Netrin G1 Is a Novel Target Antigen in Primary Membranous Nephropathy

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ABSTRACT

Background Primary membranous nephropathy (MN) is caused by circulating autoantibodies binding to antigens on the podocyte surface. PLA2R1 is the main target antigen in 70%–80% of cases, but the pathogenesis is unresolved in 10%–15% of patients.

Methods We used native western blotting to identify IgG4 autoantibodies, which bind an antigen endogenously expressed on podocyte membranes, in the serum of the index patient with MN. These IgG4 autoantibodies were used to immunoprecipitate the target antigen, and mass spectrometry was used to identify Netrin G1 (NTNG1). Using native western blot and ELISA, NTNG1 autoantibodies were analyzed in cohorts of 888 patients with MN or other glomerular diseases.

Results NTNG1 was identified as a novel target antigen in MN. It is a membrane protein expressed in healthy podocytes. Immunohistochemistry confirmed granular NTNG1 positivity in subepithelial glomerular immune deposits. In prospective and retrospective MN cohorts, we identified three patients with NTNG1-associated MN who showed IgG4-dominant circulating NTNG1 autoantibodies, enhanced NTNG1 expression in the kidney, and glomerular IgG4 deposits. No NTNG1 autoantibodies were identified in 561 PLA2R1 autoantibody–positive patients, 27 THSD7A autoantibody–positive patients, 27 THSD7A autoantibody–positive patients, and 77 patients with other glomerular diseases. In two patients with available follow-up of 2 and 4 years, both NTNG1 autoantibodies and proteinuria persisted.

Conclusions NTNG1 expands the repertoire of target antigens in patients with MN. The clinical role of NTNG1 autoantibodies remains to be defined.


Primary membranous nephropathy (MN) is an immune-mediated disease caused by the binding of circulating antibodies to endogenous antigens, which are expressed on glomerular podocytes. The formation of immune complexes leads to the activation of the complement system and results in proteinuria. This pathomechanistic concept of MN initiation was initially described in the animal model of passive Heymann nephritis and also applies for human podocytic antigens, like neutral endopeptidase, phospholipase A2 receptor 1 (PLA2R1), and thrombospondin type 1 domain containing protein 7A (THSD7A). In the last years, a number of novel potential target antigens have been described in patients with MN who do not exhibit antibodies against PLA2R1 or THSD7A. These proteins include exostosin 1/2 (EXT1/2), neural EGFL-like protein 1 (NELL1), high-temperature requirement serine protease A1 (HTRA1), semaphorin 3B, protocadherin 7 (PCDH7), neural cell adhesion molecule 1 (NCAM1), contactin-1, TGFβ receptor type 3, and protocadherin FAT1. In contrast to PLA2R1 and THSD7A, some of these antigens, such as NELL1, PCDH7, and
NCAM1, have not been shown to be expressed in podocytes, whereas others, such as HTRA1, are not membrane proteins but are endogenously expressed and secreted by podocytes.

In patients with PLA2R1- and THSD7A-induced MN, autoantibodies of the IgG4 subclass are dominant in the glomerular immune deposits and in the sera of these patients. The antigens are unknown, representing 10% of patients with MN in whom the antigens are unknown, representing an unsolved task in MN research.

The main scope of this study was to identify MN antigens on the basis of the pathomechanistic concept of primary MN as shown for the detection of PLA2R1 and THSD7A as endogenous antigens in MN. On the basis of this hypothesis, the antigen would be a membrane protein that shows an endogenous glomerular expression and reactivity to circulating IgG4 in patients with MN. For this, we combined the methodologic approaches of applying serum from patients with MN as a primary antibody in native western blot analyses and immunoprecipitation followed by mass spectrometry analyses.

**METHODS**

**Patient Cohorts**

In this study, four patient cohorts (A–D) were investigated.

