Netrin G1 is a novel target antigen in primary membranous nephropathy

- Supplemental Material -

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Supplemental Methods:

Preparation of human glomerular extracts

For the preparation of human glomerular extract (HGE), isolated glomeruli were thawed on ice and resuspended in resuspension buffer (50 mM Tris-HCl pH 8.5, 20% glycerol, 1X compete, EDTA-free protease inhibitor cocktail (Roche)). The cells were lysed by five rounds of sonication on ice (10s, 10% power, 3 pulses). For solubilization, 0.5%(w/v) n-dodecyl-D-maltoside (DDM) or 0.5%(w/v) lauryl maltose neopentyl glycol (LMNG) was added and the sample was incubated for 1 h at 4°C under gentle rotation. The cell debris was removed by centrifugation at 16.000 x g at 4°C. The supernatant was applied to protein G resin and incubated for 2 h at 4°C under gentle rotation. The supernatant was collected and applied through a Spin-X centrifuge tube filter (0.45 µm pore cellulose acetate membrane; Costar, #8163). The flow-through presents the solubilized human glomerular extract (HGE). It was aliquoted, flash frozen in lqN_2 and stored at -20°C until use.

Preparation of the plasma membrane fraction of HGE

For the isolation of the human glomerular plasma membrane fraction (HG-PM), isolated glomeruli were thawed on ice and resuspended in resuspension buffer II (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 20% glycerol, 1X compete, EDTA-free protease inhibitor cocktail (Roche)) and lysed by five rounds of sonication on ice (10s, 10% power, 3 pulses). The sample was centrifuged for 10 min at 16,000 x g at 4°C to remove the cell debris. The supernatant was collected and transferred into a fresh ultracentrifugation tube. The cell debris was resuspended in resuspension buffer II and the sonication and centrifugation cycle was repeated. The resulting supernatant was added to the first one. The joined supernatant was ultracentrifuged for 35 min at 100.000 x g at 6°C in a Ti70 rotor (Beckmann). The supernatant was collected and presents the cytoplasmic fraction. The pellet was resuspended in resuspension buffer

supplemented with 1% DDM or LMNG and was incubated for 1h at 4°C. The ultracentrifugation step was repeated. The supernatant was collected and presents the HG-PM. It was aliquoted, flash frozen in lqN_2 and stored at -20°C until use. Western blot analysis of the cell debris collected after the centrifugation at 16,000 x g revealed that the cell debris contains high amounts of the target antigen (as well as plasma membrane proteins such as PLA_2R1 and THSD7A).

Preparation of a membrane fraction of HGE

For the immune precipitation experiments prior to mass spectrometry, considerably larger amounts of the target antigen, i.e. membrane fraction, were needed. Considering the limited amount of human material, and in order to make the best use of this precious material, a modified protocol for the HGE membrane fraction preparation was applied. Hereby, the glomeruli were resuspended in resuspension buffer and lysed by five rounds of sonication on ice (10s, 10% power, 3 pulses). The cell debris was separated from the cytoplasmic fraction by centrifugation for 10 min at 20.000 x g at 4°C. The supernatant represents the cytosolic fraction. The cell debris was resuspended in resuspension buffer and homogenized by one round of sonication. The membrane proteins were than solubilized in resuspension buffer supplemented with 0.5% DDM or LMNG, incubated for 1h at 4°C, followed by removal of unsolubulized material by centrifugation for 10 min at 20.000 x g at 4°C. The collected sample contains a whole membrane fraction of HGE including plasma membrane and intracellular membranes, which was aliquoted, flash frozen in lqN_2 and stored at -20°C until use.

Native Western blot

All experimental steps were performed using cooled buffer solutions. The running and transfer units were placed in ice containers to allow a continuous cooling of the solutions. A 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (15-well, 15 µl, Biorad, #4561086) was placed

in the running unit, without loading protein sample. The inner chamber was filled with Tris/glycine (TG) dark cathode buffer (25 mM Tris base, 192 mM glycine, 0.02% coomasie G250), and the outer chamber was filled with cold TG anode buffer (25 mM Tris base, 192 mM glycine) up to the highest possible filling level. A pre-run (80 V, 8 mA) was performed for 10 min. The wells were flushed with TG dark cathode buffer and 5 µl of sample were loaded per lane. The run was continued with the same setting, until the running front has passed through 1/3 of the gel (approx. 40 min total run time). The run was stopped and the TG dark cathode buffer in the inner chamber was replaced by TG light cathode buffer (25 mM Tris base, 192 mM glycine, 0.002% coomasie G250). The wells were flushed again and the run was continued until the end of the gel was reached (approximately 3 h 45 min total run time).

