Supplementary Information Titles

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Article Title: Podocyte secreted Angiopoietin-like 4 mediates proteinuria in glucocorticoid sensitive nephrotic syndrome

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Supplementary Figure 2: Experimental studies in Angptl4 -/- mice, Angptl4 transgenic mice, wild type rats, and Angptl4 transgenic rats. (a) Albuminuria in Angptl4 +/+ and Angptl4 -/- mice 48 hours after injection of γ2-NTS. (b) Electron microscopy of the glomerular capillary loop in NTS injected Angptl4 +/+ and Angptl4 -/- mice. Angptl4 -/- mice had 30.2 ± 2.4 % (mean ± SE) foot process effacement compared to diffuse effacement in Angptl4 +/+ mice. (c) Digoxigenin labeled in situ hybridization for Angptl4 expression in PAN Day 6 and control rats. Signal for Angptl4 expression (dark brown / black) in a peripheral distribution is indicated by arrows. (d) Confocal imaging of Angptl4 transgenic mouse glomeruli showing co-localization of Angptl4 and CD2AP expression. (e) Fold increase in kidney cortical mRNA expression in Angptl4 transgenic mice. (f) Real time PCR tracings for studies on glomerular and adipose tissue expression in Angptl4 transgenic rats shown in Fig. 2f. Glomerular control was Wt, adipose tissue control was GAPDH. Scale bars (b) 0.5 µm (c) 10 µm (d)10 µm. * P<0.05, *** P<0.001.
Supplementary Figure 3: Characterization of Angptl4 transgenic rats. (a) Low power electron micrograph of a 5 month old homozygous NPHS2-Angptl4 transgenic rat. Arrows point towards effaced foot processes. (b) Early exposure in situ hybridization image for Angptl4 expression in NPHS2-Angptl4 transgenic and wild type rat glomeruli. Arrows point towards a prominent peripheral pattern in transgenic rats consistent with increased podocyte expression. A faint signal was noted in wild type rats. Tubular expression was not increased in transgenic rats. (c) Albuminuria in one month old NPHS2-Angptl4 transgenic rats. (d) Densitometry of the intact albumin 70 kDa band expressed as a percentage of whole lane densitometry in Fig. 3d. Percentage of intact albumin in urine was significantly higher in NPHS2-Angptl4 transgenic rats compared to all other groups (P < 0.001), except MCD relapse (N. S., not significant). (e) Blood pressure (BP) and pulse rate in 5 month old male wild type and heterozygous NPHS2-Angptl4 transgenic rats. MAP, mean arterial pressure. Scale bars (a)1 µm (b) 20 µm. * P < 0.05, *** P < 0.001.
Supplementary Figure 4: Serum Angptl4 levels in transgenic (TG) rats, and urine Angptl4 in rats with experimental nephrotic syndrome. (a) 2D gel and Western blot assessment of circulating Angptl4 in serum from wild type and Angptl4 transgenic rats. Area magnified in panel b is demarcated by green rectangles. (b) Magnified images of blots in panel a show presence of Angptl4 fragments and oligomers in the circulation. (c) Densitometry of circulating Angptl4 seen in panel b in wild type and Angptl4 transgenic rats. (d) Ponceau red stained nitrocellulose membrane images (equal loading and transfer controls) taken immediately after transfer of 2D gels shown in panels a and b. (e) Western blot of urine from rats injected with γ2–NTS showed presence of low order Angptl4 oligomers. (f) Similar oligomers were noted in urine from PAN Day 6 rats. ** P < 0.01, *** P < 0.001
Supplementary Figure 5: Assessment of GBM charge in Angptl4 transgenic (TG) rats and mice. (a) Alcian blue staining of the GBM in 3 month old wild type and Angptl4 transgenic mice. (b) Densitometry of glomerular alcian blue staining in 3 month old Angptl4 transgenic rats and mice, expressed as a percentage difference from their wild type counterparts. (c) Confocal assessment of GBM heparan sulfate proteoglycan expression in 5 month old wild type and NPHS2-Angptl4 transgenic rats. (d) Assessment of GBM charge in 5 month old wild type and NPHS2-Angptl4 transgenic rats by the polyethyleneimine method. Arrows in the electron micrographs point towards the normal GBM charge in the subepithelial and subendothelial regions. Loss of GBM charge, effacement of foot processes and clustering of cell surface charge were noted in transgenic rat glomeruli. Scale bars (a) 10 µm (c) 10 µm (d) 0.33 µm. *** P<0.001