Cohort A was a prospective cohort that included 406 consecutive patients with a newly diagnosed, biopsy-proven MN, of whom 306 (75.4%) patients had PLA2R1-associated MN and 13 (3.2%) patients had THSD7A-associated MN. The remaining 87 (21.4%) patients had neither PLA2R1- nor THSD7A-associated MN. The whole cohort was screened for PLA2R1 (NTNG1) autoantibodies. None of the patients in cohort A had received an immunosuppressive treatment at the time of study inclusion and first serum collection. First serum collection was performed within 6 months of kidney biopsy and diagnosis of MN in all patients. In all patients, PLA2R1 autoantibodies were measured by indirect immunofluorescence test (IIFT; Euroimmun; FA 1254–1010–1) and ELISA (Euroimmun; EA 1254–9601 G) following the manufacturers’ protocols, and if both test results were negative, they were also measured by nonreducing western blot as described previously. THSD7A autoantibodies were measured by IIFT (Euroimmun; FA 1254–1010–1) and nonreducing western blot. Clinical characteristics of parts of this cohort have been reported earlier. Follow-up visits occurred every 3 months and included data collection of PLA2R1 autoantibodies, THSD7A autoantibodies, proteinuria, serum creatinine, and treatment.

Cohort B was a retrospective cohort that included 405 patients with biopsy-proven MN. This cohort included 255 (63.0%) PLA2R1 autoantibodies–positive sera, 14 (3.5%) THSD7A autoantibodies–positive sera, and 136 (33.6%) sera negative for PLA2R1 autoantibodies and THSD7A autoantibodies. All sera were tested for PLA2R1 autoantibodies by IIFT and ELISA, and if both test results were negative, they were also tested by nonreducing western blot. THSD7A autoantibodies were measured by IIFT and nonreducing western blot.

Cohort C was a control cohort that included 77 patients with a biopsy-proven diagnosis of glomerulonephritis (GN), excluding MN, as well as seven healthy controls. This cohort consisted of six patients with C3 glomerulopathy, 12 patients with membranoproliferative GN, 18 patients with IgA nephropathy, 19 patients with FSGS, and 22 patients with minimal change disease.

Cohort D was a histologic cohort that included 383 patients with biopsy-proven MN not included in cohorts A–C. In this cohort, 242 biopsies negative for PLA2R1 and THSD7A, as well as 15 THSD7A-positive biopsies and 126 PLA2R1-positive biopsies, were stained for NTNG1. Of note, 29 of the 242 biopsies with negative staining for PLA2R1 and THSD7A were diagnosed with lupus MN. All biopsies were stained for NTNG1, PLA2R1, and THSD7A.

The study was approved by the local ethics committee of the chamber of physicians in Hamburg. The study was conducted in accordance with the ethical principles stated by the Declaration of Helsinki.

**Preparation of Human Glomerular Extracts**

Kidney tissues from patients undergoing tumor nephrectomy were used to isolate human glomeruli by sieving of healthy kidney tissue sections. The sieved glomeruli were used to produce human glomerular extracts (HGEs) or the membrane and cytosolic fractions of HGE. Details are given in Supplemental Material.

**Native Western Blot**

For sample preparation, the proteins in HGE or recombinant NTNG1 protein (Sino Biologic; 12313-H08H; 4 ng per lane) diluted in resuspension buffer (50 mM Tris-HCl [pH 8.5] and 20% glycerol) were directly loaded as antigens into the wells. The native western blot protocol was adjusted from Wittig et al. In short, proteins were separated using blue native PAGE followed by transfer to polyvinylidene difluoride membranes using a tank blot system and standard immunodetection methods. The detailed
procedure is described in Supplemental Material.

**Immunoprecipitation of the Target Antigen from the Membrane Fraction of Healthy Human Glomeruli**

IgG4 antibodies of the index patient and of a healthy donor were isolated using the CaptureSelect IgG4 (Hu) affinity matrix (Thermo Scientific; 294290200). The IgG4 antibodies were eluted using IgG elution buffer (Pierce; 21004), applied through a Spin-X centrifuge tube filter (0.45-μm pore cellulose acetate membrane; Costar; 8163), and neutralized using 1 M Tris-HCl (pH 8.5; Jena Bioscience; BU-124L-85). The neutralized, purified IgG4 antibodies were concentrated and buffer exchanged to buffer C1 (Invitrogen; 14321D) by ultrafiltration using a 50-kD molecular mass cutoff. The concentrated, purified IgG4 antibodies were then coupled to dynabeads M-270 epoxy beads following the manufacturer’s protocol (Invitrogen; 14321D). Uncoupled IgG4 antibodies were removed by washing the beads three times using 100 mM glycine (pH 2.3) supplemented with 0.5% Lauryl Maltose Neopentyl Glycol (LMNG). The immunoprecipitation was performed as described in the manufacturer’s protocol. The membrane fraction of HGE solubilized in LMNG was first applied to the IgG4 coupled beads from the healthy donor. The resulting flow through was then applied to the IgG4 coupled beads from the index patient. The elution buffer was supplemented with 10% glyceral and 0.25% LMNG. The eluates were applied through a Spin-X filter, and the flow through was neutralized using 1 M Tris-HCl (pH 8.5). The eluted antigen was concentrated by ultrafiltration (molecular mass cutoff 10 kD).