The proteins were transferred to PVDF membranes using a tank blot system. The PVDF membrane was activated for 30 s in 100% methanol, followed by an incubation for 2 min in distilled water, and > 5 min in TG transfer buffer (25 mM Tris base, 192 mM glycine). Only one cassette was placed within one running unit. The proteins were transferred for 3 h at 35 V and 30 mA. After transfer, the membrane was placed in destaining buffer (PBS supplemented with 0.3%(v/v) Tween-20) and destained over night at 4°C under gentle agitation. The destaining buffer was replaced the next morning until the membrane only shows a weak bluish background color. The membrane was transferred into blocking buffer (3.5% milk in PBS supplemented with 0.1% Tween-20 (PBS-T)) and incubated for 2 h at 4°C under gentle agitation. The membrane was incubated over-night in primary antibody: human serum (1:100 dilution in dilution buffer (0.5% milk in PBS-T)) or anti-NTNG1 (abcam, #ab133732; 1:500 in dilution buffer). As secondary antibody, HRP-conjugated mouse anti-human IgG4 Fc (SouthernBiotech; #9200-05; 1:30,000 in blocking buffer), mouse anti-human IgG Fc (SouthernBiotech; #9040-05; 1:20,000 in blocking buffer), mouse anti-human IgG3 Hinge (SouthernBiotech; #9210-05; 1:20,000 in blocking buffer) or goat anti-rabbit IgG (Sigma Aldrich; #A9169; 1:20,000 dilution in blocking buffer) were incubated for 1 h at 4°C, or HRP-coupled mouse monoclonal anti-human IgG1 hinge heavy chain (abcam, #ab99774; 1:10,000

in blocking buffer) or anti-human IgG2 Fc (abcam, #ab99779; 1:20,000 in blocking buffer) were incubated over night at 4°C. Chemiluminescence was developed using Clarity™ Western ECL substrate (Biorad, #1705060).

Dot blot

The dot blot method was used to evaluate the steps of the IgG4 purification and coupling of the purified IgG4 to the magnetic beads.

A PVDF membrane was activated for 30 s in 100% methanol, followed by an incubation for 2 min in distilled water and > 5 min in PBS. The PVDF membrane was then placed on filter paper which was moisturized with PBS. Samples were dotted by pipetting 1 µl of sample onto the membrane. After dotting the membrane was blocked in blocking buffer for 1 h at room temperature. The IgG4 was directly detected by incubation with HRP-conjugated mouse anti-human IgG4 Fc (SouthernBiotech; #9200-05; 1:10,000 in blocking buffer), which was incubated for 1 h at room temperature. After washing 3x with PBS-T, chemiluminescence was developed using Clarity™ Western ECL substrate (biorad, #1705060).

Mass spectrometry of immunoprecipitated antigen

a) Sample preparation

Reduction and alkylation of disulphide bridges in cysteine containing proteins was performed with dithiothreitol at 56°C for 30 min, followed by the addition of 2-chloroacetamide to the samples and incubation at room temperature in the dark for 30 min. Samples were prepared using the SP3 protocol[1,2]. Trypsin (sequencing grade, Promega) was added in an enzyme to protein ratio 1:50 for overnight digestion at 37°C in 50 mM HEPES at pH 8.5. Peptide recovery was performed by collecting the supernatant. A second elution was performed by washing the beads again with 50 mM HEPES at pH 8.5. Both supernatants were combined.

Peptides were labelled with TMT6plex[3] Isobaric Label Reagent (ThermoFisher) according the manufacturer's instructions. In brief, 0.8 mg reagent of each label was dissolved in 42ul acetonitrile (100%) and 8 ul of stock was added to the samples and incubated for 1h at room temperature. The reaction was quenched with 5% hydroxylamine for 15min at RT. Samples were combined and cleaned up with the OASIS® HLB µElution Plate (Waters) system.

b) Mass spectrometry

An UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping cartridge (µ-Precolumn C18 PepMap 100, 5µm, 300 µm i.d. x 5 mm, 100 Å) and an analytical column (nanoEase™ M/Z HSS T3 column 75 µm x 250 mm C18, 1.8 µm, 100 Å, Waters) was coupled directly to an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo) using the Nanospray Flex™ ion source in positive ion mode.

Trapping was carried out with a constant flow of trapping solution (0.05% trifluoroacetic acid in water) at 30 µL/min onto the trapping column for 4 minutes. Subsequently, peptides were eluted via the analytical column running solvent A (0.1% formic acid in water, 3% DMSO) with a constant flow of 0.3 µL/min, with increasing percentage of solvent B (0.1% formic acid in acetonitrile, 3% DMSO) from 2% to 8% in 2 min, then 8% to 28% for a further 42 min, in another 4 min. from 28% to 40%, followed by an increase of B from 40-80% for 4 min. and a re-equilibration back to 2% B for 4 min.

The peptides were introduced into the Fusion Lumos via a Pico-Tip Emitter 360 µm OD x 20 µm ID; 10 µm tip (New Objective) and a spray voltage of 2.4 kV was applied. The capillary temperature was at 275°C. MS1 spectra were acquired with mass range of 375-1500 m/z in profile mode in the orbitrap with a resolution of 60000. The fill time was set at maximum of 50 ms. Data dependent acquisition (DDA) was performed in the Orbitrap with a resution of 15000, a fill time of 54 ms and a limitation of 1x10^5 ions. A normalized collision energy of 36 was applied. MS2 data was acquired in profile mode. Activation Type was HCD, and a fixed first mass at 110 m/z was set.

c) Mass spectrometry data analysis

To process the RAW data IsobarQuant[4] and Mascot (v2.2.07) were used. The search was conducted against the Uniprot Homo sapiens proteome database (UP000005640) including common contaminants and reversed sequences. Carbamidomethyl (C) and TMT6 (K) were set as fixed modifications, Acetyl (Protein N-term), Oxidation (M) and TMT6 (N-term) as variable modifications. For the full scan (MS1) a mass error tolerance of 10 ppm and for MS/MS (MS2) spectra of 0.02 Da was set. Trypsin was used as protease with a maximum of two missed cleavages and minimum peptide length of seven amino acids was allowed. At least two unique peptides were required for a protein identification. The false discovery rate on peptide and protein level was 0.01.

