.52</td>
</tr>
<tr>
<td>Serum ANGPTL3 (ng/ml)</td>
<td>33.01±14.226</td>
<td>70.23±37.045</td>
<td>8.23</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Note: CHO cholesterol, TG triglyceride, LDL low density lipoprotein, HDL high density lipoprotein.

**Table 2. The correlation between serum ANGPTL3 and lipid**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Serum ANGPTL3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r/r_s</td>
</tr>
<tr>
<td>CHO</td>
<td>0.34</td>
</tr>
<tr>
<td>TG</td>
<td>0.25</td>
</tr>
<tr>
<td>LDL</td>
<td>0.50</td>
</tr>
<tr>
<td>HDL</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Note: CHO cholesterol, TG triglyceride, LDL low density lipoprotein, HDL high density lipoprotein.

**Figures**
In physical condition, lipid level and proteinuria with knocked-out angptl3 gene C57 mice shows nearly normal. a, b, c: the levels of triglycerides, cholesterolemia and proteinuria there were no differences in the both groups. (P > 0.05). d: under the light microscope, there was no observed changes between the wild-type and the angptl3/- mice of the liver structure. e, f: The glomerular structure of the knockout mice was
also normal under light microscope and electron microscope. WT: wild type mice, KO: Angptl3-/- mice. * P<0.05 ** P<0.01 the P values were subjected to independent-sample t tests.

**Figure 2**

The knockout of angptl3 gene did not affect the liver and kidney function of mice in physiological state and play an important role in LPS-treated nephropathy. a, b, c: the levels of triglycerides, cholesterolemia and proteinuria in the three groups. d: the lipid droplet deposition in each group liver tissues of mice, and
the magnification is 400 X. e: the electronic changes in the different group, and the magnification is 5,000 X. WT: wild type mice, WL: wild type mice with LPS treatment, KL: Angptl3-/− mice with LPS treatment. Each group included 5 mice. *P<0.05**P<0.01the P values were subjected to one-way ANOVA tests.

**Figure 3**

Angptl3-tg mice developed hyperlipidemia and varying degrees of proteinuria. a: Triglyceride level in angptl3-tg mice markedly increased at different age points. b: The cholesterol level in angptl3-tg mice also
significantly higher than that in wild type mice at every time point. c: The changes of proteinuria level in these three group. d: Random EM photographs of the glomerular basement membrane surrounded by epithelium and endothelium were taken at a magnification of 5,000 X. WT: wild type mice, WL: wild type mice with LPS treatment. Each group included 5 mice. *P<0.05; **P<0.01 the P values were subjected to one-way ANOVA tests.

Figure 4
Observe the effects of ANGPTL3 on hepatocytes in nephrotic state in vitro. a: Red oil staining area changes in the different group, and the magnification is 400 X. b: the OD data were from the red oil staining. c: ELISA data showed LDL in the different group. d: The TG level changed in the different group. WT: wild type hepatocytes, miRNA: angptl3 gene-knockdown hepatocytes, miRNA+LPS: hepatocytes treated with LPS after the knockdown of angptl3, Teg: hepatocytes transfected angptl3. Each group included 5 mice. *P<0.05 **P<0.01 the P values were subjected to one-way ANOVA tests.
ANGPTL3 may affect the occurrence of PNS hyperlipidemia via impacting LPL expression in mice liver tissue. a,b,c: the expression of LPL in wild or Angptl3-/- mice with physiological and nephritis state. d,e,f: ANGPTL3 expression changes in wild or Angptl3-/- mice with physiological and nephritis state. WT: wild type mice, WL: wild type mice with LPS treatment, KO: Angptl3-/- mice, KL: Angptl3-/- mice with LPS treatment. Each group included 5 mice. *P<0.05**P<0.01***P<0.001the P values were subjected to one-way ANOVA tests.

**Supplementary Files**

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- supplementFigurelegends.docx
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