**Silver Staining**

The concentrated samples from one immunoprecipitation experiment were loaded on a 4%-15% Mini-PROTEAN TGX Precast Protein Gel, and SDS-PAGE was performed following standard procedures. The protein bands were visualized by silver staining following the manufacturer’s protocol (Pierce Silver Stain Kit; 24612).

**Mass Spectrometry of Immunoprecipitated Antigen**

The concentrated samples from the immunoprecipitation experiments were sent for mass spectrometry analysis at the Proteomics Core Facility at European Molecular Biology Laboratory (EMBL) Heidelberg, Germany. The immunoprecipitation experiments were repeated three times using serum for the index patient or healthy control. For each experiment, the isolated purified target antigen was identified by mass spectrometry analysis using tandem mass tagged-based relative quantification. The detailed procedure is described in Supplemental Material. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE21,22 partner repository with the database identifier PXD032713.

**Mass Spectrometry Analysis of Human Glomeruli**

Sieved human glomeruli from healthy tissue of tumor nephrectomy specimens were used for mass spectrometry analysis in order to perform a relative quantification of glomerular proteins. Details are presented in Supplemental Material.

**NTNG1 Autoantibodies-Specific ELISA**

Recombinant NTNG1 (Sino Biologic; 12313-H08H) was diluted in carbonate bicarbonate buffer (Sigma-Aldrich; C3041) to a concentration of 0.5 ng/μl. For the coating of 96-well plates (Greiner; 655101), 100 μl per well were applied, and the plate was incubated overnight at 4°C. The wells were washed twice with PBS, and they were then blocked using 4% milk in PBS supplemented with 0.05% Tween-20 (PBS-T) for 2 hours. Sera were diluted 1:100 in 2% milk in PBS-T. To each well, 100 μl of serum dilution was added and incubated for 2 hours under gentle agitation. The wells were washed five times with 300 μl PBS-T. As a secondary antibody, anti-human IgG (Southern Biotech; 9040-05) diluted 1:30,000 in 4% milk in PBS-T was incubated for 1 hour at room temperature. The wells were washed five times with 300 μl PBS-T. For development, 3,3’,5,5’-tetramethylbenzidine solution was added and incubated 8 minutes in the dark. The reaction was stopped by the addition of 1 M H₂PO₄. Absorption was measured at 450 nm.

The presence of NTNG1 autoantibodies was analyzed in all sera of cohorts A–C. Cohort C (patients without MN and healthy controls) was used to define the cutoff values of three and five SDs above the mean. All sera that showed a normalized ELISA value below three SDs were considered negative. Sera that showed a normalized ELISA value above this cutoff but below five SDs were considered “intermediate,” whereas sera with antibody levels above five SDs were considered positive in the ELISA.

**Histologic and Immunohistochemical Analyses of Kidney Biopsies**

One- to two-micrometer-thin sections of formalin-fixed, paraffin-embedded kidney biopsies were stained with periodic acid–Schiff and trichrome (Masson-Goldner-Elastica). Immunohistochemical analyses for PLA2R1, THSD7A, IgG, C1q, C3, and fibrinogen/fibrin were performed as previously reported.18,23 For NTNG1 immunohistochemical analyses, slides were deparaffinized and subjected to 600-W microwave treatment in EDTA buffer (pH 9.0) for 17 minutes. Afterward, slides were incubated for 10 minutes with normal horse serum (Vector Laboratories; Vector S2000) followed by anti-NTNG1 autoantibodies (D-2; mAb mouse; 1:20; Santa Cruz; sc-271774) overnight at 4°C. The slides were then washed with PBS, incubated with Post-Block (Zytomed; Zytocsm-Plus AP Polymer-Kit, POLAP-100), rinsed with PBS, and incubated with AP-Polymer (mouse/rabbit; Zytomed; Zytocsm-Plus AP Polymer-Kit, POLAP-100). After washing in PBS, slides were stained in new fuchsin naphthol As-Bi phosphate substrate mixture (30 minutes), rinsed in tap water, and left in 1% hydrochloric acid for
15 minutes. Slides were then rinsed in tap water followed by 1 minute of nuclear staining in hemalaun (Mayer).