Mass spectrometry analysis of healthy glomeruli

Pellets of sieved glomeruli were lysed in 8 M urea, 50 mM ammonium bicarbonate, in the presence of protease inhibitors. Pellets were homogenized at 30 Hz for 1 min using a TissueLyser II (Qiagen), before sonication at 10% intensity, 10 pulses of 0.1 seconds on, 0.9 seconds off) to shear DNA. Insoluble material was removed via centrifugation and protein concentration was measured using BCA (Thermo Scientific). Samples were reduced using 5 mM DTT for 1 h at 37 °C and alkylated using 10 mM IAA for 30 minutes in the dark at room temperature. Urea concentration was adjusted to 1.5 M using 50 mM ammonium bicarbonate before trypsin was added at 1:50 enzyme to substrate ratio and samples digested at 37 °C overnight. The tryptic digests were fractionated and desalted using high pH reverse phase fractionation in stage tips. Proteomics data acquisition of fractions was carried out on a quadrupole Orbitrap mass spectrometer (QExactive; Thermo Fisher Scientific, Bremen, Germany) coupled to a nano UPLC (nanoAcquity system, Waters) with an inline trap column for desalting and purification (180 µm × 20 mm, 100 Å pore size, 5 µm particle size, Symmetry C18, Waters) followed by a 25 cm C18 reversed-phase column for peptide separation (75 µm × 200 mm, 130 Å pore size, 1.7 µm particle size, Peptide BEH C18, Waters). Peptides

were separated using an 80-min gradient with linearly increasing ACN concentration from 2% to 30% ACN in 65 min using a two-buffer system (buffer A: 0.1% FA in water, buffer B: 0.1% FA in ACN). The mass spectrometer was operated in data-dependent acquisition (DDA) mode with the top 12 ions by intensity per precursor scan (1 × 106 ions, 70,000 Resolution, 240 ms fill time) being selected for MS/MS (HCD at 25 normalized collision energy, 1 × 105 ions, 17,500 Resolution, 50 ms fill time) in a range of 400-1200 m/z. A dynamic precursor exclusion of 20 s was used.

LC-MS/MS data were searched against the uniprot human reference proteome (downloaded August 2021) using MaxQuant (version 1.6.17.0) with default parameters. The match between runs (MBR), LFQ, IBAQ and classical normalization features were enabled. Protein expression as per IBAQ were visualized using R (version 4.0.4) with the ggplot2 package (version 3.3.3). Raw proteomics data will be made available through researchers upon request.

Supplemental Results:

