Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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SUPPLEMENTARY METHODS

Patients

The diagnosis of membranous nephropathy (MN) was made by renal biopsy. In the European cohort, all patients were without previous immunosuppressive therapy at the time the first serum was taken. The Boston cohort was more heterogeneous in that some had already been treated with immunosuppressive regimens by the time the serum was collected. Patients were classified as idiopathic or secondary according to clinical characteristics: A patient was considered to have secondary MN in the presence of antinuclear antibodies above a titer of 1:80, anti-double stranded DNA antibodies, histological signs of lupus membranous nephritis (type V lupus nephritis), or positive hepatitis B or C serology. If a malignancy was present and the diagnosis of MN appeared to be temporally associated, the patient was also considered to have secondary MN. In addition, in the Boston cohort, patients with MN associated with mercury exposure, IgG4-related systemic disease, or very atypical biopsy findings (such as microspherical substructure to the deposits), were all considered to have secondary disease. All other patients were classified as idiopathic MN (iMN).

Minimal change disease (MCD), focal-segmental glomerulosclerosis (FSGS), IgA nephropathy (IgAN), ANCA vasculitis, and acute tubular necrosis (ATN) were also diagnosed by renal biopsy. Patients with systemic lupus erythematosus were diagnosed according to ARA criteria and lupus nephritis was diagnosed by renal biopsy. All cases of lupus nephritis other than type V were classified as "other glomerular disease". In the Boston cohort, patients with idiopathic MN were assembled from three primary sources. The majority of the patients (n=204) had been enrolled at Boston University Medical Center as part of ongoing studies into the pathogenesis of MN. These idiopathic MN samples were taken from a larger, cross-sectional cohort of serum samples from

patients with MN. A proportion of the subjects had been treated with immunosuppression by the time the sample was obtained. All patients in the idiopathic MN group had biopsy-proven MN and no evidence of a diagnosis that would be considered a secondary cause of MN (as detailed above). Samples were excluded if the patient was <18 years old; if gender, age, and proteinuria data were unavailable; or if proteinuria at the time of sample collection was < 1 g/d. There were 33 additional subjects from this cohort who fit the above criteria but whose MN was more likely due to a secondary cause. Another large group of idiopathic MN cases (n=117) was assembled as part of the Limburg Renal Registry in the Netherlands, and serum was obtained at the time of renal biopsy prior to any immunosuppressive treatment. Finally, 44 patients from Sweden made up the remainder of the cohort. All sera were screened by Western blot at 1:25 against native and recombinant PLA2R1 (as described in Beck et al, 2009[1]). Only samples reactive with a band in glomerular extract but not with recombinant PLA2R1 were screened for reactivity against THSD7A. Numbers are shown in table S1.

Preparation of human renal tissue

Macroscopically healthy parts of kidneys from patients who underwent nephrectomy or from kidneys that were deemed unsuitable for transplantation were used for the preparation of glomeruli. These latter samples are tipically without blood supply for 12-24 hours. After cross-clamping and surgical removal, they are treated identically to kidneys that would be used for transplantation. Some are stored on ice, and some are attached to a kidney perfusion pump, which circulates University of Wisconsin (nutrient-containing) perfusion solution through the kidney. About half of the kidneys were received boxed (on ice) and the other half on the pump.

First, the capsule was removed and the cortex was cut in pieces. Glomeruli were isolated by graded sieving and the glomerular pellet was resuspended in 50 mM Tris pH

7.4, 0.32 M sucrose, 10 mM EDTA, protease inhibitor cocktail (Roche, Mannheim, Germany), thereby using a volume around 10x the volume of the glomerular pellet. The solution was then homogenized for 1 min at 28,000 rpm using Polytron PT 2500 (Kinematica, Luzern, Switzerland) and debris was removed by a gentle centrifugation at 500 rpm for 3 minutes. Subsequently, the preparation was centrifuged for 1 h at 4 °C, 100,000 x *g* (Optima Max-XP ultracentrifuge, Beckman Coulter, Indianapolis, USA). The supernatant containing soluble glomerular proteins, mostly cytosolic proteins was refered to as the *soluble fraction (FS)*. The pellet was resuspended in 3 volumes of the initial glomerular pellet volume in 50 mM Tris pH 7.4, 0.32 M sucrose, 10 mM EDTA and then solubilized by addition of an equal volume of 50 mM Tris pH 7.4, 150 mM NaCl, 0.1%SDS, 1% NP-40, 0.5% Na-deoxycholate. The preparation was homogenized by 10 rounds of manual douncing and then incubated for 30 minutes at 4 °C with constant rotation. The insoluble fraction was removed by a 100,000 x *g* centrifugation for 1 h at 4 °C. The supernatant containing solubilized glomerular proteins, e.g. membrane proteins, was refered to as the *solubilized fraction (FSz)*. Immunoglobulin G was removed from both preparations, FS and FSz, by overnight incubation with MACS Protein A microbeads and subsequent extraction of microbeads with bound IgG by magnetic separation columns (both Miltenyi Biotec, Bergisch Gladbach, Germany). Western blot analysis revealed no significant amounts of human IgG in the preparations. Alternatively, soluble and membrane proteins were not separated by ultracentrifugation, mainly for the purpose of a higher protein yield. In this case, glomerular proteins were obtained as described previously[1] with minor modifications: glomerular pellets were resuspended in an equal volume of 100 mM Tris pH 8.0, 1 mM $MgCl_2$, and freeze-thawed at -80 °C for 20 minutes. An equal volume of 50 mM Tris pH 7.4, 150 mM NaCl, 0.1%SDS, 1% NP-40, 0.5% Na-deoxycholate was added with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA). Glomeruli were manually dounced 15x followed by a

break of 5 minutes. This procedure was repeated three times. Then the solution was incubated for 30 minutes at 4 °C with constant rotation followed by a 10 minutes centrifugation at 14,000 rpm at 4 °C. Human IgG was removed as described above. Protein concentration of the different preparations was determined by the Bradford method using bovine serum albumin as a standard. For Western blot analysis, 10 µg of total proteins were loaded per lane if not indicated otherwise.

Cell culture

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, USA) with addition of 10% fetal bovine serum (Thermo Scientific, Cramlington, United Kingdom) and 1% Penicillin/Streptomycin (Life Technologies, Grand Island, USA).

Cell transfection and recombinant protein expression

Human cDNAs coding for THSD7A and the three paralogs of PLA2R1, *i.e.* MRC1, MRC2, and LY75, were cloned by PCR using standard methods and according to the NCBI sequences or purchased from companies (Gene Coppoeia and OriGene). All cDNAs were verified by sequencing after subcloning into mammalian expression vectors and addition of specific HA or DDK tags (pLPCX or pCMV6 entry).

We performed transient transfection of HEK293 cells using a Ca/PO4 homemade transfection kit (see below). For each 10-cm dish of cultured HEK cells, we used 15 µg of plasmid DNA mixed with 36 µL of 2 M $CaCl_2$ and diluted with sterile water up to a volume of 300 µL. This solution was gently mixed with an equal volume of 2x Hepes buffered saline (HBS, 275 mM NaCl, 55 mM Hepes, pH 7.0) and incubated 30 minutes at RT. The resulting solution was added dropwise to HEK cells. Medium was exchanged

24 h after transfection. Cells were scraped, centrifugated at 1,500 rpm for 5 minutes, washed with PBS, and centrifugated again at 1,500 rpm for 5 minutes. Cells were then lysed in 20 mM Tris pH 7.4, 2 mM EDTA after addition of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA), sonicated, and centrifugated at 100,000 x *g* and 4 °C for 1 hour. The supernatant corresponded to the *soluble fraction* of the protein. The pellet was solubilized in 50 mM Tris pH 7.4, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate after addition of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA), dounced, sonicated again, incubated for 1 hour at 4 °C, and centrifugated again at 100,000 x *g* and 4 °C for 1 hour. The supernatant corresponded to the *solubilized fraction* of the protein.

Protein concentration was determined using the Bradford assay and the expression of the different recombinant proteins was validated in comparison to mock transfection using specific antibodies.

Deglycosylation

Peptides contained in the protein samples were deglycosylated using neuraminidase, *N*-glycopeptidase F (both Roche Diagnostics, Mannheim, Germany) or a combination of both. One µL of the enzyme solution was added to approximately 400 µg of total protein and samples were incubated overnight at 37 °C and subsequently analyzed by Western blot.

Western blot analysis

Protein samples were prepared for Western blot analysis by addition of 5x Laemmli buffer (1.5 M Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 1% bromophenol blue) and subsequent heating to 95 °C for 10 minutes. If reducing conditions were desired, 20% beta-mercaptoethanol was added to the 5X loading buffer. Proteins were separated by

electrophoresis either in homemade gels containing sodium dodecyl sulfate (SDS) and 7% acrylamide, or in precast 4-12% gradient gels (Bio-Rad, Hercules, USA) in an electrophoresis chamber (Bio-Rad, Hercules, USA) in the presence of a migration buffer (25 mM Tris, 192 mM glycine, 0.1% SDS; Amresco, Solon, USA). Proteins were then transferred to methanol-soaked PVDF membranes (Millipore, Billerica, USA) under semi-dry conditions in the presence of 25 mM Tris pH 8.5, 192 mM glycine, ethanol 20% using Transblot Turbo (Bio-Rad, Hercules, USA) at 25 V constant for 35 min.
Membranes were blocked overnight at 4°C in 5% dry milk with PBS-Tween 0.05% (PBS-T) and then incubated with primary and secondary antibodies for 2 h at RT. Primary antibodies were diluted with 0.5% dry milk in PBS-T and horseradish peroxidase (HRP)-conjugated secondary antibodies in PBS-T alone. Membranes were washed three times for 5 minutes in PBS-T after incubation with primary and secondary antibodies.
If serum was used as the primary antibody, a dilution of 1:100 was used unless indicated otherwise. Secondary antibodies were HRP-conjugated mouse anti-human IgG (SouthernBiotech, Birmingham, USA). Dilutions for total anti-human IgG and anti-IgG4 were 1:20,000 and 1:30,000, respectively.
For specific detection of thrombospondin type 1 domain containing 7A we used a commercial rabbit polyclonal antibody (Sigma, St. Louis, USA) in a dilution of 1:1,000. HRP-conjugated goat anti-rabbit IgG (SouthernBiotech, Birmingham, USA) in a dilution of 1:5,000 was used as a secondary antibody in this case.
For detection of the paralogs of PLA2R1, we used monospecific antibodies to PLA2R1 (Sigma, St. Louis, USA), MRC1 (Sigma, St. Louis, USA), MRC2 (R&D Systems, Minneapolis, USA) and LY75 (Exbio, Vestec, Czech Republic).
Determination of IgG subclasses was performed using anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4 antibodies as secondary antibodies, all at a dilution of 1:20,000 (all SouthernBiotech, Birmingham, USA).

For the detection of protein bands, membranes were incubated in a chemiluminescent substrate (Millipore, Billerica, USA) for 90 seconds followed by luminescence detection with a Fujifilm LAS imager 3000. Exposure time was incremental and typically between 20 seconds and 2 minutes. When ultrahigh sensitivity detection was desired, Western Lightning Ultra (PerkinElmer, USA) was used.

For some experiments, bound primary antibodies were stripped by incubating membranes in 0.25 M glycine, 0.1% SDS, 1% Tween, pH 2.2 for 10 minutes at room temperature. Membranes were then washed twice in PBS for 10 minutes and twice in PBS-T for 5 minutes. Subsequently, membranes were blocked with 5% milk in PBS-T overnight at 4°C an incubated with primary and secondary antibodies as described above.