The index patient was a 64-year-old man diagnosed with PLA2R1 and THSD7A autoantibodies--negative MN (Table 1, Supplemental Figure 1). The serum of the patient showed an IgG4 reactivity with the membrane fraction but not the cytoplasmic fraction of healthy human glomeruli in native western blot (Figure 1A, Supplemental Figure 2). Circulating IgG4 from this serum was used to immunoprecipitate the target antigen from the membrane fraction of healthy human glomeruli (Supplemental Figure 3). The purified target antigen was visualized on an SDS-PAGE using silver staining and identified as NTNG1 by mass spectrometry analysis using tandem mass tagged–based relative quantification (Figure 1, B and C, Supplemental Figure 4, Supplemental Material).

NTNG1 is an approximately 50-kD secreted glycoprotein that is attached to the cell surface via a glycosylphosphatidylinositol anchor (Figure 1D). Proteomic analysis of snap-frozen human glomeruli obtained by sieving confirmed the NTNG1 expression in healthy glomeruli. Interestingly, these proteomic analyses resolved >4500 proteins from human glomeruli of each patient (Supplemental Material), among which NTNG1, PLA2R1, and THSD7A were abundantly detected (Figure 1E), whereas the potential antigens NELL1, PCDH7, and EXT1/2 were not detected in any of the samples.

The presence of NTNG1 autoantibodies in the serum of the index patient was confirmed by native western blot and ELISA (Figure 2A, Supplemental Figure 5). In native western blot analyses, IgG4 reactivity was detected for the index patient but not for healthy subjects or patients with PLA2R1 or THSD7A autoantibodies–positive MN. The kidney biopsy of the patient showed typical granular positivity along the glomerular basement membrane for NTNG1 and IgG4 (Figure 2, B and C).

Deposition of subepithelial electron-dense material and effacement of podocyte foot processes were confirmed by electron microscopy (Figure 2D). In contrast, no granular NTNG1 positivity was observed in patients with PLA2R1 autoantibodies–positive MN (Figure 2E).

Four patient cohorts were investigated to identify additional patients with NTNG1-associated MN. The specificity of circulating NTNG1 autoantibodies detection for the diagnosis of NTNG1-associated MN was investigated in two cohorts of patients with MN (prospective cohort A and retrospective cohort B) and control cohort C. Using an in-house ELISA and native western blot, two NTNG1 autoantibodies–positive patients were identified: index case 1 from prospective cohort A and case 2 from retrospective cohort B. Both patients were negative for PLA2R1 autoantibodies and THSD7A autoantibodies. In control cohort C, all patients were NTNG1 autoantibodies negative (Supplemental Figure 5, Supplemental Material).

In prospective cohort A, kidney biopsies were available and stained for NTNG1 in 80 patients with PLA2R1-associated MN and 47 patients with neither PLA2R1 nor THSD7A-associated MN. NTNG1 positivity was found only in the biopsy of the index patient. In histologic cohort D, 383 kidney biopsies from patients with MN were stained for NTNG1. NTNG1 positivity was found in one of 242 biopsies that were negative for PLA2R1 and THSD7A. This biopsy also stained positive for IgG4 (case 3) (Figure 3, A and B). All PLA2R1- and THSD7A-positive biopsies and the 29 biopsies with lupus nephritis stained negative for NTNG1.

Of note, in case 2, an NTNG1 staining of the kidney biopsy could not be performed because of the absence of glomeruli in the remaining biopsy material.

In this study, we identified three patients with NTNG1-associated MN (Table 1). In all three patients, NTNG1 autoantibodies were predominantly of the IgG4 subclass both in the circulation (Supplemental Figure 6) and in the kidney biopsy (Figures 2C and 3B, Supplemental Figure 7). Clinical, serologic, or histomorphologic sings of secondary MN were not present in any of these three patients.