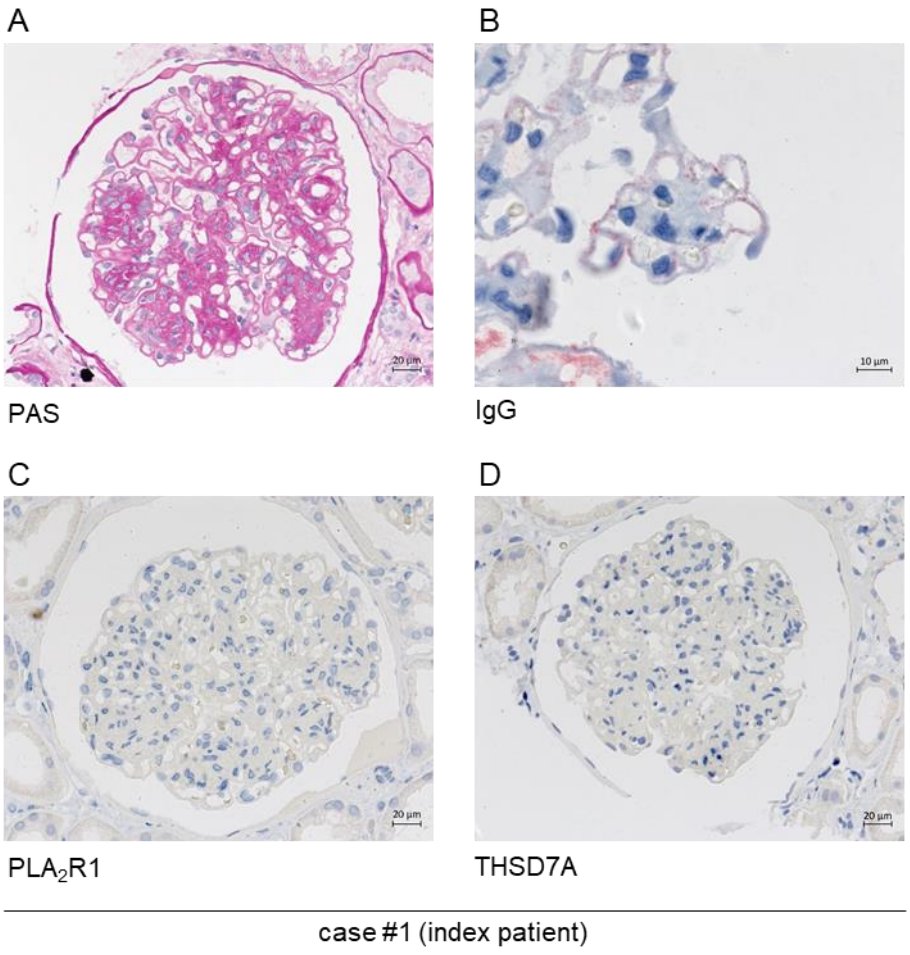
NTNG1-antibody specific ELISA

An in-house ELISA was developed for the detection of circulating NTNG1-ab in three patient cohorts (Supplemental Figure 5). In the prospective cohort A NTNG1-ab were positive in 1 (1.1%) out of 87 patients with MN not associated with PLA_2R1 or THSD7A (the index patient, case #1) and 1 (7.7%) out of 13 patients with THSD7A-associated MN. An “intermediate” result for NTNG1-ab was found in 1 (0.3%) out of 306 PLA_2R1-ab positive patients and 1 (1.1%) patient with MN not associated with PLA_2R1 or THSD7A. All remaining 402 patients were NTNG1-ab negative. All 4 sera which showed an intermediate or positive result for NTNG1-ab by ELISA were analyzed by native Western blot (Supplemental Figure 5B). Only the index serum (case #1) resulted positive for NTNG1-ab in the native Western blot experiment, while the remaining 3 sera (case A-C) had no detectable NTNG1-ab in native Western blot analyses (Supplemental Figure 5B).

In the retrospective cohort B, NTNG1-ab were detected by ELISA in 1 out of 405 sera (case #2). This patient was negative for PLA_2R1-ab and THSD7A-ab (Supplemental Figure 5A). Intermediate levels of NTNG1-ab were found in 1 (0.4%) out of 255 PLA_2R1-ab positive sera, 0 out of 14 THSD7A-ab positive sera and 1 (0.7%) out of 136 PLA_2R1-ab and THSD7A-ab negative sera (Figure 4A). All remaining 402 sera were NTNG1-ab negative. Native Western blot analyses confirmed NTNG1-ab positivity in the serum which resulted positive in the ELISA, while both sera with “intermediate” ELISA results tested negative by native Western blot (case D-E; Supplemental Figure 5B).

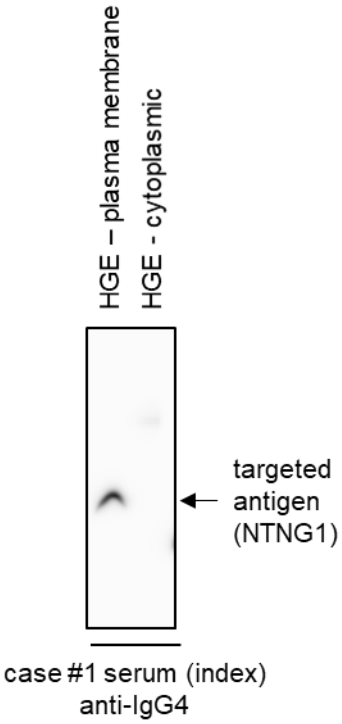
The control cohort C included 1 patient with IgA nephropathy (case F), who showed a low positivity in ELISA, but did not have detectable antibodies in the native Western blot analysis (Supplemental Figure 5A,B).

Supplemental figures:



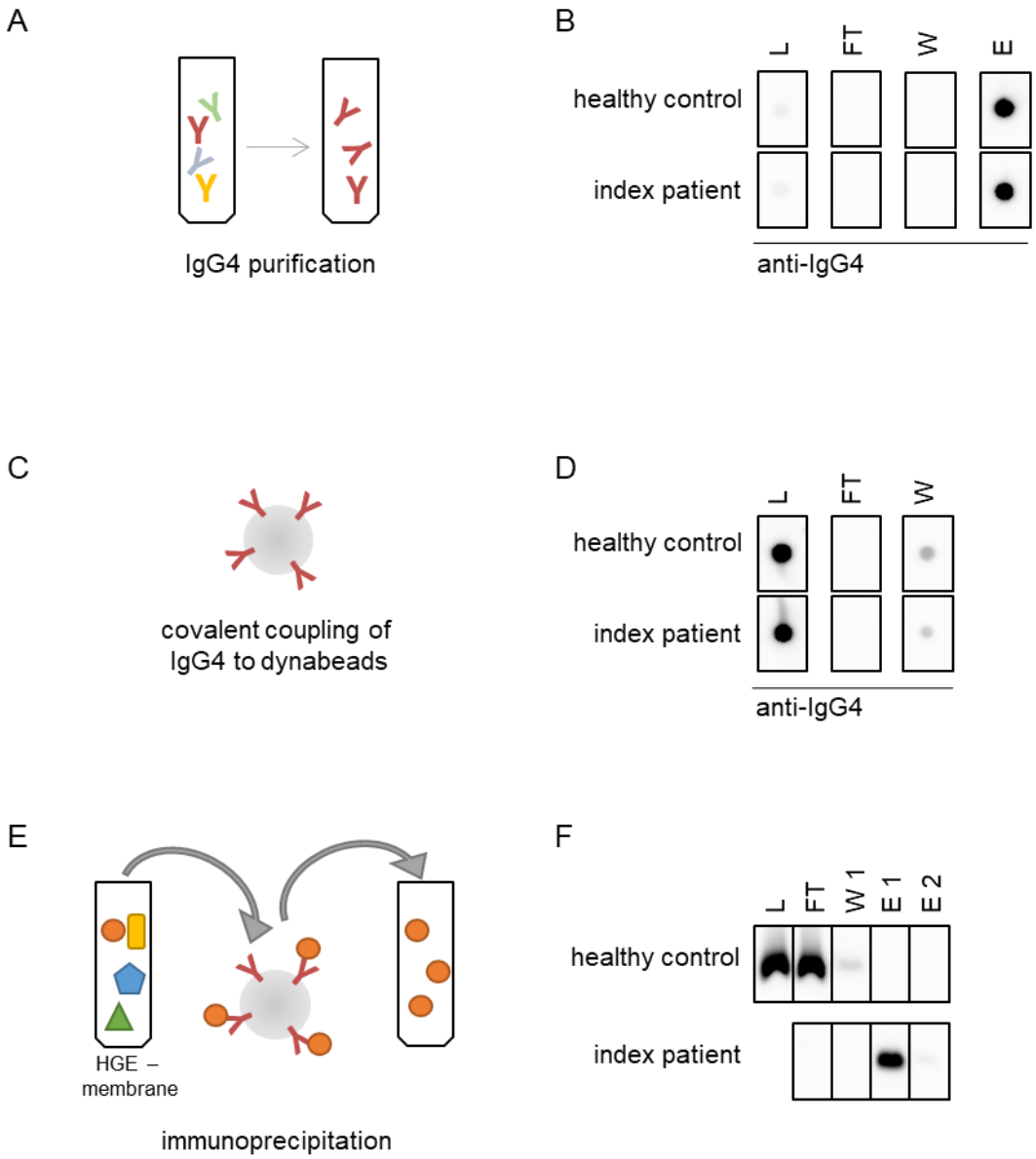
Supplemental Figure 1: Morphologic characterization of the kidney biopsy of the index patient (case #1).

