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 auto-antibodies 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 autoantibodies–positive patients, 27 THSD7A autoantibodies–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.

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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. With the exception of EXT1/2, circulating antibodies were detected for all other antigens, but these antibodies are mostly of the IgG1/IgG3 subclases. These findings might suggest that the pathomechanisms of disease onset could be different from those of PLA2R1-induced MN and in part, align to the concept of secondary MN initiation. Despite the detection of a number of new antigens, there are still 10%–15% 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 suffered from 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-Cl [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

Significance Statement
Membranous nephropathy (MN) is an autoimmune disease caused by circulating antibodies, which target antigens on podocyte surfaces. We identified NTNG1 as a membrane protein that podocytes express and that circulating (mainly IgG4 subclass) autoantibodies target in patients with primary MN. Staining the target antigen in the biopsy and measuring circulating antibodies to determine the immunologic activity of disease provide a molecular characterization that improves diagnosis and clinical management of MN.
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% glycerol 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 PRIDE⁴¹,²² 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 PLA₂-R₁, THSD7A, IgG, Clq, 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 from mouse, 1:20; Santa Cruz; sc-271774) overnight at 4°C. The slides were then washed with PBS, incubated with Post-Block (Zytomed; Zytocell-Plus AP Polymer-Kit, POLAP-100), rinsed with PBS, and incubated with AP-Polymer (mouse/rabbit; Zytomed; Zytocell-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
Table 1. Clinical characteristics of NTNG1 autoantibodies–positive patients

<table>
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<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>
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<td>n.a.</td>
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</table>

All patients were negative for PLA2R1 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).

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, Supplementary 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 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 signs 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
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.24,25 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.26 Unlike other Netrin-family members, NTNG1 is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor.27 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.28,29 Together, they promote thalamocortical axon outgrowth,28 induce and maintain excitatory synapse formation,30 and contribute to subdenritic segmentation in the hippocampus and cortex.29 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.31,32 NTNG1 can also be associated with schizophrenia.33 Also, the deletion of its ligand, NLG-1 in mice is associated with neurobehavioral disorders.34,35 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 mouse podocytes. It has been shown that podocytes and neurons share a number of proteins involved in critical structural and signaling components, such as Robo2/Slit2, synaptopodin, and UCHL-1. 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 and the good clinical characterization of the cohorts, including patients with newly diagnosed, untreated MN.

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, translatio-nale Entzündungsforschung. M.M. Rinschen reports honoraria from JASN and Physiological Genomics and advisory or leadership roles with the editorial board of American Journal of Physiology Renal Physiology, the editorial board of JASN, and the editorial board of Physiological Genomics. S. Sethi reports a consultancy agreement with Novartis and 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 EUROMMUNE (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.

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AUTHORS CONTRIBUTIONS

DATA SHARING STATEMENT

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

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2022050608/-/DC. 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 membrane 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