Mass spectrometry proteomics analysis to identify THSD7A as a new MN antigen

We designed two different strategies to determine the molecular identity of the novel antigen. In the first strategy, we performed gel electrophoresis of native glomerular proteins and glomerular proteins digested with *N*-glycopeptidase F alone or with both N-glycopeptidase and neuraminidase. Coomassie blue-stained gel regions corresponding to the Western blot signals at 250, 225 and 200 kDa, were excised with the expectation that the polypeptide backbone of the novel antigen would be present in all regions. The greater part of the proteins from each treatment was stained with Coomassie blue dye and the other part was transferred to PVDF and probed with a patient's serum positive for antibodies against the novel antigen. For in-gel digestion, gel pieces containing the proteins of interest were excised and destained by adding 100 µL of H_2O/ACN (1/1). After 10 min incubation with vortexing the liquid was discarded. This procedure was repeated 2 times. Gel pieces were then rinsed (15 min) with acetonitrile and vacuum-dried. Gel pieces were rehydrated in 60 µL of 100 mM DTT in 100 mM NH_4HCO_3 and

incubated for 30 min at 56°C followed by cooling down to RT. The DTT solution was replaced with 60 µL of 55 mM iodoacetamide in 100 mM NH_4HCO_3. After 15 min incubation at RT the solution was discarded and gel pieces were washed by adding successively i) 100 µL of H_2O/ACN (1/1), repeated 2 times and ii) 100 µL of 100% ACN. Next, gel pieces were rehydrated in 60 µL of 50 mM NH_4HCO_3 containing 10 ng/µL of trypsin (modified porcine trypsin sequence grade, Promega) and incubated for one hour at 4°C. The solution was then removed and replaced by 60 µL of 50 mM NH_4HCO_3 buffer (without trypsin) and incubated 18 hours at 37°C. After trypsin digestion the solution was transferred into a microcentrifuge tube and tryptic peptides were isolated by extraction with i) 50 µL of 1% formic acid in water (10 min at RT) and ii) 50 µL acetonitrile (10 min at RT). Peptide extracts were pooled, concentrated under vacuum, and solubilized in 10 µL of 0.1% TFA (trifluoroacetic acid) in water. Peptide separation was carried out using a nanoHPLC offline (DIONEX, U3000) coupled with a MALDI-TOF/TOF mass spectrometer (4800 *plus*, Applied Biosystems). Peptide solutions were concentrated on a µ-Precolumn Cartridge Acclaim PepMap 100 C_{18} (i.d. 5 mm, 5 µm, 100 Å, DIONEX, LC Packings) at a flow rate of 20 µL/min and using a solvent containing H_2O/ACN/TFA 98%/2%/0.04%. Next, peptide separation was performed on a 75 µm i.d. x 150 mm (3 µm, 100 Å) Acclaim PepMap 100 C_{18} column (DIONEX, LC Packings) at a flow rate of 200 nL/min and with detection at 214 nm. Solvent systems were: (A) 100% water, 0.05% TFA, (B) 100% acetonitrile, 0.04% TFA. The following gradient was used t = 0min 100% A; t = 3 min 100% A; t = 63min, 80% B; t = 64min, 100% B; t = 68min 100% B (temperature was regulated at 30°C). For offline nanoHPLC-MALDI-TOF/TOF-MS and MS/MS analyses, fractions were collected on an Opti-TOF LC/MALDI target (123x81mm, Applied Biosystems) and fractionation was done using a Probot fractionation robot (DIONEX, LC Packings). Matrix solution (α-cyano-4-hydroxycinnamic acid, 2.5 mg/mL in 50% water, 50% acetonitrile, 0.1% TFA solution) and nanoHPLC

fractions were mixed (in rate 4:1, matrix:fractions) and collected every 20s (208 fractions were collected per run). MALDL-TOF/TOF-MS analysis: MS spectra were recorded automatically in a mass range of 700-4,000 Da resulting from 200 laser shots of constant intensity. Data were collected using 4000 series Explorer (Applied Biosystems) allowing for an automatic selection of peptide masses for subsequent MS/MS experiments. Each MS/MS spectra acquired using 1,000 laser shots were further processed using 4000 series Explorer. Finally, all the raw data were transferred into the ProteinPilot software (Applied Biosystems, MDS Analytical Technologies) and protein identification was processed using the Paragon™ Algorithm.

In the second strategy, we took advantage of the differential sensitivity of PLA2R1 and the novel antigen to proteolysis with trypsin. A fraction of human glomeruli containing soluble proteins and shed microparticles was generated by incubating glomeruli freshly isolated from human kidney cortex (derived from a deceased donor kidney deemed unsuitable for transplantation) 1:1 with phosphate-buffered saline, pH 7.4 for 1 hr at 37°C in the absence of protease inhibitors. Glomeruli and larger cellular debris were centrifugated 5 min at 14,000 rpm in a tabletop microcentrifuge and the supernatant frozen in aliquots at -80°C. This fraction is known to contain PLA2R1 and the novel antigen by Western blot analysis with serum samples from MN patients reactive with each antigen (data not shown). In order to increase the yield of (non-glycosylated) peptide spectra that could potentially be identified by mass spectrometric analysis, the following steps were carried out both in the presence and absence of protein *N*-glycopeptidase F (PNGase F) treatment. Limited proteolysis of these fractions was carried out with 0.005% trypsin for 10 min at 37°C. The reaction was stopped by the addition of 4x non-reducing gel loading buffer and heating for 5 min at 95°C. Polyacrylamide gels were loaded with the PNGase F-treated and partially-proteolyzed glomerular fraction and electrophoresed as detailed above. A portion of the gel was

transferred to nitrocellulose membranes and Western blotted for PLA2R1 and the unidentified antigen to confirm their relative positions. Once confirmed, the region of the gel (approximately 150-200 kDa) containing the minimally proteolyzed novel antigen and no detectable PLA2R1 was cut and sent for mass spectrometric analysis. Two duplicate SDS-PAGE gel bands containing candidate target antigens were processed for liquid chromatography-mass spectrometric analysis separately using trypsin and chymotrypsin (Promega) as previously described with modifications to address the requirement of Ca++ for chymotrypsin[2-4]. The digest supernatant and gel piece extracts were combined, lyophilized and re-dissolved in 2% acetonitrile / 0.1% formic acid and fractioned off-line using strong cation exchange (SCX) chromatography (SCX MicroTrap™ (Michrom-Bruker, Auburn, CA, USA)) to yield eight fractions. SCX fractions were further resolved using an EASY n-LC (ThermoElectron, Waltham, MA), UHPLC system with a Dionex RSLC Pepmap 100 reversed phase column and introduced by nanospray Nanospray Flex source (ThermoElectron), into a LTQ-Orbitrap ELITE (ThermoElectron) mass spectrometer. Tandem mass spectra were collected using HCD and ETD fragmentation using an Nth Order Double Play with ETD Decision Tree method was created in Xcalibur v2.2. [5] Data dependent spectra were acquired and searched using Proteome Discoverer (v1.3.0.330) using Mascot (v2.1) and SageN Sequest Sorcerer using UniprotKB Homo sapiens reference proteome canonical and isoform sequences (version 12/10/2013). In order to estimate the false discovery rate, a decoy database was generated from this database with the program decoy.pl (from matrixscience.com). The search strategy included dynamic modifications of methionine oxidation and asparagine deamidation with fixed modification of cysteine carbamidomethylation. The enzyme Tryp/Chymo (cleavage after KFRWY at C-terminus; restricted by P residues) was used with up to two missed cleavages allowed. The resulting .msf files from Proteome Discoverer were loaded into Scaffold (v3.6.5). Scaffold was used to calculate the false discovery rate

(<1%) using the Peptide and Protein Prophet algorithms. The results were annotated with human gene ontology information from the Gene Ontology Annotations Database (ftp.ebi.ac.uk).

Mass spectrometric results were filtered according to size (170-250 kDa) and likelihood that they would be available to autoantibodies (*i.e.,* transmembrane or extracellular proteins). A number of intracellular and transmembrane proteins were identified by this analysis (see Table S3), many of which were common to both mass spectrometric analyses. A limited list of candidates, including the complement receptor type 1, agrin, fibronectin, laminin gamma1 subunit, and thrombospondin type 1 containing 7A, were chosen for further study. Based on expected size and cellular location, THSD7A emerged as a promising candidate.

Immunoprecipitation

25 µL of the glomerular protein lysate was mixed with 100 µL of 20 mM Tris pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$ and 5 µL of serum (MN anti-THSD7A positive, MN anti-PLA2R1 positive, MN non-reactive, other glomerular disease, healthy control) or 5 µL of water. Samples were incubated 2 h at 4 °C on a rotator. Then 20 µL of IgG4 affinity matrix (CaptureSelect, Life Technologies, Leiden, Netherlands) was added and samples were incubated overnight at 4 °C on a rotator. Samples were then spun down for one minute at low speed and the supernatant was discarded. The pellet was washed three times with 20 mM Tris pH 7.4, 140 mM NaCl, 0.8 mM $CaCl_2$, 0.02% SDS, 0.2 % NP-40, 0.1% Na-DOC and subsequently resuspended in Laemmli buffer containing beta-mercaptoethanol, loaded on polyacrylamide gels and electrophoresed. Immunoprecipitates were detected with a rabbit polyclonal anti-THSD7A antibody (Atlas).

Histological analysis

For immunofluorescence analyses, 2 µM paraffin sections of the healthy pole of a human tumor nephrectomy specimen were deparaffinized and rehydrated in water. Antigen retrieval was obtained by boiling in citrate buffer pH 6.1 (30 min at constant 98°C). Nonspecific binding was blocked with 5% horse serum (Vector, Burlingame, USA) with 0.05% Triton X-100 (Sigma, St. Louis, USA) in PBS for 30 min at RT prior to incubation at 4°C overnight with primary antibodies in blocking buffer. Staining was visualized with fluorochrome-conjugated secondary antibodies (Jackson Immunoresearch, Dianova, Hamburg, Germany; 1:400, 30 min RT in 5% horse serum). Nuclei were visualized using DRAQ5 (Molecular Probes, Life Technologies, Grand Island, USA). Negative controls were performed omitting primary antibodies. Stained sections were evaluated by confocal microscopy using the Laser Scanning Microscope 510 and appropriate software (all Zeiss, Oberkochen, Germany).

For immunohistochemistry, 1 µM paraffin sections of renal biopsies from patients with MN were deparaffinized and rehydrated. Antigen retrieval was obtained by boiling in DAKO antigen retrieval buffer, pH 9 (15 min at 98°C) and subsequent cooling at RT for 15 min. Nonspecific binding was blocked with 5% horse serum (Vector) with 0.05% Triton X-100 (Sigma) in PBS for 30 min at RT prior to incubation at 4°C o/n with rabbit anti-THSD7A (1:400, Atlas) in blocking buffer. Staining was visualized with the ZytochemPlus AP Polymer kit (Zytomed Systems) according to the manufacturer's instruction. Nuclei were counterstained with hemalaun and sections were mounted with gum Arabic (Sigma). Negative controls were performed omitting primary antibodies. Stainings were evaluated with an Axioskop using the Axiovision software (all Zeiss).

Primary antibodies used for histological analyses were: rabbit anti-THSD7A (Atlas, 1:400), goat-anti-THSD7A (Santa Cruz, 1:200), anti-PLA2R1 (Atlas, 1:8000), anti-IgG4 (Binding Site, 1:1000), guinea-pig anti-nephrin (Acris, 1:100), goat anti-collagen Type IV

(SouthernBiotech, 1:400), and sheep anti-fibronectin (DAKO, 1:500). All secondary antibodies were fluorochrome-conjugated affinity purified donkey antibodies (Jackson Immunoresearch, Dianova, Hamburg, Germany, 1:400).