A) PAS staining of the kidney biopsy of the index patient showing typical signs of diabetic nephropathy. B) Immunohistochemical staining for IgG shows a discrete granular positivity, indicating the diagnosis of early-stage MN. C, D) Immunohistochemical stainings for PLA_2R1 (C) and THSD7A (D) are not enhanced.



Supplemental Figure 2: Reactivity of the index patient serum with a protein of the plasma membrane fraction.

Native Western blot analysis shows an IgG4-specific reactivity of the index patient serum (case #1) with a protein present in the plasma membrane fraction, but not the cytosolic fraction of healthy glomeruli extract. The plasma membrane fraction was isolated using differential centrifugation including ultracentrifugation.

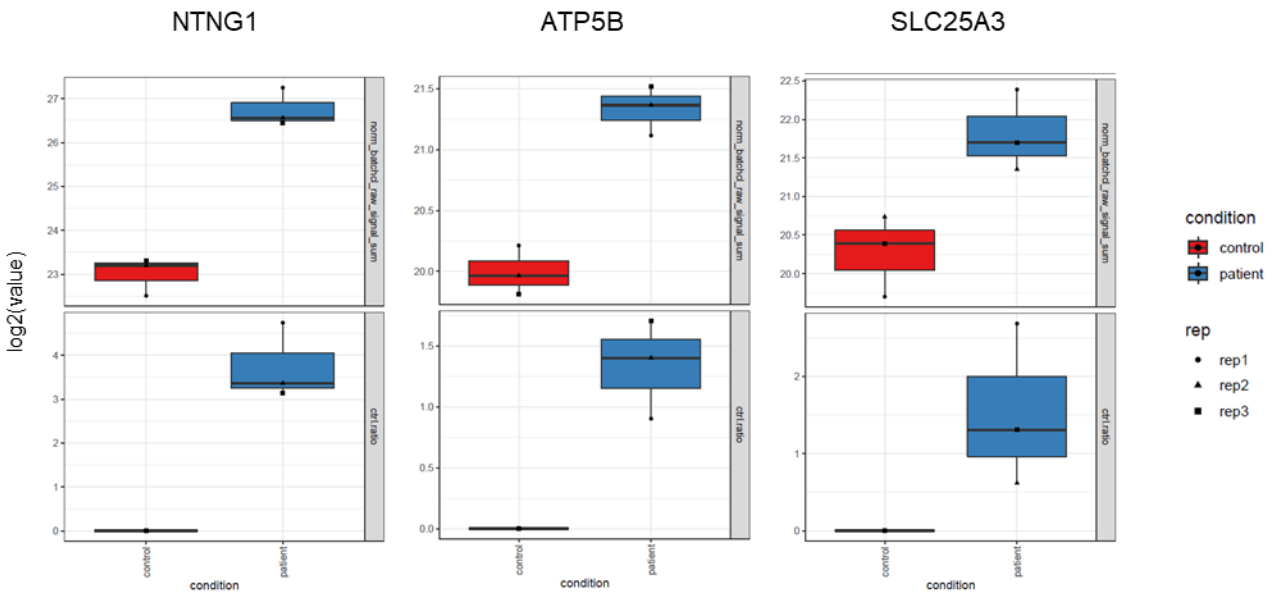


Supplemental Figure 3: Experimental procedure and validation.