For case 1 and case 2, follow-up, including clinical data and serum samples, was available for 48 and 24 months, respectively. In both cases, no immunosuppressive treatment was initiated during the observational period. Both cases showed persisting proteinuria and persisting circulating NTNG1 autoantibodies (Supplemental Figures 5 and 8).

In primary human MN, subepithelial immune complexes develop when circulating autoantibodies, mostly belonging to the IgG4 subclass, bind to an endogenous antigen on the podocytes. On the basis of these known pathogenic characteristics of PLA2R1- and THSD7A-associated MN, we identified NTNG1 as a new target antigen in three patients with MN who do not have antibodies against other antigens and do not have any other autoimmune diseases. In this context, NTNG1 seems to exhibit properties that are similar to PLA2R1 and THSD7A because it is expressed in healthy human glomeruli, is a

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**Table 1. Clinical characteristics of NTNG1 autoantibodies–positive patients**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Proteinuria at Study Start, g/d</th>
<th>Serum Creatinine at Study Start, mg/dl</th>
<th>IgG4 Positivity in Kidney Biopsy</th>
<th>NTNG1 Autoantibodies IgG4 Dominant</th>
<th>Other Diseases</th>
<th>Immunosuppressive Treatment</th>
<th>Follow-Up Time, mo</th>
<th>Serum Creatinine at End of Follow-Up, mg/dl</th>
<th>Remission at End of Follow-Up</th>
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<td>1</td>
<td>64</td>
<td>Male</td>
<td>12.0</td>
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<td>Yes</td>
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<td>48</td>
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<td>3*</td>
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<td>1.8</td>
<td>1.2</td>
<td>Yes</td>
<td>Yes</td>
<td>Hypertension, obesity</td>
<td>No</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

All patients were negative for PLA2R1 autoantibodies and THSD7A autoantibodies. DM2, diabetes mellitus type 2; n.a., not available.

*For case 3, inclusion in the study and serum collection were performed after NTNG1 was identified as the target antigen in MN (2 years after the diagnosis of MN was made).
plasma membrane protein, and is targeted by IgG4 antibodies in all three described cases. None of the patients had clinical diagnoses corresponding to secondary MN. Whether these similarities might also indicate that similar pathogenetic mechanisms are active in these patients remains to be shown. Taken together, the clinical and histopathologic evaluations of these patients correspond to the diagnosis of primary MN.

NTNG1 is a 50-kD neuronal protein that was shown to be expressed in podocytes. The protein has an N-terminal signal peptide and an N-terminal Laminin N-terminal domain followed by up to four EGF-like domains, depending on the splice variant. Unlike other Netrin-family members, NTNG1 is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. NTNG1 is an adhesion molecule located on distinct axons, where it specifically interacts with the receptor NTNG1 ligand (also leucine-rich repeat-containing protein 4C) on target dendrites. Together, they promote thalamocortical axon outgrowth, induce and maintain excitatory synapse formation, and contribute to subendodritic segmentation in the hippocampus and cortex. Thus, NTNG1 is an important neuronal protein, and mutations of the protein are a rare cause of Rett syndrome, a neurodevelopmental disorder that predominantly affects women. NTNG1 can also be associated with schizophrenia. Also, the deletion of its ligand, NLG-1 in mice is associated with neurobehavioral disorders. No data on proteinuria were provided for these mice; however, functional disruption of MN target antigens in mice does not necessarily

Figure 1. Identification of NTNG1 as target antigen in MN. (A) Native western blot analysis shows an IgG4-specific reactivity of the index patient serum (case 1) with a protein present in the membrane fraction of healthy HGE. (B) As visualized by silver staining, an IgG4-specific immunoprecipitation leads to an enrichment of a protein band at approximately 50 kD in the index patient sample. (C) A volcano plot shows the specific enrichment of NTNG1 identified by tandem mass tagged–based relative quantification mass spectrometry. (D) Schematic representation of NTNG1 with labeling of the N-terminal signal peptide (SP), N-terminal Laminin domain (Lam), EGF-like domains (EGF), and the glycosylphosphatidylinositol (GPI) anchor. (E) Relative quantification of sieved glomeruli confirms that NTNG1 is endogenously expressed in healthy glomeruli. iBAQ, intensity based absolute quantification; IP, immunoprecipitation.
**Figure 2. Serologic and histologic characterization of index case 1.** (A) Native western blot analyses show that the serum of the index patient (but not control sera) specifically reacts with recombinant NTNG1. (B and C) Immunohistochemical (IHC) staining reveals a distinct granular positivity for (B) NTNG1 and (C) IgG4 along the glomerular basement membrane in the glomeruli of the index patient (case 1). (D) Small electron-dense deposits and an effacement of podocyte foot processes are visualized by electron microscopy. (E) Representative IHC staining for NTNG1 in the kidney biopsy of a patient with PLA2R1 autoantibodies–positive MN. No NTNG1 accumulation is seen in the glomerular deposits of the kidney biopsy.
lead to a podocytic phenotype as seen for PLA2R1, which is not expressed on healthy mice podocytes.\textsuperscript{36} It has been shown that podocytes and neurons share a number of proteins involved in critical structural and signaling components, such as Robo2/Slit\textsuperscript{2,37,38}, synaptopodin\textsuperscript{39,40} and UCHL-1.\textsuperscript{41,42} Thus, NTNG1 and contactin-1 add to this list as potential target antigens of MN.