Elution of immunoglobulins

The remaining frozen biopsy core from a subject (MN D) whose sera was reactive with the novel antigen was used for IgG elution as described in Beck et al, 2009. Eluted IgG was also available from two subjects with PLA2R1-associated MN and one subject with lupus-associated MN. Western blots were run with extracts from cells expressing recombinant PLA2R1 and THSD7A, and the region of the membrane containing both proteins was excised and blocked in milk. The eluted IgG was diluted 1:1 with blocking solution and incubated overnight with the blots. Eluted IgG was detected with sheep antibodies to IgG4, followed by peroxidase-conjugated anti-sheep antibodies, followed by chemiluminescent detection.

Assays to detect circulating soluble THSD7A and THSD7A-containing immune complexes

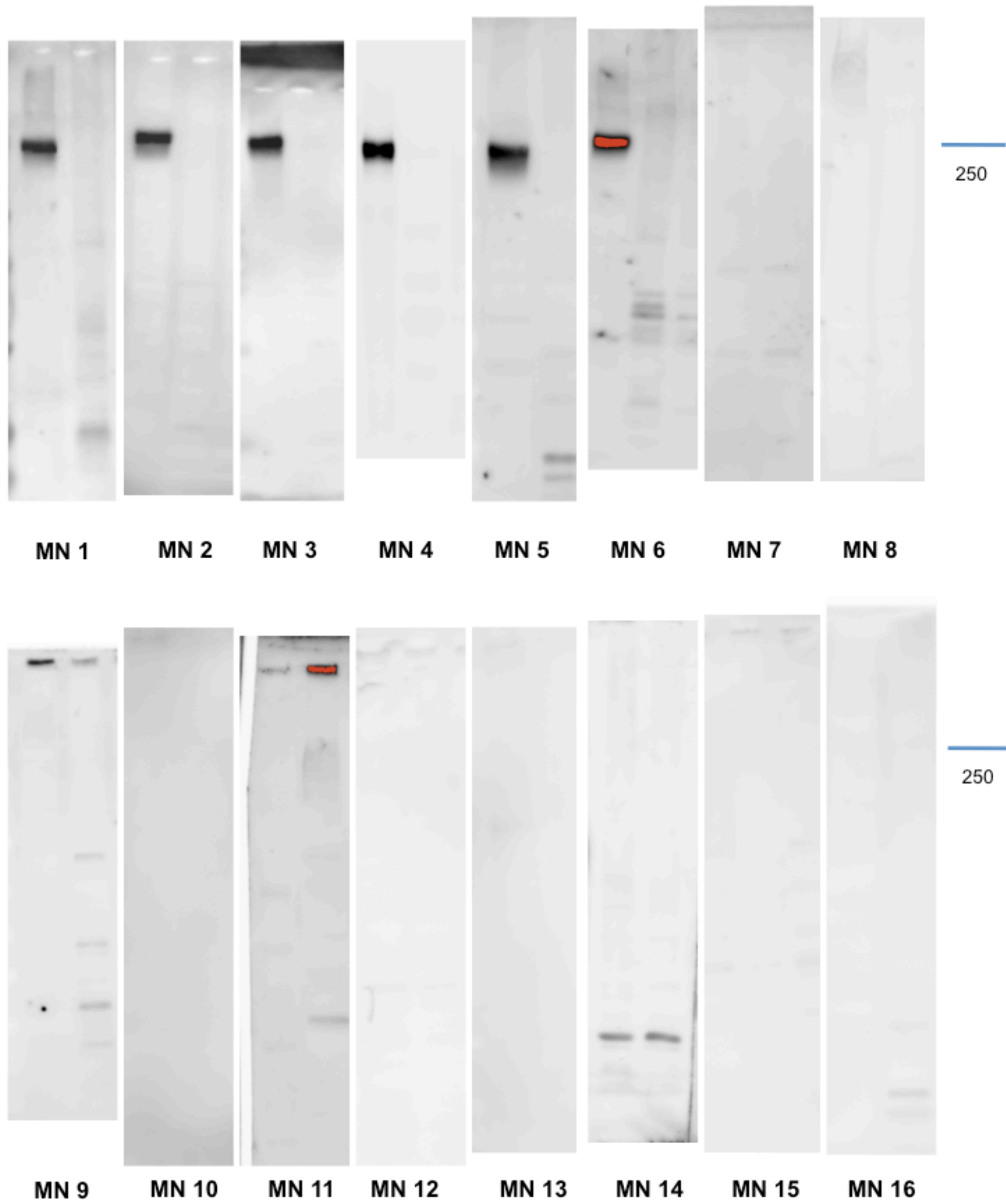
In order to detect a circulating soluble form of THSD7A as well as THSD7A-anti-THSD7A immune complexes, we performed two different assays. In the first one, 30 µL of anti-THSD7A positive, anti-PLA2R1 positive and healthy control sera were diluted 1:10 with 20 mM Tris pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$ and mixed with wheat germ agglutinin (WGA) beads. As a positive control to ensure that THSD7A could bind WGA beads in the presence of other serum glycoproteins and can further be detected by Western blot, we used a detergent-solubilized fraction of recombinant THSD7A protein obtained from transfected HEK293 cells. THSD7A was “spiked” in the different sera in different amounts (50, 5 and 0.5 µg of HEK293 membrane proteins) to determine the

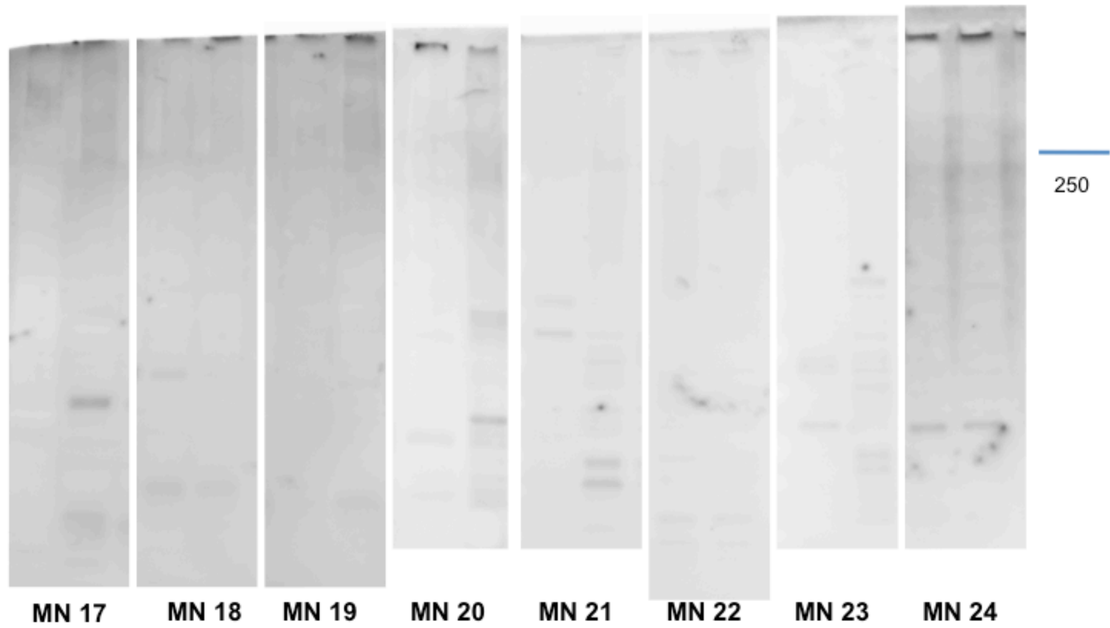
sensitivity of the assay. As an additional positive control, recombinant THSD7A was incubated with WGA beads alone. Samples were incubated overnight at 4 °C. WGA beads were then collected by centrifugation, vigorously washed three times with 400 µL of 20 mM Tris pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$, mixed with reducing Laemmli buffer, and boiled for 7 minutes. Samples were centrifuged and the supernatant was loaded on SDS-PAGE gels, electrophoresed, transferred to PVDF membranes, and detected with anti-THSD7A antibody (Atlas) using ultrahigh sensitivity ECL detection (Western Lightning Ultra, PerkinElmer, Waltham, USA).

In the second assay, we aimed to detect circulating immune complexes consisting of soluble THSD7A and IgG4 anti-THSD7A autoantibodies. Thirty µL of sera from patients with anti-THSD7A positive MN, anti-PLA2R1 positive MN, double negative MN, and from one healthy control patient were diluted 1:10 with 20 mM Tris pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$ and mixed with 20 µL of MACS IgG4 affinity matrix (CaptureSelect, Life Technologies, Leiden, Netherlands). As a positive control, sera were spiked with small amounts of solubilized recombinant THSD7A as described above. Samples were incubated overnight at 4°C. IgG4 beads were then collected by centrifugation, vigorously washed three times with 250 µL of 20 mM Tris pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$, mixed with reducing Laemmli buffer, and boiled for 7 minutes. Samples were centrifuged and the supernatant was loaded on SDS-PAGE gels, electrophoresed, transferred to PVDF membranes, and detected with anti-THSD7A antibody (Atlas) using ultrahigh sensitivity ECL detection (Western Lightning Ultra, PerkinElmer, Waltham, USA).

SUPPLEMENTARY FIGURES

Figure S1. Reactivity of iMN sera with human glomerular extracts and recombinant PLA2R1