Supplementary Figure 6
Supplementary Figure 6: Assessment of glomerular Angptl4 sialylation. (a) 2D gel electrophoresis and Western blot of protein from perfused glomeruli in control, PAN Day 6, and glucocorticoid treated PAN Day 6 rats revealed presence of small amounts of fragments (red arrow) and monomers (blue arrow), and larger amounts of low order oligomers (pink, orange arrows) migrating at neutral or high pI. Magnified images of Angptl4 oligomers enclosed in red boxes are shown in Fig. 4a. Neutral pI Angptl4 fragments, but not oligomers, were reactive with anti-phosphothreonine antibodies. (b) Ponceau red stained nitrocellulose membrane images (equal loading and transfer controls) taken immediately after transfer of 2D gels shown in panel a and Fig. 4a. (c) Equal loading and transfer controls for Fig. 4c (as described for panel b). (d) Equal loading and transfer controls for Fig. 4d (as described for panel b). (e) Equal loading and transfer controls for Fig. 4e (as described for panel b). (f) 2D gel electrophoresis and Western blots for glomerular podocalyxin expression on Day 12 of experiment described in Fig. 4e. In addition, podocalyxin expression in wild type rats was also assessed. (g) Densitometry studies from blots in panel f. (h) Equal loading and transfer controls for panel f (as described for panel b). N. S., not significant, TG, transgenic
Supplementary Figure 7: Characterization of Angptl4 secreting HEK293 and mouse GEC stable cell lines. (a) Analysis of Angptl4 mRNA upregulation in the Angptl4-HEK293 stable cell line. Expression in the control (empty vector) stable cell line was given an arbitrary value of 1. (b) 2D gel electrophoresis and Western blot for Angptl4 in supernatant from Angptl4-HEK293 and control-HEK293 stable cell lines collected under serum free conditions. (c) A molecular weight marker gel (top image) was superimposed with the right lower image from panel b to generate a composite image (bottom image) using Adobe Photoshop. Most of the intact 70 kDa Angptl4 monomer secreted by the Angptl4-HEK293 stable cell line migrates between pI 8.0 and 8.5. (d) Analysis of Angptl4 mRNA upregulation in the Angptl4-GEC stable cell line. (e) Western blot and GelCode blue analysis of recombinant protein secreted by 2 different clones of the Angptl4-GEC stable cell line. *** P < 0.001
Supplementary Figure 8: Albuminuria in ManNAc - treated NPHS2-Angptl4 transgenic (TG) rats, and oligomerization of recombinant and transgene expressed Angptl4. (a) Representative tracings of albuminuria from pilot studies, in which increasing doses of ManNAc were shown to reversibly reduce albuminuria in NPHS2-Angptl4 transgenic rats with moderate to heavy albuminuria. (b) Individual tracings of albuminuria from four NPHS2-Angptl4 transgenic rats that competed both the ManNAc and washout phase of the 36 day study. Two urine collections 12 days apart were conducted prior to the start of the study to ensure that these rats developed increasing albuminuria with time. (c) Non-reducing SDS PAGE and Western blot to assess for oligomerization of circulating and glomerular expressed Angptl4 from transgenic rats, and recombinant Angptl4 from the HEK293 stable cell line. Gels were run for 1 hour (left) or 2.5 hours (right) to study all oligomeric forms (low, middle and high order oligomers). α2 macroglobulin (A2M) was used as a high molecular weight marker (720 kDa).
Supplementary Figure 9: Confocal assessment of glomerular Angptl4 expression in human minimal change disease (MCD). (a) Increased expression and co-localization of Angptl4 with podocyte (nephrin), GBM (laminin) and endothelial cell (PECAM) markers in the biopsy of an individual with MCD. (b) Biopsies from 4 additional individuals with MCD were stained for Angptl4 and other glomerular capillary loop markers listed above. Arrows in high power images point towards overlap of staining patterns. Lowermost panels show presence of Angptl4 in proximal tubules (marked by aquaporin 1), most likely due to tubular uptake, since upregulation of Angptl4 mRNA expression is not seen in tubules in experimental MCD (puromycin nephrosis) by in situ hybridization. Scale bars 10 μm.