NTNG1 autoantibodies detection and NTNG1 positivity in the kidney biopsy were very specific for NTNG1-associated MN, as shown in our extended serologic and histomorphologic control cohorts including 888 and 383 patients, respectively. The large number of patients included in this study and the good characterization of the cohorts, including patients with newly diagnosed, untreated MN, represent important strengths of the study. In the two patients with available clinical follow-up data, NTNG1 autoantibodies persisted in parallel with proteinuria. However, the low number of patients described here is a limitation, and therefore, no final conclusions can be made on the role of NTNG1 autoantibodies for the course of the disease. For this, a larger number of patients needs to be studied in the future. Also, it needs to be considered that these data show an association but do not provide evidence of a causal relationship between NTNG1 autoantibodies and disease activity. To provide evidence of pathogenicity in MN, antigen-specific animal models are needed.

The description of novel target antigens in MN has resulted in substantial progress in the morphologic and clinical diagnoses of patients in the last years. Therefore, the aim will remain to characterize every patient with MN by the responsible molecular disease pathogenesis. This will allow us to better predict the prognosis and clinical outcome of patients and therefore help to make better decisions on the need for immunosuppressive treatment.

DISCLOSURES

J.H. Bräsen reports honoraria from Alexion. E. Hoxha received fees from Morphosys, Planegg (Germany) and Novartis (Basel, Switzerland) for advisory board activities; reports consultancy agreements with Morphosys AG and Novartis; and reports research funding from Deutsche Forschungsgemeinschaft. M. Machalitza reports other interests or relationships through a stipend from the Else Kröner-Fresenius-Stiftung iPRIME programme, M.M. Rinschen acknowledges support from the Young Investigator Award through Novo Nordisk Fonden grant NNF19OC0056043.

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AUTHORS CONTRIBUTIONS

E. Hoxha, L. Reinhard, and R.A.K. Stahl conceptualized the study; J.H. Bräsen, F. Drömann, N. Ferru, H.-J. Gröne, E. Hoxha, M. Lassé, M. Machalitza, L. Reinhard, M.M. Rinschen, P.M. Rob, and T. Wiech were responsible for data curation; J.H. Bräsen, N. Ferru, H.-J. Gröne, E. Hoxha, M. Lassé, M. Machalitza, L. Reinhard, M.M. Rinschen, R.A.K. Stahl, and T. Wiech were honoraria for teaching, grand rounds, lectures, UpToDate, and reviewing slides for a study for Novartis; R.A.K. Stahl received fees from Morphosys and Planegg (Germany) for advisory board activities; reports consultancy agreements with Morphosys and Planegg (Germany); and reports patents or royalties from EUROIMMUNE (Germany). T. Wiech reports honoraria from Bayer, GlaxoSmithKline GmbH, and Novartis and an advisory or leadership role with Retrophin. All remaining authors have nothing to disclose.

Figure 3. Serologic and histologic characterization of case 3. (A) One patient with NTNG1-positive MN (case 3) was identified in histologic cohort D using immunohistochemical staining for NTNG1. (B) The patient also had a granular deposition of IgG4 along the glomerular basement membrane. (C) Serum of the patient collected 2 years after diagnosis showed the presence of circulating NTNG1 autoantibodies.
responsible for formal analysis; E. Hoxha and R.A.K. Stahl provided supervision; E. Hoxha and L. Reinhard wrote the original draft; and J.H. Bräsen, F. Doermann, N. Ferru, H.-J. Gröne, E. Hoxha, M. Lassé, M. Machalitza, L. Reinhard, M.M. Rinschen, P.M. Rob, S. Sethi, R.A.K. Stahl, and T. WIECH reviewed and edited the manuscript.