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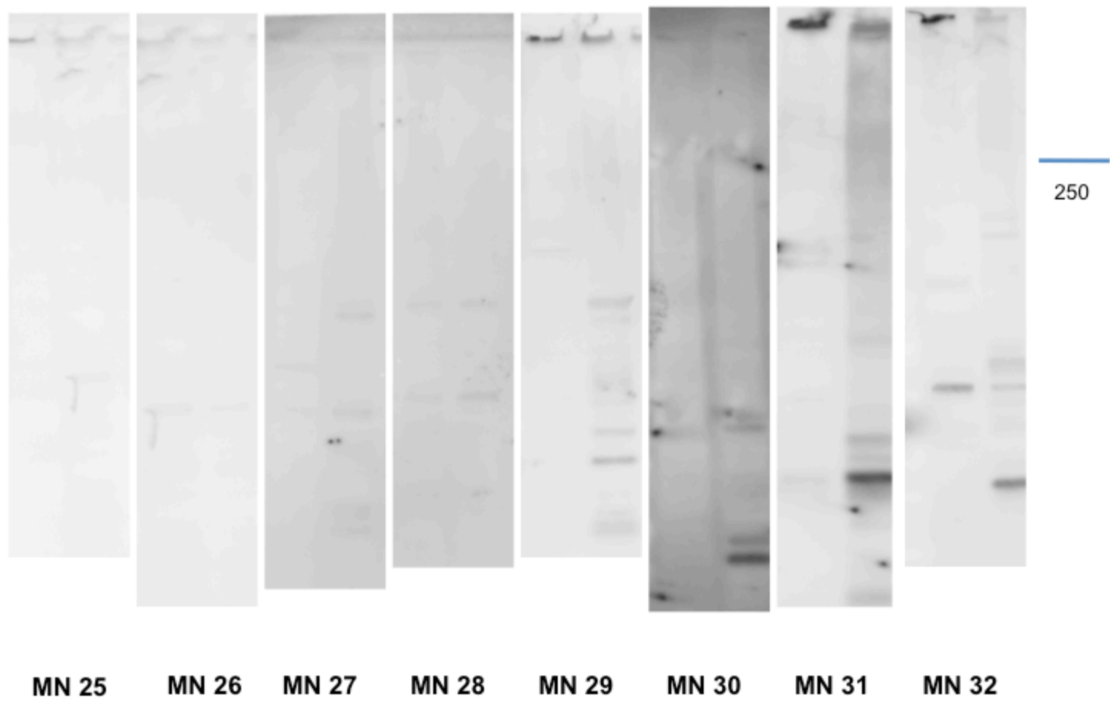
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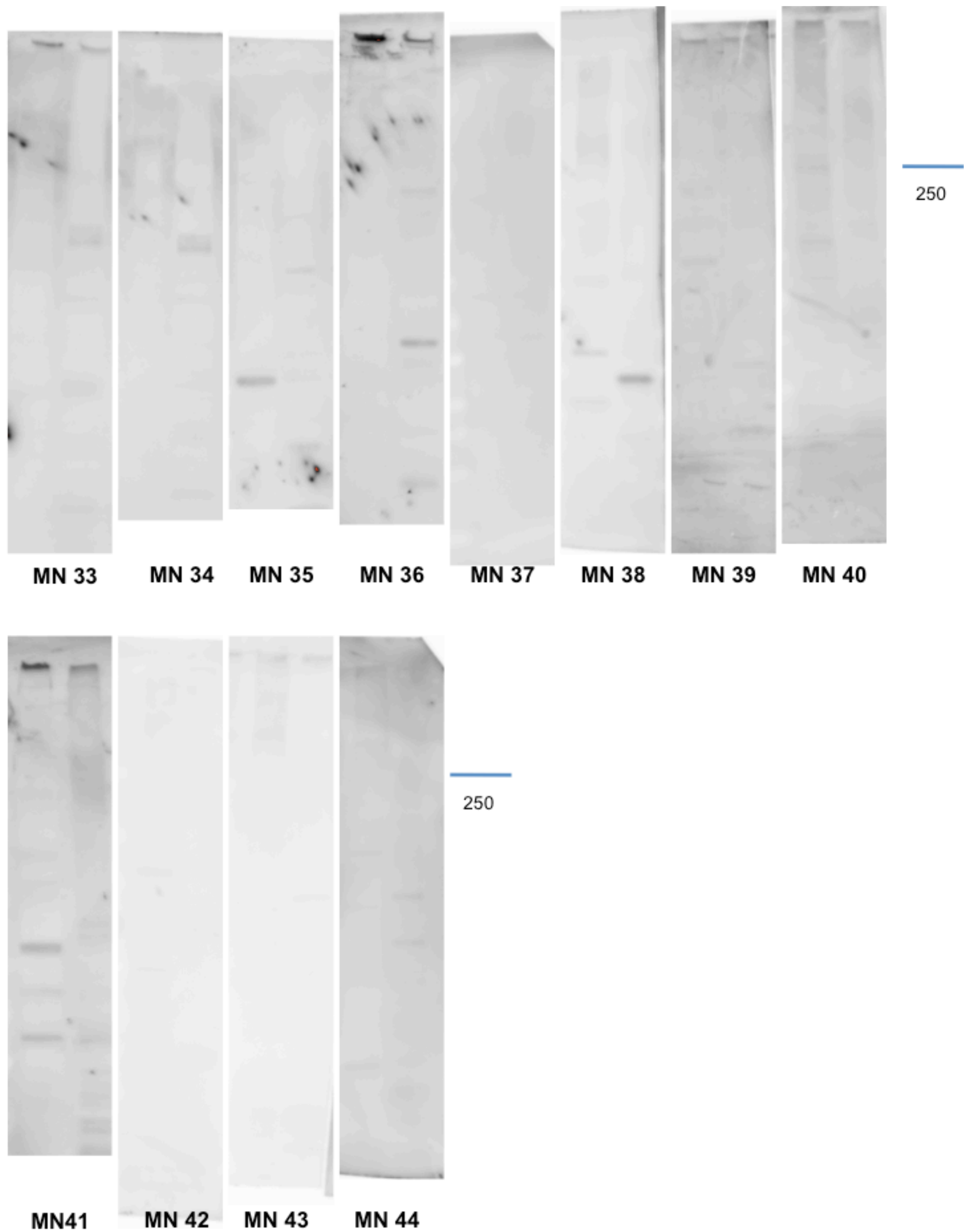
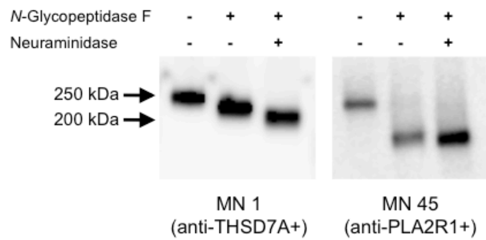


Figure S1. Original membrane strips of Western blot analyses of all sera from iMN patients negative for anti-PLA2R1 antibodies (MN 1 to MN 44) blotted with human glomerular extracts (left lane) and recombinant PLA2R1 (right lane). The first seven sera all recognized a protein of 250 kDa in size. The strips may differ in size due to slightly different migration times during gel electrophoresis and small differences in camera zoom. All strips are aligned at the 250 kDa molecular weight marker. This figure depicts that the sera did not show any reactivity with PLA2R1 and no other significant reactivity with proteins present in HGE other than THSD7A.

Figure S2. Biochemical characterization of the novel antigen

A

B



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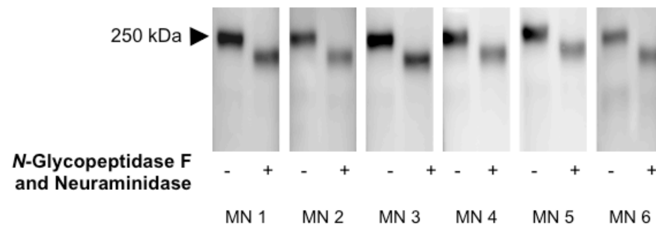

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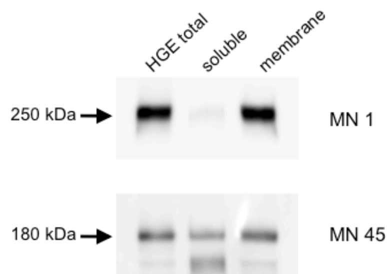
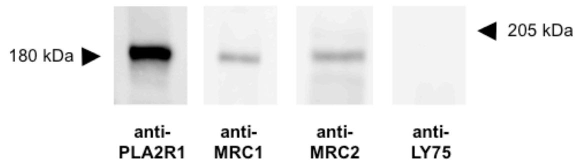


Figure S2. In order to differentiate the novel, still unknown antigen from PLA2R1, we ran the human glomerular extracts (HGE) on SDS-PAGE gels with low acrylamide percentage to fully separate the two proteins, then prepared Western blots and sequentially incubated the membranes with sera reactive against PLA2R1 or the novel

antigen. As expected, two distinct bands with a size difference of about 70 kDa were revealed (Panel A), suggesting the likely presence of either two different antigens or alternatively two isoforms of the same antigen with distinct epitopes and posttranslational modifications. To further characterize the reactive protein in comparison with PLA2R1, we enzymatically deglycosylated HGE. *N*-glycopeptidase F decreased the size of the putative novel antigen to approximatively 225 kDa, and the addition of neuraminidase, an enzyme that removes sialic acid, caused a further shift to 200 kDa (Panel B). On the other hand, PLA2R1 migrated to approximatively 145 kDa after the addition of *N*-glycopeptidase F, as described previously,[1] but no further shift was seen after addition of neuraminidase (Panel B), further supporting the hypothesis of two different antigens. All sera reacting with the fully glycosylated 250 kDa protein also recognized the deglycosylated forms at the same molecular mass, suggesting that all sera recognize the same protein (Panel C). Moreover, both the novel antigen and PLA2R1 are present in the membrane fraction of HGE, but only PLA2R1 is present in the soluble fraction (Panel D).

Figure S3. Paralogs of PLA2R1

A



B

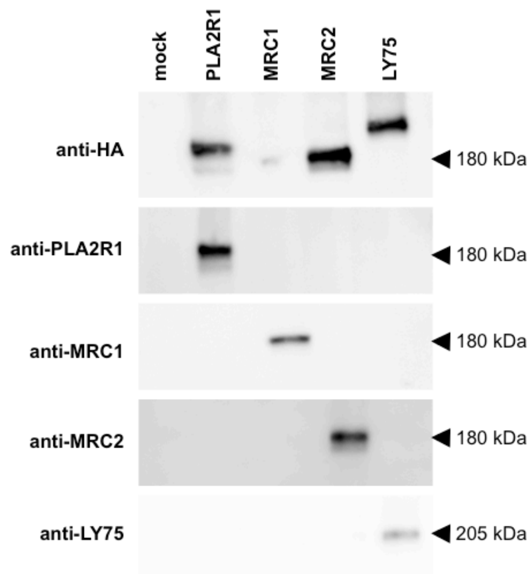


Figure S3. In a first approach towards the identification of new antigens in MN, we initially screened the sera on the paralogs of PLA2R1, which are all members of the mannose receptor family. Apart from PLA2R1, this comprises the macrophage mannose receptor (MRC1), the endocytic receptor 180 (MRC2) and the dendritic cell receptor 205 (LY75).[6] The four transmembrane proteins share a similar overall structure with an N-terminal cysteine-rich domain, a fibronectin type II domain and eight to ten C-type lectin-like domains.[6] We hypothesized that these paralogs might have similar immunogenicity to PLA2R1 or might even share common epitopes. There is also evidence from the literature that these proteins are expressed in kidney glomeruli.[6,7] Indeed, we found expression of MRC1 and MRC2, but no or very weak expression of LY75, in normal human glomeruli using commercially available antibodies (Panel A). The members of the mannose receptor family were recombinantly expressed: HEK293 cells were transiently transfected with full-length, HA-tagged cDNA of PLA2R1, MRC1, MRC2, and LY75. Expression was validated with an anti-HA antibody as well as monospecific antibodies (Panel B).

Figure S4. Reactivity of MN sera with paralogs of PLA2R1

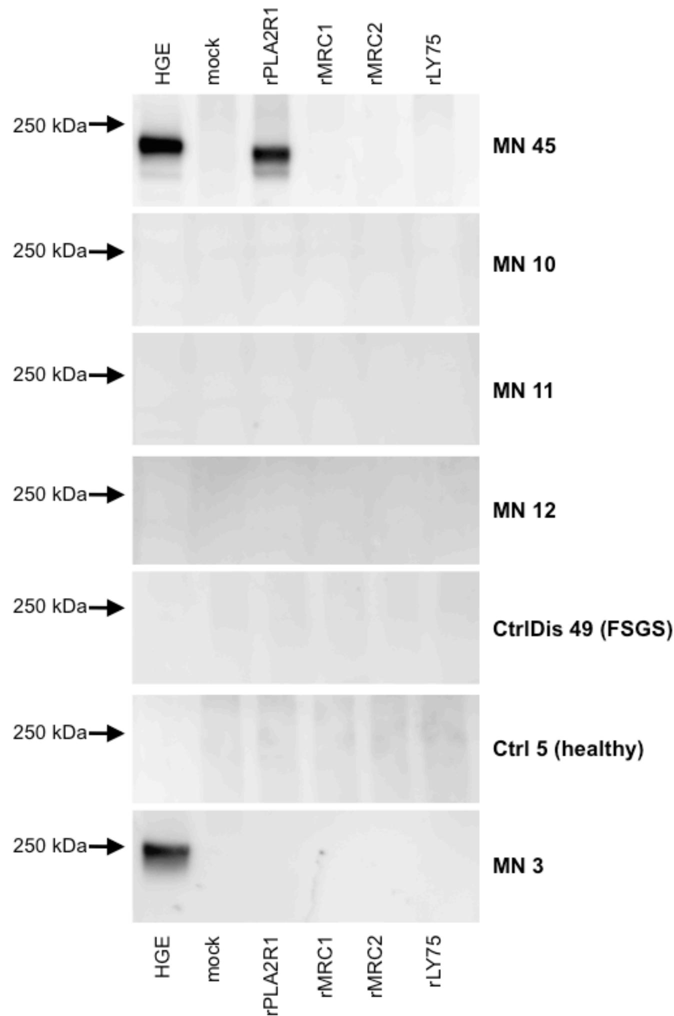


Figure S4. Results of Western blotting of human glomerular extracts (HGE) as well as members of the mannose receptor family (phospholipase A2 receptor, PLA2R1; macrophage mannose receptor, MRC1; endocytic receptor 180, MRC2; dendritic cell receptor 205, LY75) with sera from patients with MN and controls. This figure shows representative images of different patients: MN 45 is a typical anti-PLA2R1 positive patient whose serum recognizes both PLA2R1 present in HGE and recombinant PLA2R1 (top). All sera from patients with MN (represented by MN 10 to MN 12), all patients with other kidney disease (represented by CtrlDis 49) or healthy controls (represented by Ctrl 5) did not show any reactivity with the paralog proteins. However, seven patients reacted with a 250 kDa protein present in HGE, but not with any of the paralogs (represented by MN 3).