Supplementary Figure 10: Plasma and urine Angptl4 in human primary glomerular disease. Clinical data shown in Supplementary Table 1. (a) Reducing SDS PAGE and Western blot of urine shows presence of Angptl4 oligomers in individuals with minimal change disease (MCD), and fragments in focal and segmental glomerulosclerosis (FSGS) patients. (b) 2D gel electrophoresis and Western blot of urine (left panels) confirms the presence of Angptl4 oligomers and fragments in MCD, and only fragments in FSGS. Ponceau red stained nitrocellulose membrane images (equal loading and transfer controls) taken immediately after transfer of 2D gels are also shown (right panels). (c) Representative 2D gel electrophoresis and Western blot of plasma from individuals with MCD, FSGS and membranous nephropathy (MN). Only individuals with MCD in relapse had elevated circulating levels of 55 - 70 kDa pI 8 - 8.5 Angptl4 protein (red oval). Increased circulating neutral pI monomers and oligomers were noted in individuals with MCD in relapse (red arrow), and monomers only in MN (green arrow). (d) Densitometry analysis of circulating Angptl4 represented in panel c. (e) Equal loading and transfer controls for blots shown in panel c (as described for panel b). *** P < 0.001, comparison with MCD remission.
Supplementary Figure 11: Schematic representation of the role of podocyte secreted Angptl4 in nephrotic syndrome. Sequence of events arranged from top to bottom. Podocytes secrete neutral and high pI Angptl4 that binds to the GBM to alter protein-protein interactions, resulting in proteinuria. Over time, Angptl4 reaches up to the endothelial surface. Progressive accumulation and clustering of Angptl4 in the GBM likely activates signals at the podocyte-GBM interface, induces foot process effacement and further increase in proteinuria. Circulating Angptl4 secreted from other organs in disease states e.g. adipose tissue, forms medium and high order oligomers that are bound to HDL particles, migrate at neutral or low-neutral pI, and do not enter the GBM or cause proteinuria.
| Patient | Diagnosis   | age (years) | sex | proteinuria          | primary treatment (initiated or past)         |
|---------|-------------|-------------|-----|----------------------|-----------------------------------------------|
| MCD 6   | MCD - relapse | 14          | female | nephrotic             | glucocorticoids                               |
| MCD 7   | MCD - relapse | 14          | female | nephrotic             | glucocorticoids + cyclosporin                 |
| MCD 8   | MCD - relapse | 4           | male  | nephrotic             | glucocorticoids                               |
| MCD 9   | MCD - relapse | 10          | male  | nephrotic             | glucocorticoids                               |
| MCD-R1  | MCD remission | 12          | male  | < 0.5 gm/24 hours    | glucocorticoids + cyclophosphamide            |
| MCD-R2  | MCD remission | 5           | male  | < 0.5 gm/24 hours    | glucocorticoids + cyclophosphamide            |
| MCD-R3  | MCD remission | 9           | male  | < 0.5 gm/24 hours    | glucocorticoids + cyclophosphamide            |
| MCD-R4  | MCD remission | 3           | male  | < 0.5 gm/24 hours    | glucocorticoids                               |
| FSGS 1  | FSGS        | 49          | male  | sub nephrotic         | AEC inhibitor                                 |
| FSGS 2  | FSGS        | 29          | female | nephrotic             | ACE inhibitor                                  |
| FSGS 3  | FSGS        | 9           | male  | sub nephrotic         | cyclosporin                                   |
| FSGS 4  | FSGS        | 8           | male  | nephrotic             | glucocorticoids                               |
| MN 1    | MN          | 59          | male  | sub nephrotic         | ACE inhibitor                                 |
| MN 2    | MN          | 63          | male  | sub nephrotic         | ACE inhibitor                                 |
| MN 3    | MN          | 31          | female | nephrotic             | unknown                                       |
| MN 4    | MN          | 52          | male  | sub nephrotic         | ACE inhibitor                                 |