A) The serum of the index patient and of a healthy donor were used to affinity purify IgG4 subclass antibodies. B) Dot blot analyses were performed to evaluate the successful purification and enrichment of IgG4 subclass antibodies. The following fractions of the purification process were dotted: L: load (serum sample); FT: flow through fraction; W: wash fraction; E: eluted IgG4 antibody. C) The purified Ig4 subclass antibodies were covalently coupled to dynabeads. D) The coupling process was validated by dot blot analyses. The majority of applied antibody was successfully coupled to the dynabeads. Access of non-covalently attached IgG4 antibody was removed in the wash fraction. E) The target antigen

was immunoprecipitated from the membrane fraction of human glomerular extract (HGE). F) The purification process was validated by native Western blot. The membrane fraction of HGE was first applied to dynabeads containing the IgG4 antibodies from the healthy donor (negative control). The resulting flow-through was then applied to dynabeads containing the IgG4 antibodies from the index patient. The target antigen was successfully eluted from the later immunoprecipitation.

A



B

>NTNG1 (Q9Y2I2; sequence coverage: 21.3%)

MYLSRFLSIH ALWVTVSSVM QPYPLVWGHY DLCKTQIYTE EGKVWDYMAC QPESTDMTKY
LKVKLDPPDI TCGDPPETFC AMGNPYMCNN ECDASTPELA HPPELMFDFE GRHPSTFWQS
ATWKEYPKPL QVNITLSWSK TIELTDNIVI TFESGRPDQM ILEKSLDYGR TWQPYQYYAT
DCLDAFHMDP KSVKDLSQHT VLEIICTEEY STGYTTNSKI IHFEIKDRFA FFAGPRLRNM
ASLYGQLDTT KKLRDFFTVT DLRIRLLRPA VGEIFVDELH LARYFYAISD IKVRGRCKCN
LHATVCVYDN SKLTCECEHN TTGPDCGKCK KNYQGRPWSP GSYLPIPKGT ANTCIPSISS
IGNCECFGHS NRCSYIDLLN TVICVSCKHN TRGQHCELCR LGYFRNASAQ LDDENVCIEC
YCNPLGSIHD RCNGSGFCEC KTGTTGPKCD ECLPGNSWHY GCQPNVCDNE LLHCQNGGTC
HNNVRCLCPA AYTGILCEKL RCEEAGSCGS DSGQGAPPHG SPALLLLTTL LGTASPLVF

>ATP5A2 (P25705; sequence coverage: 10.5%)

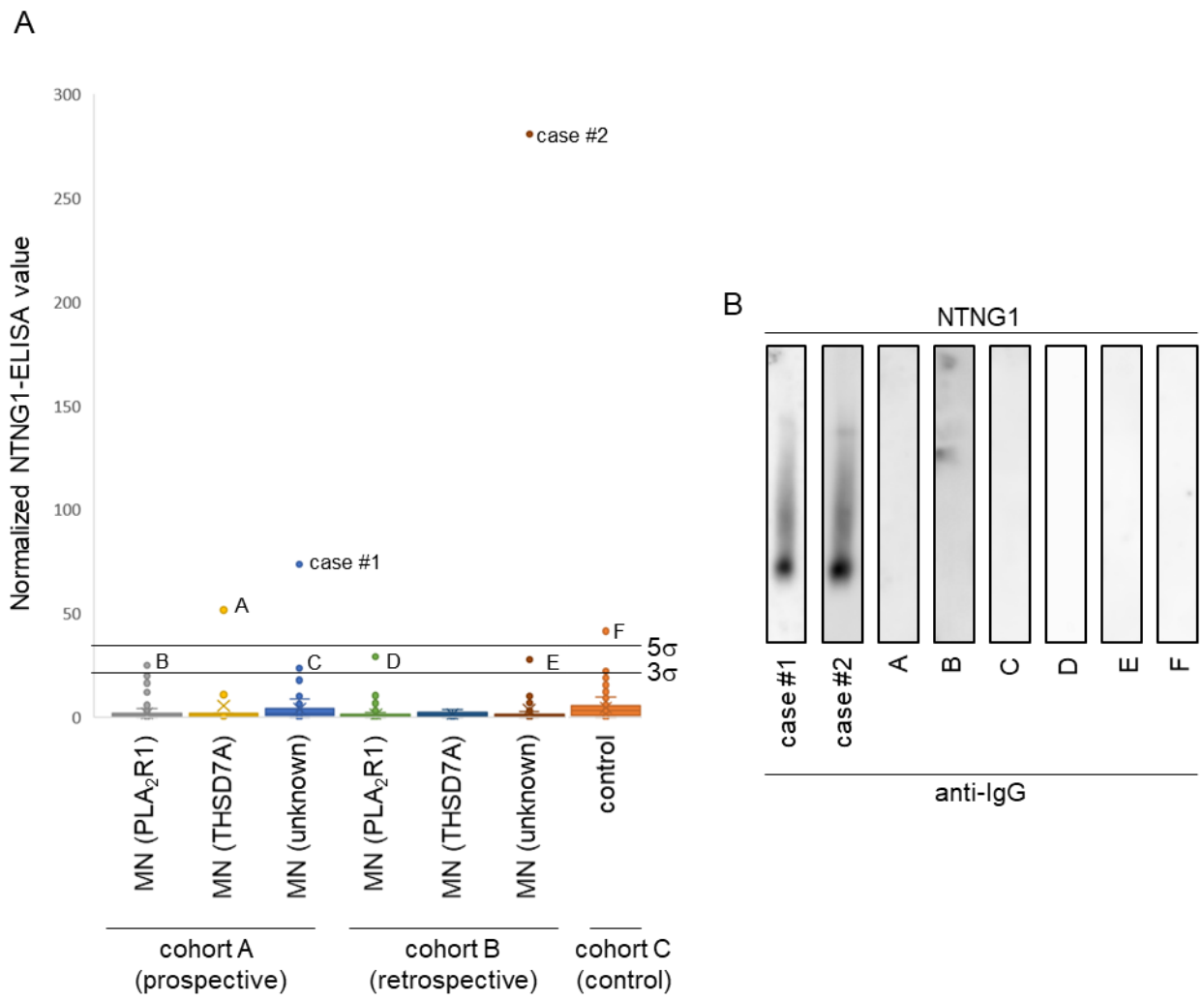
MLSVRVAAAV VRALPRRAGL VSRNALGSSF IAARNFHASN THLQKTGTAE MSSILEERIL
GADTSVDLEE TGRVLSIGDG IARVHGLRNV QAEEMVEFSS GLKGMSLNLE PDNVGVVVFG
NDKLIKEGDI VKRTGAIVDV PVGEELLGRV VDALGNAIDG KGPIGSKTRR RVGLKAPGII
PRISVREPMQ TGIKAVDSLV PIGRGQRELI IGDRQTGKTS IAIDTIINQK RFNDGSDEKK
KLYCIYVAIG QKRSTVAQLV KRLTDADAMK YTIVVSATAS DAAPLQYLAP YSGCSMGEYF
RDNGKHALII YDDLSKQAVA YRQMSLLLRR PPGREAYPGD VFYLHSRLLE RAAKMNDAFG
GGSLTALPVI ETQAGDVSAY IPTNVISITD GQIFLETELF YKGIRPAINV GLSVSRVGSA
AQTRAMKQVA GTMKLELAQY REVAAFAQFG SDLDAATQQL LSRGVRLTEL LKQGQYSPMA
IEEQVAVIYA GVRGYLDKLE PSKITKFENA FLSHVVSQHQ ALLGTIRADG KISEQSDAKL
KEIVTNFLAG FEA