Figure S5. Glycosylation of THSD7A

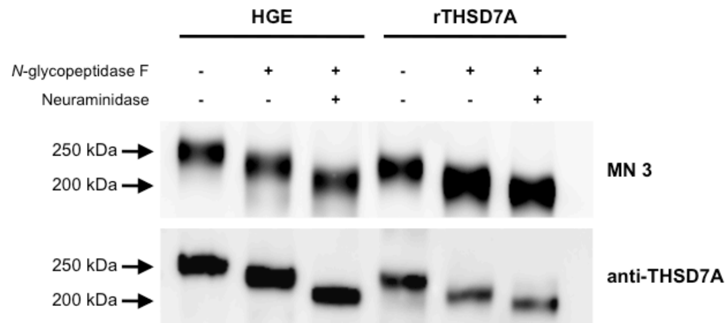


Figure S5. Western blotting of HGE and recombinant THSD7A before and after deglycosylation. Blots were incubated with serum from a patient positive for anti-THSD7A antibodies (MN 3) as well as a polyclonal rabbit-antibody against THSD7A. Native THSD7A and recombinant THSD7A expressed in HEK293 cells show the same pattern of glycosylation when probed with the patient serum and the monospecific antibody, suggesting that the protein recognized within HGE is indeed THSD7A.

Figure S6. Expression of THSD7A, collagen type IV and nephrin

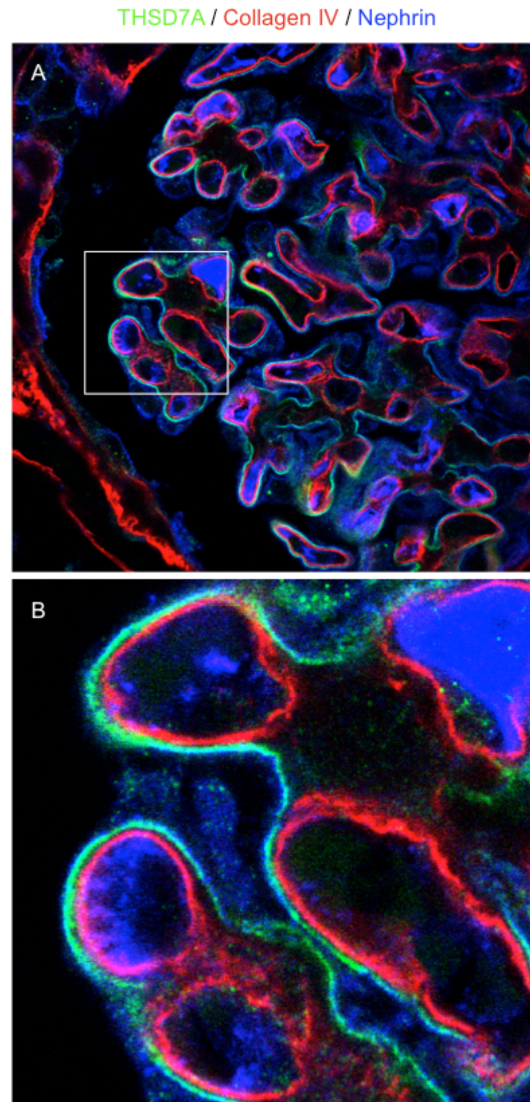


Figure S6. Histological analyses of healthy kidney areas from a patient who underwent nephrectomy due to a renal tumor. THSD7A (green) strongly co-localized with nephrin (blue), a transmembrane protein expressed in the region of the intercellular slit diaphragm of podocyte foot processes. On the other hand, collagen type IV (red) as a marker of the glomerular basement membrane is clearly located towards the inner aspect of the renal filtration barrier. This histological analysis suggests that THSD7A is expressed in podocyte foot processes in normal human kidneys. The blue staining that is present intraluminally (seen in Panel B) is likely artefactual due to ‘pooling’ of the antibody during the staining process.

Figure S7. Expression of THSD7A and fibronectin

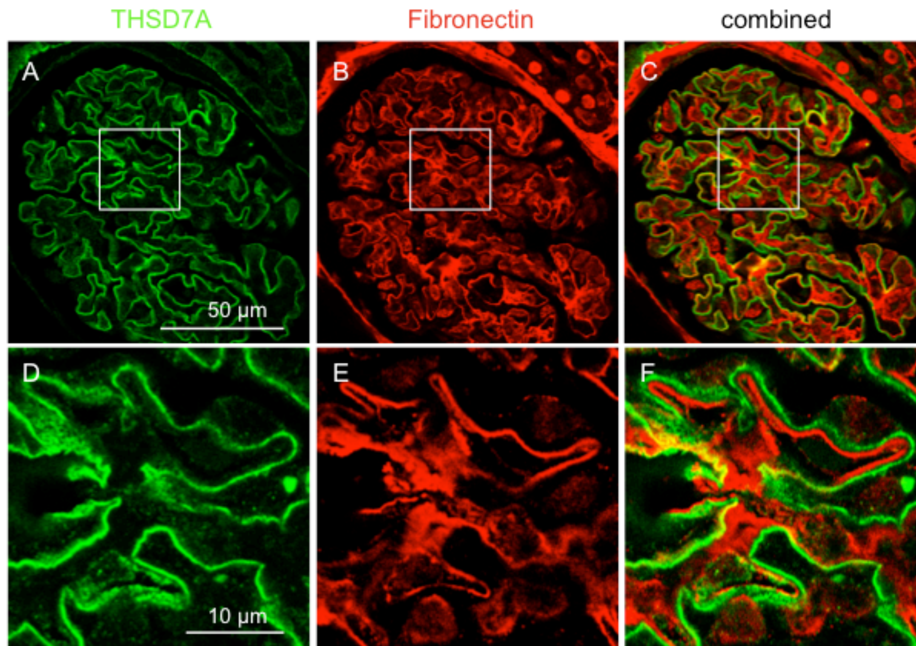


Figure S7. Staining for THSD7A (green) and fibronectin (red) as a marker of the glomerular basement membrane and mesangium in the healthy area of the kidney of a patient who underwent nephrectomy due to a renal tumor. There is no co-localization of THSD7A and fibronectin. THSD7A is located abluminally of fibronectin, suggesting that it is expressed on podocyte foot processes rather than in the glomerular basement membrane.

Figure S8. Expression of THSD7A and CD34

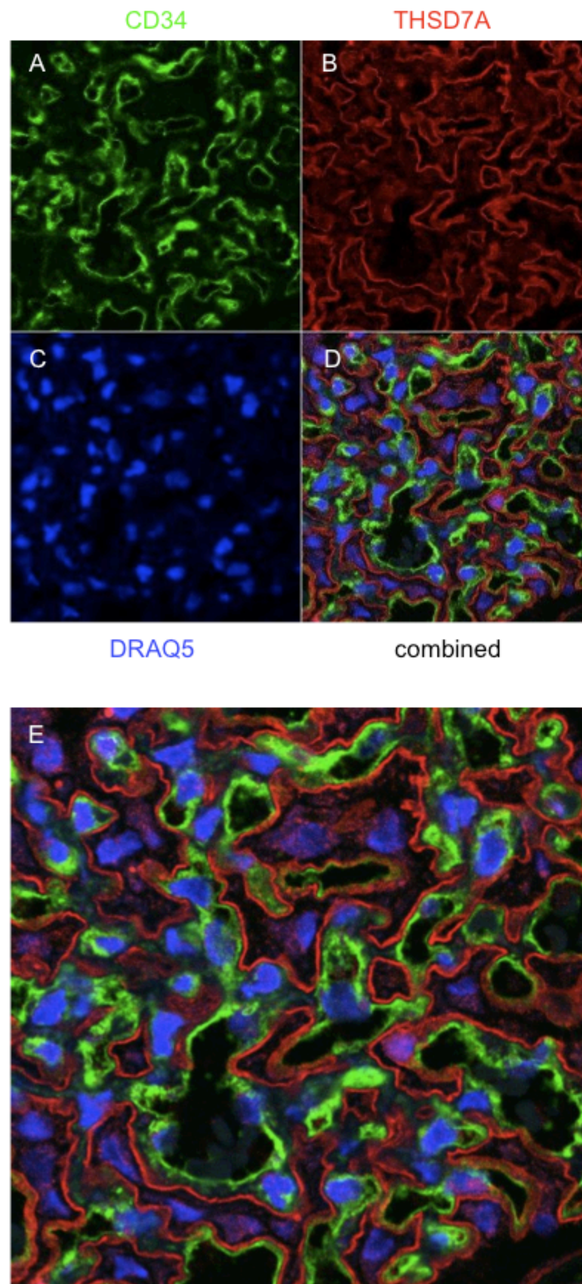


Figure S8. Staining for THSD7A (red) and CD34 as a marker of endothelial cells in the healthy area of the kidney of a patient who underwent nephrectomy due to a renal tumor. There is no co-localization of THSD7A and CD34, suggesting that THSD7A is not expressed in glomerular endothelial cells. An unstained area can be detected between CD34 and THSD7A, supposedly representing the glomerular basement membrane. Panel E is an enlargement of panel D.

Figure S9. Co-localization of IgG4 and THSD7A

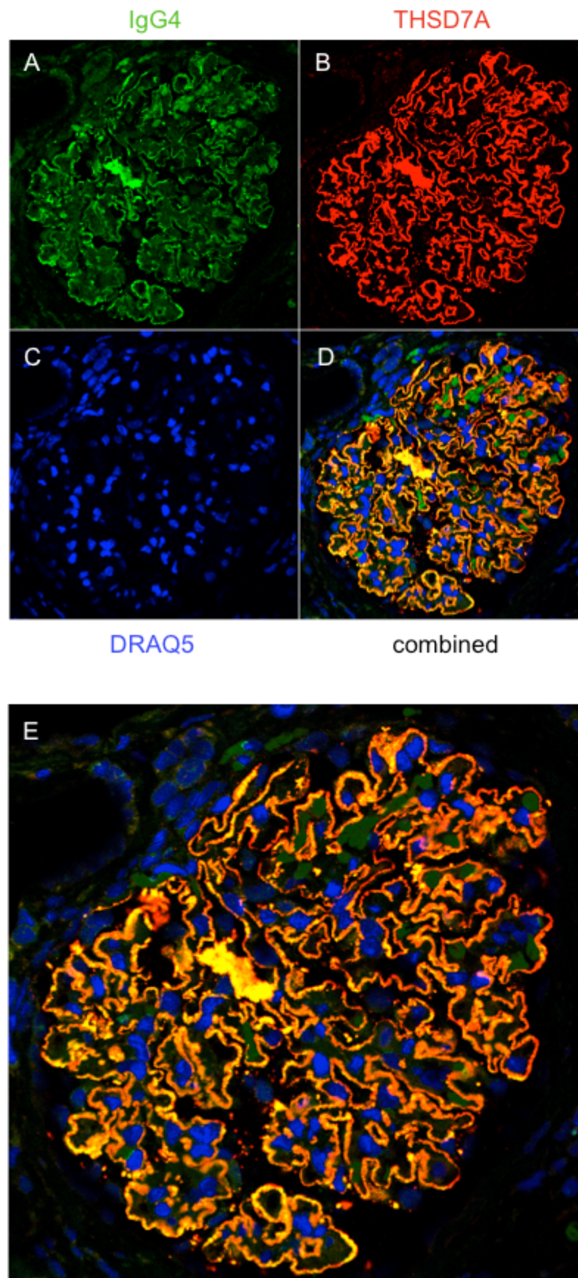


Figure S9. Confocal microscopy analysis of the biopsy of a patient with membranous nephropathy (MN) and serum autoantibodies against THSD7A. IgG4 (Panel A, green) and THSD7A (Panel B, red) are both present in this biopsy specimen. Panel C shows staining for DRAQ5 (blue), a marker of cell nuclei. Panel D shows co-localization of IgG4 and THSD7A in the peripheral capillary wall and the glomerular basement membrane. Panel E is an enlargement of Panel D.

