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Contactin-1 is a novel target antigen in membranous nephropathy associated with chronic inflammatory demyelinating polyneuropathy.

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Abstract

Primary membranous nephropathy (MN) is an autoimmune glomerular disease in which autoantibodies are directed against podocyte proteins. In about 80% of cases the main targeted antigen is the phospholipase A2 receptor 1 (PLA2R1). Anti-PLA2R1 antibodies are mainly immunoglobulin G type 4 (IgG4). However, the antigenic target remains to be defined in 20% of cases. MN can be associated with chronic inflammatory demyelinating polyneuropathy, an autoimmune disease of the peripheral nervous system where a common antigenic target has yet to be identified. To ascertain a possible novel target antigen, we analyzed kidney biopsies from five patients positive for anti-contactin 1 antibodies and presenting with MN combined with chronic inflammatory demyelinating polyneuropathy. Eluted IgG from biopsy sections against contactin 1 and nerve tissue were screened. Western blot revealed contactin 1 expression in normal kidney glomeruli. Confocal microscopic analysis showed the presence and colocalization of contactin 1 and IgG4 on the glomerular basement membrane of these patients. Glomerular contactin 1 was absent in patients with anti-PLA2R1 associated MN or membranous lupus nephritis or a healthy control. The eluted IgG from contactin 1 positive biopsy sections but not the IgG eluted from patients with PLA2R1 MN bound contactin 1 with the main eluted subclass IgG4. Eluted IgG could bind paranodal tissue (myelinated axon) and colocalized with commercial anti-contactin 1 antibody. Thus, contactin 1 is a novel common antigenic target in MN associated with chronic inflammatory demyelinating polyneuropathy. However, the precise pathophysiology remains to be elucidated.

Key words: Membranous nephropathy, nephrotic syndrome, Contactin-1, Immunoglobulin G type 4, Chronic inflammatory demyelinating polyneuropathy

Translational Statement

Some cases of primary membranous nephropathy (MN) are associated with chronic inflammatory demyelinating polyneuropathy (CIDP) although the mechanisms are unknown. We demonstrate here for the first time that CNTN1 is a shared antigenic target in both diseases and that it may be a marker of disease severity.

Our findings could constitute a breakthrough in the diagnosis and management of patients with MN/CIDP and identify patients of poor prognosis who may benefit from a first-line combined multi-target therapy. Future work is required to understand the role of anti-CNTN1 antibodies in the pathogenicity and to define its place in isolated MN.

INTRODUCTION:

Membranous nephropathy (MN) is a rare autoimmune disease affecting the kidney glomerulus and more specifically the podocytes. It is characterized by the subepithelial formation of immune deposits outside the glomerular capillary wall, close to the podocytes. These deposits are responsible for proteinuria and MN is a common cause of nephrotic syndrome in adults with a peak incidence in males aged 50 to 60 years. In around 80% of the cases, MN occurs in the absence of an established cause and is called primary membranous nephropathy. Alternatively, MN occurs in association with a clinical condition – infections (hepatitis B), lupus erythematosus, cancer, or drug intoxication – and is called secondary MN. In MN, podocyte antigens are the major targets of the autoimmune response. Immunoglobulin G type 4 (IgG4) is the prominent deposited immunoglobulin subclass in MN, while IgG1, IgG2, and IgG3 are more prominent in cases of secondary MN^{1,2,3}. In 2009, Beck et al identified the phospholipase A2 receptor (PLA2R1) as being the main antigenic target involved in human primary MN⁴. Anti-PLA2R1 antibodies are involved in about 80% of cases of primary MN and anti-Thrombospondin type 1 domain-containing 7 A (THSD7A) in 3-5% of cases⁵. Other autoantibodies, such as anti-neural epidermal growth factor-like 1 protein (NELL-1) and anti-Semaphorine 3B have also recently been reported^{6,7}. However, in about 15% of cases of primary MN, the target antigen remains unknown.