>SLC25A5(P05141; sequence coverage: 7.4%)

MTDAAVSFAK DFLAGGVAAA ISKTAVAPIE RVKLLLQVQH ASKQITADKQ YKGIIDCVVR
IPKEQGVLSF WRGNLANVIR YFPTQALNFA FKDKYKQIFL GGVDKRTQFW LYFAGNLASG
GAAGATSLCF VYPLDFARTR LAADVGKAGA EREFRGLGDC LVKIYKSDGI KGLYQGFNVS
VQGIIIYRAA YFGIYDTAKG MLPDPKNTHI VISWMIAQTV TAVAGLTSYP FDTVRRRMMM
QSGRKGTDIM YTGTLDCWRK IARDEGGKAF FKGAWSNVLR GMGGAFVLVL YDEIKKYT

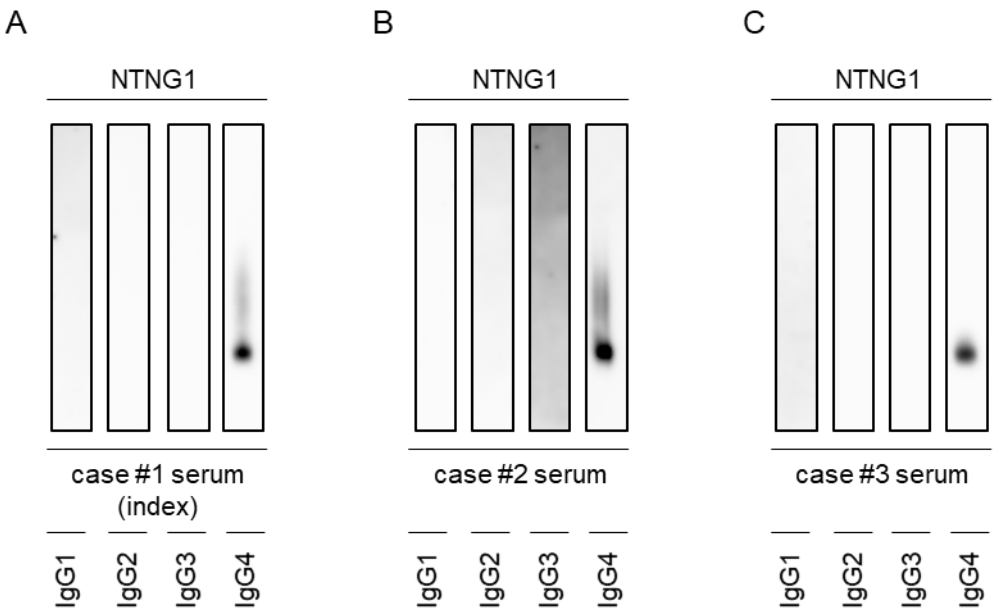
Supplemental Figure 4: Identification of NTNG1 by mass spectrometry analysis using TMT-based relative quantification.