**Supplementary Table 1:** Brief clinical profile of individuals whose plasma and urine were assessed for presence and patterns of Angptl4 expression by Western blot.

| Gene / transgene | Species | Forward primer | Reverse primer | Taqman probe          |
|------------------|---------|----------------|----------------|-----------------------|
| Angptl4          | rat     | tctggatctcaccatcttttg | tcaccgtcatcatcttttac | caactgtgagagacttc     |
| Angptl4          | rat     | cgccaccaagtacca | cagaggcttttctgaaaagt | tggcagtttttctt        |
| NPHS2-Angptl4 construct | rat | tacagcgttttttctgta | aaccggggtctgctag | cactgaggcttctagca     |
| aP2-Angptl4 construct | rat | tcgtgcttttttctgta | aggggcttttctgcatttg | cagcagctctc          |
| Prolactin (genomic) | rat |cttgaagggattgaaagatattgc | cctgagtgaaagatattgc | aggtgagatccttctcttg  |

**Supplementary Table 2.** List of primers and probes used for Taqman real time PCR.

**Supplementary Tables**
Supplementary Methods

Cloning of full length rat Angptl4, and generation of antibody against full length recombinant Angptl4: We cloned the full length rat Angptl4 open reading frame from our previous experiments (3), excluding the stop codon, into pcDNA3.1/V5-HisB for eukaryotic expression, and into pET28a for prokaryotic expression. The E. Coli expressed purified full length protein was used to generate a polyclonal antibody in rabbits (Proteintech group, Inc., Chicago IL USA) that was tested by ELISA and Western blot. We excised antibody reactive bands from GelCode blue stained gels and confirmed the presence of Angptl4 peptide sequences by MALDI-TOF/TOF. Part of the antiserum was affinity purified to the antigen. All studies described used this antibody. We raised an additional polyclonal antibody against the N-terminal part of rat Angptl4 (amino acids 7 – 86 excluding signal peptide) in rabbits, and used this for confirmation studies.

We purchased the following antibodies: goat anti-mouse CD2AP (Santa Cruz Biotechnology Inc., Santa Cruz CA USA), guinea pig anti-human nephrin (Fitzgerald Industries Int., Acton MA USA), mouse anti-rat β2γ1 laminin (Abcam, Cambridge MA USA), mouse anti-rat PECAM-1 (BD Pharmingen, San Diego CA USA), mouse anti-human aquaporin 1 (Santa Cruz Biotechnology Inc.), goat anti-mouse podocalyxin (Alpha Diagnostic International, San Antonio TX USA).

Induction of proteinuria in animal models of human glomerular disease. All animal studies using rats were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Alabama at Birmingham, Instituto Nacional de Cardiologia and Northwestern University. Induction of animal models of proteinuria (n = 4 rats/group) in
wild type rats are described in previous publications in parenthesis: PAN (3), PHN (3), PAN with glucocorticoids (15), non-HIV collapsing glomerulopathy (14), nephrotoxic serum induced heterologous phase proteinuria (3). Anti-Thy1.1 nephritis was induced by injection of 200 μg of anti-Thy1.1 (Ox-7 hybridoma) or control IgG IV into different groups of male Wistar rats (100-125 gm, n = 4 rats/group), and rats euthanized after 24 and 72 hours.

The following techniques are described in prior publications: Taqman real time PCR (23), confocal imaging (3), in situ hybridization (24), promoter – reporter studies (3), immunogold electron microscopy using 10nM gold particles (23), glomerular extraction and processing for Western blot (23), assessment of charge by polyethyleneimine method (25). Real time PCR studies for screening were performed with a minimum of 3 templates, and if positive, were confirmed with a minimum of 6 templates. In rat models of proteinuria, 3-fold change in glomerular gene expression was considered significant (3). In the rat model of non-HIV collapsing glomerulopathy, we assessed glomerular gene expression in sieved and laser captured glomeruli. Taqman real time PCR primers and probes are listed in Supplementary Table 2. For in situ hybridization (n = 2 rats / condition), we generated a digoxigenin labeled probe for rat Angptl4 that included bp 1 to 548 of the open reading frame. Unless otherwise stated, all 2D gel electrophoresis was performed using 11 cm Immobiline pH 3–11 strips (GE Healthcare, Uppsala, Sweden) and Criterion 8–16% Tris HCl Precast Gels (Bio-Rad Laboratories, Hercules CA USA), using 200 μg protein loaded in the first phase (n = 3 gels / condition). Densitometry of 2D gel Western blots was assessed using Gel-Pro Analyzer software (Media Cybernetics, Inc., Bethesda MD, USA). For alcian blue staining, the pH of the
staining solution was adjusted to 2.5 using acetic acid, and 0.1% nuclear fast red solution was used as a counterstain. Densitometry of glomerular basement membrane alcian blue stain (20 glomeruli / rodent, 3 rodents / group) was assessed using Image-Pro software (Media Cybernetics, Inc.).