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a disease of the peripheral nervous system which has a chronic progressive course over more than 8 weeks. Diagnosis is mostly based on electrophysiological and clinical investigations⁸. The severity of this disease is highly heterogeneous. Auto-antibodies against nodal and paranodal proteins such as neurofascin 155 (NF155), neurofascin 186 (NF186), contactin-associated protein 1 (Caspr1), or contactin-1 (CNTN1), mainly of the IgG4 subclass, have recently been reported in severe cases⁹. These IgG4 autoantibodies are pathogenic and induce the disorganization of the septate-like junctions at the paranodes causing a nodo-paranodopathy^{10,11}. In the case of anti-CNTN1 IgG4, the antibodies have a function-blocking activity and inhibit the interaction between CNTN1 and NF155 at the paranodes^{10,12}. The incidence of these autoantibodies in CIDP is low, varying from 2% in Europe to 10% in Asian countries¹³⁻¹⁵. A few case reports have described the association of MN with anti-CNTN1 related CIDP^{13,14,16-18}.

To date, nine cases of MN have been described in CIDP patients with anti-CNTN1 IgG4^{13,14,16-18}. Although the hypothesis of a shared antigenic target, expressed by nerves and podocytes, has been put forward, no such target has yet been identified.

In this study, we describe CNTN1 as a novel shared target antigen involved in both CIDP and MN. We also present the incidence of combined diseases in a national cohort of CNTN1-positive CIDP patients.

RESULTS

1. Characteristics of patients positive for anti-CNTN1 antibodies with neuro-renal syndrome or isolated CIDP

Since the discovery of the involvement of antibodies directed against the CNTN1 protein in the pathophysiology of inflammatory demyelinating diseases of the peripheral nervous system, 1500 sera from patients with acute polyneuritis or CIDP have been tested in the French reference center of neurologic autoimmune diseases in Marseille. Among these patients, 11 were positive for anti-CNTN1 antibodies (Figure 1) and all were diagnosed with CIDP. The mean age at the onset of CIDP disease was 67 years (range: 39 to 85 years), and the male-to-female ratio was 10: one patient had tobacco exposure, and two were treated for hypertension. All patients had a typical and severe form of CIDP. The median titer of anti-CNTN1 was 1/4450 (1/500 to 1/52700), and the anti-CNTN1 antibody subclasses revealed a predominance of IgG4 subclass (Figure 2). At the time of neurological investigation, five of these 11 patients had kidney injury consisting of proteinuria with or without nephrotic syndrome (Figure 1, Table 1). All of them had a normal serum creatinine. The anti-CNTN1 titers (tested by ELISA assay) appeared higher, although not significantly, in the patients with both CIDP and MN diseases than in those with isolated disease (1/20450 to 1/2300; $p < 0.064$) (Fig S1). Sera anti-PLA2R1 antibodies were negative. The kidney biopsies revealed a stage 1 MN in four patients and a stage 2 MN in one patient. Immunofluorescence showed diffuse granular subepithelial deposits of IgG, mainly composed of IgG4. None of them had a PLA2R1 or TSHD7A positive staining.

Among the five patients with both CIDP and MN three died, as opposed to none of the patients with isolated CIDP disease. Of the three patients who died, one death was due to inhalation pneumonia 3 months after the treatment had ended, one from cardiac arrest resulting from severe dysautonomia, and one from gastric hemorrhage. All three had an uncontrolled neurological disease despite treatment. Of the three patients who did not die, partial remission was achieved for two and complete remission for one.

2. Glomerular expression of CNTN1

We performed immunoblotting of reduced and non-reduced protein extracts from normal human glomeruli with a commercial anti-CNTN1 antibody (Figure 3). Reduced or non-reduced human brain extracts were used as a positive control for CNTN1 blotting.

On both the brain extracts and non-reduced human glomerular extracts a distinctive band of approximately 125-135 kDa was detected which corresponds to the expected molecular weight of CNTN1. The band obtained on the extract of reduced human glomeruli was slightly positive. The presence of CNTN1 was unambiguously confirmed by the identification of seven unique peptides in the above-mentioned immunoblotting band using mass spectrometry.

In order to validate our immunoblotting approach, we performed immunoblotting of non-reduced normal human glomeruli protein extracts with a commercial anti-PLA2R1 antibody. As described by Beck et al, we found a strongly positive band corresponding to the expected size of PLA2R1 in non-reduced extracts of human glomeruli.

These results show that CNTN1 is expressed in normal kidney glomeruli.

RT-PCR analysis on a podocyte cell line showed that CNTN1 is expressed by podocytes (data not shown).