Figure S10. Activation of complement in a anti-THSD7A positive iMN patient

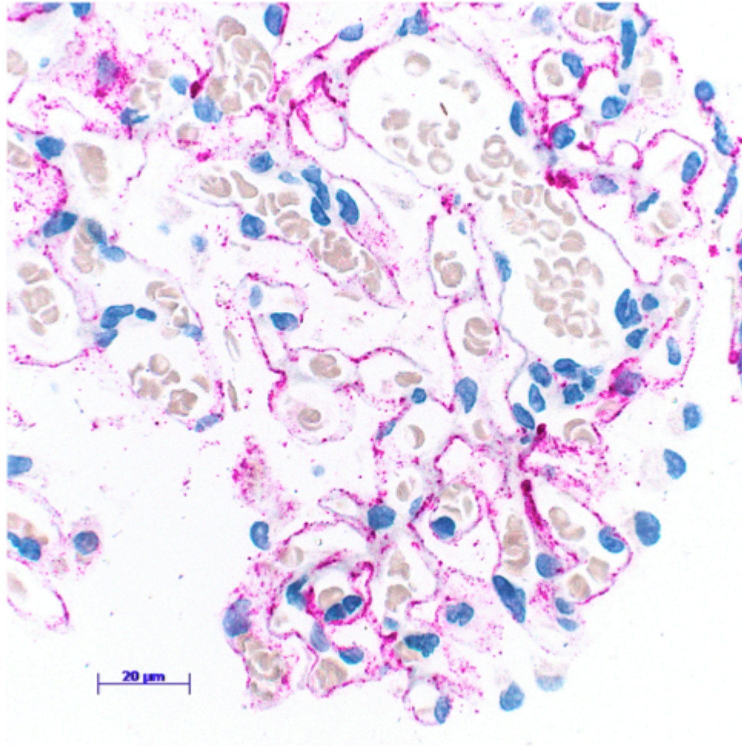


Figure S10. Immunohistochemical staining for C5b-9, the terminal complement complex, in a patient with anti-THSD7A positive MN. This image shows granular deposition of C5b-9, representing local activation of complement, as it can be found in patients with anti-PLA2R1 positive MN (data not shown).

Figure S11. Assays to detect soluble THSD7A and immune complexes

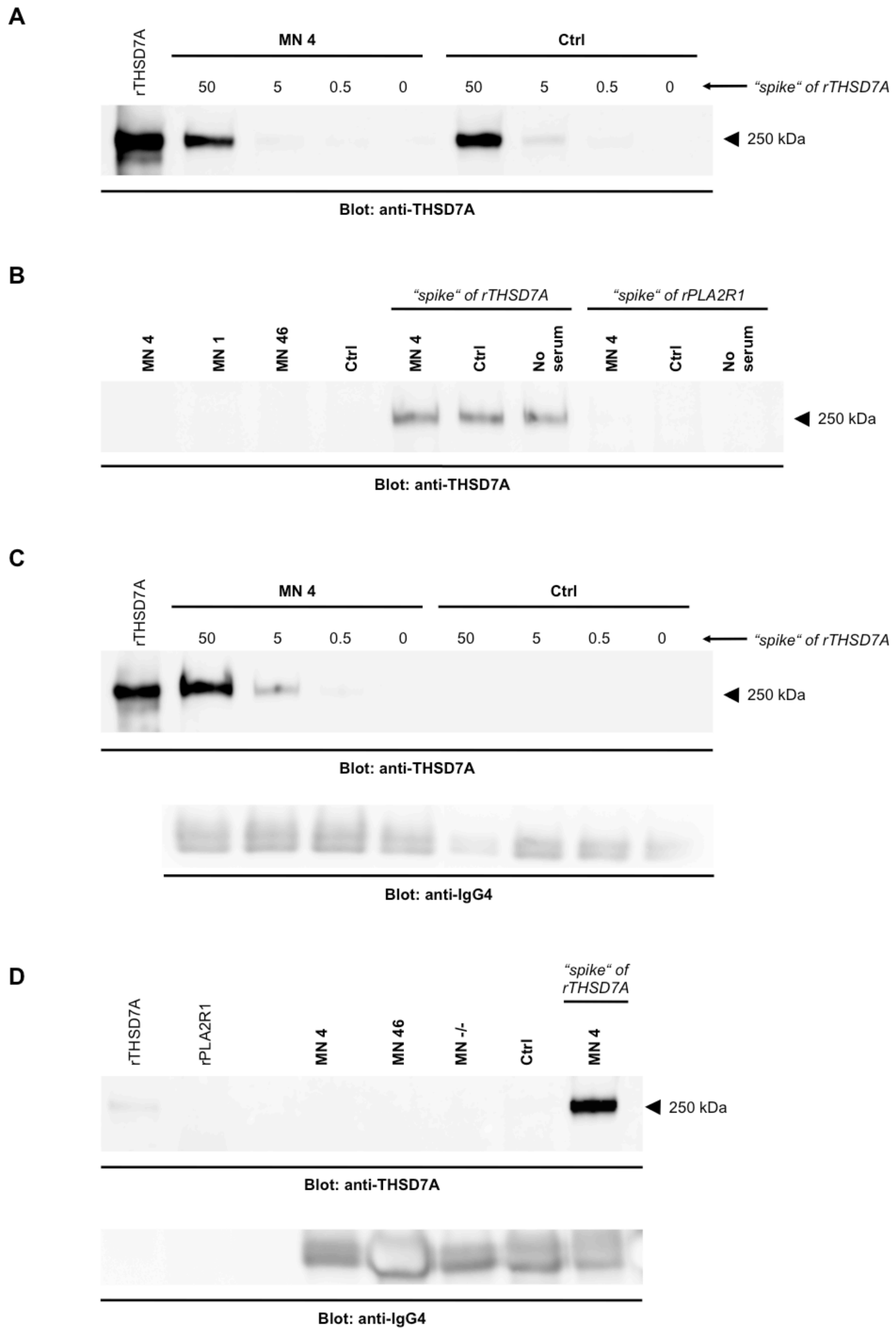


Figure S11. Even though we could show that the identified autoantigen is present in non-diseased kidneys on glomerular podocytes, there was still the possibility that circulating immune complexes reached the kidney, passed the glomerular filtration barrier, and deposited in the typical subepithelial location. To exclude this possibility, we performed two assays to address the possible presence of circulating soluble THSD7A and/or circulating THSD7A-anti-THSD7A immune complexes in various human sera.
In the first experiment, we took advantage of the *N*-glycosylation of THSD7A to purify it along other serum glycoproteins on wheat germ agglutinin (WGA) beads and then try to detect THSD7A by Western blot. We initially tested 30 µL of each one serum highly positive for anti-THSD7A and one healthy control serum. In order to demonstrate the sensitivity of our assay, we spiked the two sera with different amounts of solubilized recombinant THSD7A protein obtained from transfected HEK293 cells. Panel A shows the results of Western blotting of the purified glycoproteins with a monospecific anti-THSD7A antibody. The numbering above lanes indicates the total protein amount of HEK293 THSD7A detergent-solublized membrane extract used for each assay (50, 5 and 0.5 µg of total protein/assay). No band was seen in the condition without spiked THSD7A while we could detect the recombinant THSD7A protein when spiking 5 µg of total protein. Panel B shows more examples of Western blotting of purified glycoproteins from different sera after WGA purification. No soluble THSD7A could be detected in one more patient with anti-THSD7A positive MN (MN 1), one patient with anti-PLA2R1 positive MN (MN 46) and one more healthy control subject. As an additional control, recombinant THSD7A protein was purified on WGA beads without adding sera. These results suggest either that no soluble THSD7A is present in the above sera or that the sensitivity of the WGA assay is insufficient to detect low quantities of soluble THSD7A present in human sera.
In the second assay, we aimed to precipitate circulating THSD7A-anti-THSD7A immune complexes using IgG4-specific beads. Similar to the above experiment, we also “spiked” the sera from one patient with THSD7A-associated iMN and one healthy control with different amounts of HEK293 recombinant THSD7A. Panel C shows the results of Western blotting of the immunoprecipitates with a monospecific anti-THSD7A antibody. A faint band could be seen even when only 0.5 µg of total protein from THSD7A transfected cells was added to the iMN serum. However, no band is seen in serum without spiking, suggesting either that no circulating immune complexes are present or that the sensitivity of this assay is insufficient to detect these latters. No band is also seen when the iMN serum is replaced by a healthy donor serum. Panel D shows that no IgG4-bound THSD7A could be detected after pull-down of IgG4 from several other patients, including one iMN patient with antibodies against PLA2R1 (MN 46), one patient with double negative iMN, and one healthy control.

Figure S12

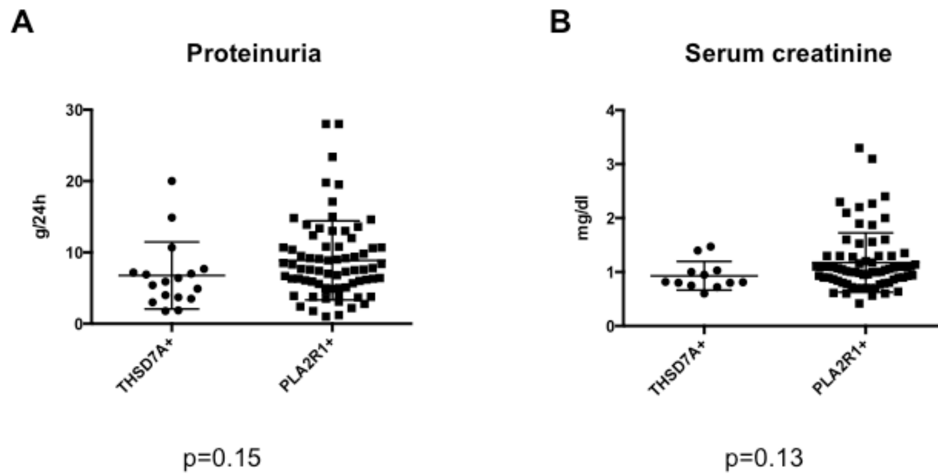


Figure S12. In order to identify potential differences in disease severity between iMN patients positive for anti-THSD7A and positive for anti-PLA2R1, we assessed several clinical parameters in these patients (also see Tabel S5 below). No significant difference could be found in regard to proteinuria (Panel A) and serum creatinine (Panel B) at the time of diagnosis.