DATA SHARING STATEMENT

Anonymized data reported in this paper of type experimental data have been deposited in the PRoteomics IDENTifications database (accession no. PXD032713).

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2020250608/-/DC Supplemental.

Supplemental Material. Methods, results, and references.

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

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

Supplemental Figure 3. Experimental procedure and validation.

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

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

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

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

Supplemental Figure 8. Clinical and serologic follow-up of cases 1 and 2.

REFERENCES


Netrin G1 is a novel target antigen in primary membranous nephropathy

- Supplemental Material -

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## Table of contents

### Supplemental Methods:

<table>
<thead>
<tr>
<th>Method</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of human glomerular extracts</td>
<td>3</td>
</tr>
<tr>
<td>Preparation of the plasma membrane fraction of HGE</td>
<td>3</td>
</tr>
<tr>
<td>Preparation of a membrane fraction of HGE</td>
<td>4</td>
</tr>
<tr>
<td>Native Western blot</td>
<td>4</td>
</tr>
<tr>
<td>Dot blot</td>
<td>6</td>
</tr>
<tr>
<td>Mass spectrometry of immunoprecipitated antigen</td>
<td>6</td>
</tr>
<tr>
<td>a) Sample preparation</td>
<td>6</td>
</tr>
<tr>
<td>b) Mass spectrometry</td>
<td>7</td>
</tr>
<tr>
<td>c) Mass spectrometry data analysis</td>
<td>8</td>
</tr>
<tr>
<td>Mass spectrometry analysis of healthy glomeruli</td>
<td>8</td>
</tr>
</tbody>
</table>

### Supplemental Results:

<table>
<thead>
<tr>
<th>Method</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTNG1-antibody specific ELISA</td>
<td>9</td>
</tr>
</tbody>
</table>

### Supplemental Figures:

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplemental Figure 1: Morphologic characterization of the kidney biopsy of the index patient (case #1).</td>
<td>11</td>
</tr>
<tr>
<td>Supplemental Figure 2: Reactivity of the index patient serum with a protein of the plasma membrane fraction.</td>
<td>12</td>
</tr>
<tr>
<td>Supplemental Figure 3: Experimental procedure and validation.</td>
<td>13</td>
</tr>
<tr>
<td>Supplemental Figure 4: Identification of NTNG1 by mass spectrometry analysis using TMT-based relative quantification.</td>
<td>15</td>
</tr>
<tr>
<td>Supplemental Figure 5: In-house ELISA and native Western blot for the identification of NTNG1-associated MN.</td>
<td>17</td>
</tr>
<tr>
<td>Supplemental Figure 6: IgG subclass analysis of patients with NTNG1-antibody positive MN.</td>
<td>18</td>
</tr>
<tr>
<td>Supplemental Figure 7: IHC staining of IgG4 in the kidney biopsy of case #2.</td>
<td>Fehler! Textmarke nicht definiert.9</td>
</tr>
<tr>
<td>Supplemental Figure 8: Clinical and serological follow-up of case#1 and case#2</td>
<td>20</td>
</tr>
</tbody>
</table>
Supplemental Methods:

**Preparation of human glomerular extract**

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 liquid nitrogen 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 lqN₂ 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 PLA₂R1 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 then solubilized in resuspension buffer supplemented with 0.5% DDM or LMNG, incubated for 1h at 4°C, followed by removal of unsolubilized 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 lqN₂ 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. 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 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 resolution 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 IsobarQuant⁴ 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 PLA2R1 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 PLA2R1-ab positive patients and 1 (1.1%) patient with MN not associated with PLA2R1 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 PLA₂R1-ab and THSD7A-ab (Supplemental Figure 5A). Intermediate levels of NTNG1-ab were found in 1 (0.4%) out of 255 PLA₂R1-ab positive sera, 0 out of 14 THSD7A-ab positive sera and 1 (0.7%) out of 136 PLA₂R1-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 PL4R1 (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 IgG4 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.
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.
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