A) Visualization of the accumulation of the top 3 candidates (NTNG1, ATP5A2 and SLC25A5) in comparison to the control samples. For the control and patient samples, three independent replicates were analyzed. Shown is the log2(value) over the normalized raw signal sum (top) as well as over the ratio to the control (bottom). A significant difference in the concentration of NTNG1 in the index patient probe compared to the healthy control was found in three independent immunoprecipitation experiments. B) The sequence coverage map of the top three candidates NTNG1, ATP5A2 and SLC25A5 identified in the TMT-based mass spectrometry analyses are shown. Amino acids highlighted over yellow background are the amino acids detected.



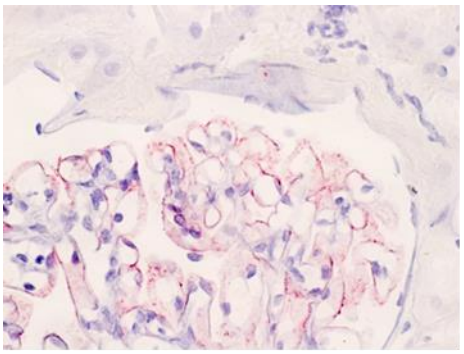
Supplemental Figure 5: In-house ELISA and native Western blot for the identification of NTNG1-associated MN.

A) An NTNG1-antibody specific ELISA was used to search for additional patients with NTNG1-antibody positivity in the cohorts A (prospective), B (retrospective) and C (control). Sera with results between 3 and 5 standard deviations above the mean were considered intermediate, and sera with an ELISA result higher than 5 standard deviations above the mean were considered positive. B) Native Western blot was used to confirm or exclude the presence of circulating NTNG1-antibodies.



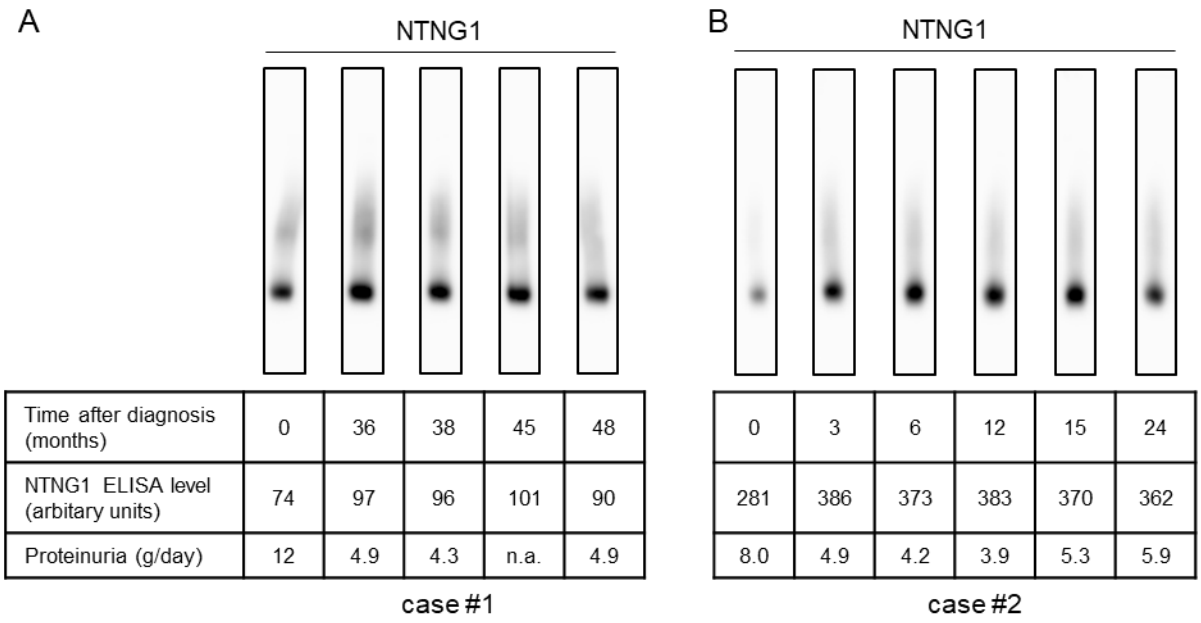
Supplemental Figure 6: IgG subclass analysis of patients with NTNG1-antibody positive MN.

Native Western blot was used to perform a IgG subclass analysis of the case #1 serum (index) (A), case #2 serum (B) and case #3 serum (C). In all three cases NTNG1 specific IgG4 antibodies were dominant.



Supplemental Figure 7: IHC staining of IgG4 in the kidney biopsy of case #2.

Immunohistochemical staining for IgG4 in the kidney biopsy of case #2 reveals a granular positivity along the glomerular basement membrane.



Time after diagnosis (months)	0	36	38	45	48
NTNG1 ELISA level (arbitary units)	74	97	96	101	90
Proteinuria (g/day)	12	4.9	4.3	n.a.	4.9

case #1

0	3	6	12	15	24
281	386	373	383	370	362
8.0	4.9	4.2	3.9	5.3	5.9

case #2

Supplemental Figure 8: Clinical and serological follow-up of case#1 and case#2.

A,B) Follow-up analyses of serum samples of case #1 (A) and case #2 (B) reveal that the patients had persisting circulating antibodies in circulation as shown by Western blot and ELISA. Also proteinuria was persisting over the complete follow-up time. n.a.: not available.

Supplemental References

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