SUPPLEMENTARY TABLES

Table S1. European Cohort (Hamburg/Nice)

Idiopathic MN, anti-PLA2R1 negative

Patient	Age	Sex	Proteinuria (g/24 h)	Months from biopsy to serum	Reactivity with HGE / rTHSD7A
MN 1	68	F	6.4	5	yes
MN 2	56	M	1.8	0.75	yes
MN 3	43	M	10.7	0.5	yes
MN 4	28	F	5.4	0.75	yes
MN 5	47	F	6.9	5	yes
MN 6	51	M	4	4.9	yes
MN 7	64	M	11.1	0.5	no
MN 8	80	F	5.5	3	no
MN 9	69	F	6.1	0.25	no
MN 10	22	F	6.3	2	no
MN 11	33	M	3.8	9	no
MN 12	75	F	7	0.5	no
MN 13	58	W	2,3	24	no
MN 14	33	M	1.7	0.25	no
MN 15	43	M	4.5	3	no
MN 16	63	M	2.2	0.25	no
MN 17	64	M	6.1	0.5	no
MN 18	45	F	7	0.25	no
MN 19	48	M	7.7	3	no
MN 20	63	M	7.6	0.75	no
MN 21	51	M	27.8	0	no
MN 22	60	M	3.7	49	no
MN 23	69	M	3.1	0.25	no
MN 24	80	M	2	4	no
MN 25	43	M	4.3	0.25	no
MN 26	58	M	6.9	0	no
MN 27	18	M	12.6	1	no
MN 28	75	F	6.3	0	no
MN 29	58	M	3.4	0	no
MN 30	80	F	13	12	no
MN 31	65	W	2.1	0	No
MN 32	43	M	4	3	no
MN 33	48	F	12.5	0	no
MN 34	45	M	11	0.5	no
MN 35	35	M	30.2	0	no

MN 36	28	M	2.8	0	no
MN 37	73	M	11	3	no
MN 38	71	M	3.6	0.5	no
MN 39	22	M	4.1	1	no
MN 40	51	M	4.7	0.5	no
MN 41	76	M	4.5	0.75	no
MN 42	52	F	11.9	0	no
MN 43	28	M	3	0.25	no
MN 44	35	M	4.8	1	no

Idiopathic MN, anti-PLA2R1 positive

Patient	**Age**	**Sex**	**Proteinuria (g/24 h)**	**Months from biopsy to serum**	**Reactivity with HGE / rTHSD7A**
MN 45	64	M	3.9	11	no
MN 46	86	F	6.3	4	no
MN 47	46	M	5.3	0.5	no
MN 48	57	F	3.4	1	no
MN 49	72	M	7.4	0.25	no
MN 50	43	M	9,8	5	no
MN 51	62	M	6,2	3	no
MN 52	67	M	2,2	2	no
MN 53	76	F	9	1	no
MN 54	55	M	10,6	0,5	no
MN 55	21	F	3.8	4	no
MN 56	44	F	6.1	0.25	no
MN 57	53	F	4.6	1	no
MN 58	37	M	7	0.25	no
MN 59	45	M	13	1	no
MN 60	34	M	3.5	1.5	no
MN 61	59	M	6.7	0.5	no
MN 62	38	M	7.6	0.5	no
MN 63	71	M	6.4	0.5	no
MN 64	54	M	12.4	1	no
MN 65	68	F	28	0	no
MN 66	78	M	7.7	0	no
MN 67	64	M	8.5	3	no
MN 68	74	M	13.6	0	no
MN 69	39	M	14.6	4	no
MN 70	57	M	6.3	0.5	no
MN 71	58	M	3.8	2	no
MN 72	59	M	3.7	3	no
MN 73	64	F	7.8	0	no
MN 74	61	F	9.5	2	no
MN 75	60	M	28	0.5	no

MN 76	38	M	17.1	2	no
MN 77	42	M	9.1	1	no
MN 78	76	F	7.5	1.5	no
MN 79	55	M	19.8	3	no
MN 80	81	F	8.4	0.5	no
MN 81	22	M	9.2	0	no
MN 82	47	F	6.8	0.5	no
MN 83	27	M	23.4	0	no
MN 84	74	M	13	0.5	no
MN 85	73	M	14.8	0.5	no
MN 86	60	F	19.5	0.5	no
MN 87	63	F	9.2	0.25	no
MN 88	45	M	15	0.5	no
MN 89	35	M	10.8	0.5	no
MN 90	41	M	10.4	1	no
MN 91	44	M	5	1	no
MN 92	47	M	7.6	0.5	no
MN 93	83	M	12	1.5	no
MN 94	44	M	10,7	1	no
MN 95	60	M	5.1	0.75	no
MN 96	64	M	3.8	5	no
MN 97	70	M	5.7	3	no
MN 98	46	M	9.4	0	no
MN 99	65	F	5.9	0	no
MN 100	57	M	6	0.5	no
MN 101	62	M	8.3	1	no
MN 102	67	F	4	0.5	no
MN 103	47	F	3.1	3	No
MN 104	84	M	10.7	0.25	no
MN 105	53	M	5.6	1	no
MN 106	42	M	1.2	0	no
MN 107	77	W	5	0.5	no
MN 108	43	M	13.4	0	no
MN 109	49	M	8.9	0	no
MN 110	58	M	2.4	0.5	no
MN 111	73	M	10.8	0	no
MN 112	32	M	2.8	0	no
MN 113	48	F	7.4	0.5	no
MN 114	24	M	4.9	3	no
MN 115	75	F	13.9	2	no
MN 116	47	M	2	1	no
MN 117	18	M	6.3	0	no
MN 118	52	M	7.3	0.5	no

Secondary MN, all anti-PLA2R1 negative

Patient	Age	Sex	Secondary cause	Proteinuria (g/24 h)	Months from biopsy to serum	Reactivity with HGE / rTHSD7A
MN 119	29	F	SLE	7	4	yes
MN 120	63	W	SLE	5.2	0.25	no
MN 121	56	M	Malignancy (bone)	4.7	0	no
MN 122	27	F	SLE	2	24	no
MN 123	54	M	Hepatitis C	4	48	no
MN 124	55	M	Hepatitis B	1.7	0	no
MN 125	42	F	SLE	3.9	0.5	no
MN 126	56	M	Malignancy (melanoma)	19	0	no
MN 127	65	M	Malignancy (prostate)	7	4	no
MN 128	37	M	Malignancy (thymoma)	13.5	0.25	no
MN 129	69	M	SLE	11.8	0	no
MN 130	38	M	SLE	7.6	0.5	no
MN 131	47	F	SLE	5.4	0	no
MN 132	58	M	SLE	8.2	4	no
MN 133	57	F	Malignancy (lungs)	1.4	2	no
MN 134	75	M	SLE	3.7	0.5	no
MN 135	67	M	SLE	4.1	1	no
MN 136	73	F	Malignancy (lungs)	3.6	5	no
MN 137	67	M	SLE	15.6	0.25	no
MN 138	76	M	SLE	7.1	1	no
MN 139	70	M	Malignancy (prostate)	7.5	0.5	no
MN 140	73	F	Malignancy (lungs)	5	0	no
MN 141	71	F	Hepatitis B	3.1	1	no
MN 142	46	M	Hepatitis B	5.4	0	no
MN 143	55	M	Hepatitis C	3.7	0	no
MN 144	50	F	SLE	22.7	0.25	no
MN 145	66	M	Multiple myeloma	6.2	0.5	no
MN 146	79	M	Non-Hodgkin lymphoma	10	0	no
MN 147	69	M	Malignancy (melanoma)	6.2	0.5	no
MN 148	67	F	Malignancy (lungs)	4.8	0.25	no
MN 149	77	F	Malignancy (breast)	3.8	0.75	no
MN 150	53	M	Malignancy (lungs)	7	0	no
MN 151	76	M	Hepatitis C	4.3	3	no
MN 152	57	F	SLE	9	0.5	no
MN 153	20	F	SLE	6.1	0.25	no

Other glomerular diseases

Patient	Age	Sex	Diagnosis	Proteinuria	Reactivity with HGE / rTHSD7A
CtrlDis 1	51	M	MCD	4	no
CtrlDis 2	20	M	MCD	0.3	no
CtrlDis 3	21	M	MCD	3.6	no
CtrlDis 4	21	F	MCD	1.5	no
CtrlDis 5	34	M	MCD	1.5	no
CtrlDis 6	26	M	MCD	4.2	no
CtrlDis 7	56	M	GPA	n/a	no
CtrlDis 8	65	M	GPA	2.2 g/g creatinine	no
CtrlDis 9	57	M	GPA	1.2 g/g creatinine	no
CtrlDis 10	62	F	GPA	1.0 g/g creatinine	no
CtrlDis 11	70	M	GPA	0.2 g/g creatinine	no
CtrlDis 12	41	F	GPA	unknown	no
CtrlDis 13	37	F	GPA	unknown	no
CtrlDis 14	71	M	GPA	anuric	no
CtrlDis 15	63	F	GPA	0.03 g/g creatinine	no
CtrlDis 16	71	F	GPA	0.07 g/g creatinine	no
CtrlDis 17	60	M	MPA	unknown	no
CtrlDis 18	76	M	MPA	0.8 g/g creatinine	no
CtrlDis 19	74	F	MPA	0.8 g/g creatinine	no
CtrlDis 20	69	M	MPA	3.3 g/g creatinine	no
CtrlDis 21	73	M	MPA	0.3 g/g creatinine	no
CtrlDis 22	69	M	MPA	0.1 g/g creatinine	no
CtrlDis 23	72	M	MPA	0.05 g/g creatinine	no
CtrlDis 24	76	M	MPA	0.3 g/g creatinine	no
CtrlDis 25	n/a	n/a	ANCA	3.7	no
CtrlDis 26	70	M	ANCA	1.1	no
CtrlDis 27	62	F	ANCA	1.25	no
CtrlDis 28	88	F	ANCA	1.3	no
CtrlDis 29	81	M	ANCA	2.3	no
CtrlDis 30	47	M	Anti GBM	4.7 g/g creatinine	no
CtrlDis 31	58	F	Anti GBM	5.1 g/g creatinine	no
CtrlDis 32	23	F	Anti GBM	1.1 g/g creatinine	no
CtrlDis 33	18	M	Anti GBM	1.8 g/g creatinine	no
CtrlDis 34	60	M	Anti GBM	5.5 g/g creatinine	no
CtrlDis 35	33	M	IgA Nephropathy	0.5	no
CtrlDis 36	43	M	IgA Nephropathy	0.4	no
CtrlDis 37	58	M	IgA Nephropathy	6.5	no
CtrlDis 38	51	M	IgA Nephropathy	2.2	no
CtrlDis 39	32	M	IgA Nephropathy	2.1	no
CtrlDis 40	n/a	n/a	IgA Nephropathy	0.4	no
CtrlDis 41	73	M	IgA Nephropathy	5.7	no
CtrlDis 42	75	F	IgA Nephropathy	1.1	no
CtrlDis 43	78	F	IgA Nephropathy	0.2	no
CtrlDis 44	27	M	IgA Nephropathy	5.7	no
CtrlDis 45	n/a	M	IgA Nephropathy	3.5	no
CtrlDis 46	35	M	FSGS	n/a	no
CtrlDis 47	41	M	FSGS	n/a	no
CtrlDis 48	46	F	FSGS	2.4	no