Injection of NTS into Angptl4−/− mice: Angptl4−/− mice were provided to Sander Kersten by Eli Lilly Corporation (Indianapolis IN USA). The Animal Ethics Committee at Wageningen University approved the study protocol. We injected 11 week old male Angptl4−/− or Angptl4+/+ mice (n = 4 mice / group) intravenously with 1.5 mg γ2-NTS or normal sheep serum (Sigma Aldrich, St. Louis MO USA), collected spot urine samples at 48 hours, euthanized the mice at 72 hours, collected plasma for biochemical measurements, and preserved kidneys for histological analysis. We assessed urine albumin by ELISA (Bethyl laboratories, Montgomery TX USA) and measured urine creatinine by mass spectrometry. To assess for foot process effacement, the mean width of foot processes was first measured in electron micrographs of control treated Angptl4+/+ mice (10 equally spaced readings / loop, 3 loops / glomerulus, 3 glomeruli / kidney, 3 kidneys / group). Effacement was defined as over 2.5 fold increase in mean width. We assessed the total number of foot processes, and the percentage effaced foot processes in NTS treated or control treated Angptl4−/− mice.

Injection of lipopolysaccharide (LPS) into Angptl4−/− mice: The study protocol was approved by the Animal Ethics Committee at Wageningen University. After a baseline spot urine collection, we injected 12 week old Angptl4−/− and Angptl4+/+ mice (n = 5 mice / group) intraperitoneally with 10 μg / gram body weight of ultrapure LPS (Sigma Aldrich, catalog number L4524) or an equal volume of PBS. Each mouse also received 0.8 ml of
normal saline at 12 and 36 hours after the LPS injection to avoid volume depletion. We collected urine at the peak of proteinuria 24 hours after LPS injection. Mice were euthanized 48 hours after LPS injection. We assessed urine albumin and creatinine concentrations as detailed for the NTS study.

**Measurement of rat blood pressure:** Blood pressure and pulse rate were measured in six 5 month old wild type and proteinuric heterozygous NPHS2-\textit{Angptl4} transgenic rats by the tail cuff method using the SC-1000 apparatus from Hetteras Instruments, Inc. (Cary NC USA). A minimum of 80 reading were analyzed per group.

**Studies on oligomerization of Angptl4:** Since 2D gel electrophoresis has limitations in resolving high molecular weight proteins, we performed non-reducing 1D SDS PAGE and Western blot were performed. We loaded 3 μg of human α2 macroglobulin (A2M, high molecular weight marker, 720 kDa), 60 μg each of αP2-Angptl4 transgenic rat plasma and protein from perfused NPHS2-Angptl4 transgenic rat glomeruli, and 35 μg of concentrated supernatant from HEK293 stable cell line in duplicate into 5% non-reducing gels, ran the for 1 hour or 2.5 hours, transferred them to PVDF membranes, and conducted Western blot studies. We also ran standard molecular weight markers in one lane.

**Studies with human samples:** We conducted immunostaining and confocal imaging of human kidney biopsies (n = 5 biopsies per condition) obtained through protocols approved by the Research Ethics Committee at the Instituto Nacional de Cardiologia, Mexico City. Control kidney biopsies used for these studies were age, sex and race matched protocol pre-transplant biopsies. We obtained human sera and urine samples
for 2D gel electrophoresis and Western blot (n = 4 samples / condition) from a previously published study (26) (select details in Supplementary Table 1).