CtrlDis 49	28	F	FSGS	6.8	no
CtrlDis 50	51	M	FSGS	n/a	no
CtrlDis 51	n/a	n/a	FSGS	4.8	no
CtrlDis 52	31	M	FSGS	8.5	no
CtrlDis 53	n/a	n/a	FSGS	6.8	no
CtrlDis 54	49	M	ATN	3.6 g/g creatinine	no
CtrlDis 55	67	M	ATN	anuric	no
CtrlDis 56	74	M	ATN	n/a	no
CtrlDis 57	76	M	ATN	n/a	no
CtrlDis 58	93	F	ATN	n/a	no
CtrlDis 59	28	F	Lupus nephritis	2.4 g/g creatinine	no
CtrlDis 60	69	F	Lupus nephritis	anuric	no
CtrlDis 61	37	F	Lupus nephritis	1.5	no
CtrlDis 62	27	F	Lupus nephritis	unknown	no
CtrlDis 63	30	F	Lupus nephritis	0.9	no
CtrlDis 64	35	W	Lupus nephritis	3	no
CtrlDis 65	30	W	Lupus nephritis	3.8	no
CtrlDis 66	21	F	Lupus nephritis	3.1	no
CtrlDis 67	34	F	Lupus nephritis	6	no
CtrlDis 68	80	M	Henoch Schoenlein purpura	1.4	no
CtrlDis 69	81	M	OD Randall	3.3	no
CtrlDis 70	46	F	Henoch Schoenlein purpura	3.8	no
CtrlDis 71	65	F	MPGN	1.2	no
CtrlDis 72	65	F	C3 glomerulonephritis	0.5	no
CtrlDis 73	48	F	Diabetic nephropathy	3.9	no
CtrlDis 74	67	M	OD SN avec nodules pulm	2.4	no
CtrlDis 75	30	M	OD LGM	4.6	no
CtrlDis 76	89	M	Nephrosclerosis	3.9	no

Healthy controls

Patient	**Age**	**Sex**	**Reactivity with HE/rTHSD7A**
Ctrl 1	31	F	no
Ctrl 2	34	F	no
Ctrl 3	31	F	no
Ctrl 4	40	F	no
Ctrl 5	37	M	no
Ctrl 6	35	M	no
Ctrl 7	27	M	no
Ctrl 8	34	M	no
Ctrl 9	25	M	no
Ctrl 10	23	M	no
Ctrl 11	41	M	no
Ctrl 12	48	M	no
Ctrl 13	49	F	no
Ctrl 14	40	F	no
Ctrl 15	31	M	no
Ctrl 16	44	M	no
Ctrl 17	52	M	no
Ctrl 18	20	M	no

Ctrl 19	42	M	no
Ctrl 20	49	M	no
Ctrl 21	57	F	no
Ctrl 22	28	M	no
Ctrl 23	54	F	no
Ctrl 24	18	F	no
Ctrl 25	53	F	no
Ctrl 26	46	M	no
Ctrl 27	52	M	no
Ctrl 28	51	M	no
Ctrl 29	23	M	no
Ctrl 30	56	M	no
Ctrl 31	27	F	no
Ctrl 32	30	M	no
Ctrl 33	30	M	no
Ctrl 34	38	M	no
Ctrl 35	33	F	no
Ctrl 36	36	M	no
Ctrl 37	33	M	no
Ctrl 38	50	M	no
Ctrl 39	42	F	no
Ctrl 40	42	F	no
Ctrl 41	49	F	no
Ctrl 42	42	M	no
Ctrl 43	38	M	no
Ctrl 44	36	M	no

Table S1. List of patients that were screened for antibodies against proteins present in human glomerular extracts (HGE) and later against recombinant THSD7A. Patients were classified according to the criteria presented in the first paragraph of the Methods section of the Supplementary Appendix.
Abbreviations: MN, membranous nephropathy; F, female; M, male; HGE, human glomerular extract; CtrlDis, disease control; Ctrl, healthy control

Table S2. Boston Cohort

Cohort	Number	(+) Anti-PLA2R1	(-) Anti-PLA2R1	(+) Anti-THSD7A	% of total	% of anti-PLA2R(-)
BUMC IMN	204	150 (74%)	54 (24%)	6	2.9%	11.1%
Dutch IMN	117	77 (66%)	40 (34%)	2	1.7%	5.0%
Sweden IMN	44	28 (64%)	16 (36%)	1	2.3%	6.3%
Total iMN	**365**	**255 (70%)**	**110 (30%)**	**9**	**2.5%**	**8.2%**
BUMC sMN	33	3 (9%)	30 (91%)	1	3.0%	3.3%

Table S2. Proportion of subjects in each cohort positive for antibodies against PLA2R1 or THSD7A. The final two columns reflect the percentage of anti-THSD7A-positive subjects within the entire cohort, and within the anti-PLA2R1-negative subgroup. iMN, idiopathic membranous nephropathy; sMN, secondary membranous nephropathy.

Table S3. Boston and European cohort (only THSD7A-positive patients).

Patient	Age	Sex	Proteinuria (g/24 h)	Months from biopsy to serum	Reactivity with HGE / rTHSD7A
MN A*	47	F	3	13.5	yes
MN B*	52	M	7.2	21	yes
MN C	33	F	1.9	1	yes
MN D	61	F	20	0.5	yes
MN E*	29	F	7.7	31	yes
MN F	80	M	5.9	87	yes
MN G	30	F	3.7	0	yes
MN H	51	F	14.9	0	yes
MN I*	66	F	3.5	30	yes
MN J**	77	M	7	0.1	yes
MN 1	68	F	6.4	5	yes
MN 2	56	M	1.8	0.75	yes
MN 3	43	M	10.7	0.5	yes
MN 4	28	F	5.4	0.75	yes
MN 5	47	F	6.9	5	yes
MN 6	51	M	4	4.9	yes
MN 119***	29	F	4	7	yes

Table S3. The 10 anti-THSD7A positive subjects amongst 365 idiopathic MN and 33 secondary MN cases collected at Boston University. Patients were classified according to the criteria presented in the first paragraph of the Methods section of the Supplementary Appendix. Abbreviations: MN, membranous nephropathy; F, female; M, male; HGE, human glomerular extract.

* indicates that cases had been treated with immunosuppressive agents prior to serum collection.

** represents the single case that was classified as secondary MN by virtue of active prostate cancer at the time of the diagnosis of MN.[8]

*** represents the single case that was classified as secondary MN due to positive antinuclear antibodies.

Table S4. Candidate antigens

Protein	Size	Glyco-sylation	Tissue expression	Cellular location
A-kinase anchor protein 12	191	No	Endothelial cells, cultured fibroblasts and osteosarcoma	Cytoplasm -> cell cortex
Activating signal cointegrator 1 complex subunit 3	251	No	N/A	N/A
Agrin	217	**N**, O	BM of lung and **kidney**	Isoform 1: secreted to extracellular space; Isoform 2: cell junction, synapse, **cell membrane** (SP II)
ALK tyrosine kinase receptor	176	**N**	Brain, CNS, small intestine, testes	**Cell membrane** (SP I)
Endocytic receptor 180	167	**N**	Ubiquitous with low expression in brain, placenta, lung, **kidney,** pancreas, spleen, thymus and colon. Expressed in endothelial cells.	**Cell membrane** (SP I)
Complement receptor type 1	223	**N**	Present on erythrocytes, leukocytes, **glomerular podocytes**, and splenic follicular dendritic cells.	**Cell membrane** (SP I)
Dysferlin	237	No	Skel. muscle, myoblast, syncytiotrophoblast of the placenta. Also in heart, brain, spleen, intestine, liver, lung, **kidney** and pancreas.	**Cell membrane** -> sarcolemma (SP II), cytoplasmic vesicle membrane (SP II)
Fibronectin	262	**N**, O	Plasma FN (soluble dimeric form) is secreted by hepatocytes. Cellular FN (dimeric or cross-linked multimeric forms) is made by fibroblasts.	Secreted -> extracellular space -> extracellular matrix
Laminin subunit gamma-1	178	**N**	Basement membrane	Secreted -> extracellular space -> extracellular matrix -> basement membrane
DEC-205	198	**N**	Expressed in spleen, thymus, colon and peripheral blood lymphocytes. Detected in myeloid and B-lymphoid cell lines.	**Cell membrane** (SP I)
Macrophage mannose receptor 1	166	**N**	N/A	Endosome membrane and **cell membrane** (SP I)
Myoferlin	235	No	Expressed in myoblast and endothelial cells. Highly expressed in card. and skel. muscles. Also present in lung, and at very low levels in **kidney**	**Cell membrane** (SP II), nucleus membrane (SP II), cytoplasmic vesicle membrane (SP II)
Myosin-9	227	No	In the **kidney,** expressed in the **glomeruli.** Also expressed in leukocytes.	Cytoplasm -> cytoskeleton, cytoplasm -> cell cortex

Nucleoprotein TPR	267	No	Esophagus, ovary, liver, skin, smooth muscles, cerebrum. Highest in testis, lung, thymus, spleen and brain, lower in heart, liver and **kidney.**	Nucleus, nucleus membrane, **cell membrane**, nucleoplasmic side, nucleus envelope.
Plexin-B2	205	**N**	N/A	**Cell membrane** (SP I)
Receptor-type tyrosine-protein phosphatase O	138	**N**	**Glomerulus of kidney.** Also detected in brain, lung and placenta.	**Cell membrane** (SP I)
Stabilin-1	275	**N**	High levels found in spleen, lymph node, liver and placenta. Also expressed in endothelial cells.	**Cell membrane** (SP I)
Talin-1	270	No	N/A	Cell projection -> ruffle membrane, **cell membrane**, cytoplasm -> cytoskeleton, cell junction -> focal adhesion
Thrombospondin type 1 domain containing 7A	185	**N**	N/A	**Cell membrane** (SP I), secreted
UDP-glucose:glycoprotein glucosyltransferase 1	177	**N**	Higher levels in pancreas, skeletal muscle, **kidney,** and brain. Low levels in lung and heart.	Endoplasmic reticulum lumen

Table S4. List of the best candidate proteins obtained from mass spectormetry analyses. Gel regions corresponding to the Western blot signals at 250 kDa (untreated), 225 kDa (treatment with N-glycopeptidase) and 200 kDa (treatment with *N*-glycopeptidase and neuraminidase) were excised and analyzed by mass spectrometry. More than 300 different proteins could be identified in total. We then compared the biochemical features of these proteins (size, glycosylation, cellular location) to the characteristics of the novel antigen. Moreover, as we expected the novel protein to be present in both the native and the deglycosylated conditions, we searched for overlapping results in the different gel regions. Table S4 presents the list of the best candidate proteins that were considered for further investigation. Characteristics that fit the protein of interest are marked in red. Abbreviations: BM, basal membrane; N/A, not available; SP I, single-pass type I (intracellular C-terminus); SP II, single-pass type II (intracellular N-terminus).

Table S5. Anti-THSD7A and anti-PLA2R1 positive MN patients

	Anti-THSD7A positive (n=17)	**Anti-PLA2R1 positive (n=74)**	***p*-value**
Age in years (± SD)	50 (± 17)	56 (± 16)	0.23
Gender male/female (% male)	6/11 (35%)	54/20 (73%)	0.005
Proteinuria in g/d (± SD)	6.8 (± 4.7)	8.7 (± 5.5)	0.15
Serum albumin in g/L (± SD)	24.8 (± 9)	25.3 (± 9.9)	0.99
Serum creatinine (± SD) in mg/dL	0.93 (± 0.3)	1.17 (± 0.55)	0.13
Treatment within 6 months yes/no (% yes)	8/7* (53%)	46/28 (62%)	0.57

Table S5. Characterisitcs and disease severity of anti-THSD7A and anti-PLA2R1 positive patiens. We compared patients with MN in regard to their characterisitcs such as age and gender as well as in regard to disease severity. The latter was assessed evaluating proteinuria, serum albumin, serum creatinine, and whether the patient was treated with immunosuppressants in the first 6 months after diagnosis. We found no significant difference between the two patient groups except that there were significantly more females in the group with anti-THSD7A positive MN. However, with this low number of patients a sampling error can not be excluded. Abbreviations: SD, standard deviation.
* information was not available for all patients

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