We also performed immunoblotting for NF155, NF186 and Caspr1 and found that all three were expressed in human glomerular extracts (date not shown).

3. CNTN1 staining on patient kidney biopsies

We then performed immunofluorescence staining for CNTN1, IgG4, PLA2R1 and THSD7A on deparaffinized kidney biopsy sections from patients presenting both MN and CIDP, from a patient with anti-PLA2R1-MN, from a patient with membranous lupus nephritis (MLN class V), and from a healthy donor (Figure 4 panel A).

CNTN1 was revealed in the glomeruli from patients presenting both MN and CIDP, but not in the other samples. Neither PLA2R1 nor THSD7A were found in patients with MN and CIDP. As expected, both IgG4 deposits and PLA2R1 were detected in the glomeruli of the patient with anti-PLA2R1-MN but not CNTN1. Neither CNTN1 nor PLA2R1 were found in the kidney specimen from the MLN class V patient. However, IgG4 deposits were detected. No significant signal was detected using the secondary antibody alone (data not shown).

Confocal microscopic analysis was performed on the four patients with an available deparaffinized kidney-tissue specimen. CNTN1 and IgG4 were colocalized in the glomerular basement membrane (Figure 4 Panel B). As previously described in Beck and al (4), a similar confocal microscopy analysis of a kidney biopsy from a patient with anti-PLA2R1-MN revealed colocalization of PLA2R1 and IgG4 in the glomeruli (data not shown).

These results demonstrate that IgG4 deposits colocalize with CNTN1 in glomeruli from patients with concomitant MN and CIDP, in the same manner as PLA2R1 and IgG4 deposits are colocalized in glomeruli from PLA2R1-MN

We then performed immunohistochemistry staining for CNTN1 on deparaffinized kidney biopsies from patients presenting both MN and CIDP, from a patient with anti-PLA2R1-MN, from a patient with membranous lupus nephritis (MLN class V), from a healthy donor, and brain tissue (Figure 5). As expected, CNTN1 was strongly expressed in the brain. As in immunofluorescence, CNTN1 was revealed in the glomeruli from patients presenting both MN and CIDP, but not in the other samples. No significant signal was detected using only the secondary antibody (data not shown).

4. IgG4 eluted from MN kidney biopsies are reactive against CNTN1 and peripheral nerve

Frozen kidney sections were available for three of the five patients with both anti-CNTN1 positive CIDP and MN. IgG was eluted from these sections using an acidic buffer. Eluted IgG were then tested for CNTN1 reactivity by ELISA. Results of IgG eluted from the three patients were compared to IgG eluted from an anti-PLA2R-positive MN, and to the last washes obtained just before elution (Fig 6 panel A).

As represented in Figure 6 panel A, eluted IgG from kidney biopsies of CIDP-MN patients recognized CNTN1, but not the IgG eluted from an anti-PLA2R1-MN kidney biopsy, or washes before the acidic elution. For all patients, IgG4 was the main representative eluted subclass of IgG (Fig 6 panel B). This is corroborated by the observation that the IgG deposits from the patients' kidneys were IgG4 raised against CNTN1 (as illustrated in Fig 4 Panel B). Because antibodies may recognize distinct forms of CNTN1 expressed in the kidney or neurons, we investigated whether the IgG eluted from kidney were able to target neuronal

CNTN1. Fig 6 Panel C shows that eluted IgG from patient 2 were able to bind paranodal tissue and colocalized with CNTN1, thus demonstrating that these antibodies target CNTN1 in both tissues.

DISCUSSION

This study is the first to provide evidence of a shared antigenic target – CNTN1 – in patients presenting with combined CIDP and MN. Furthermore, we show a high prevalence of kidney involvement in anti-CNTN1 positive CIDP patients.

The description of anti-CNTN1 antibodies in CIDP confirms the autoimmune origin of this pathology. These antibodies are pathogenic through a function-blocking effect and can block the interaction between CNTN1 and NF155^{10,12}. The role of CNTN1 in kidney physiopathology has never been studied. We, and other authors, have previously reported cases of patients with anti-CNTN1-related CIPD associated with glomerular proteinuria, and hypothesized about a common potential immunopathologic mechanism. To the best of our knowledge, only one study to date has focused on the expression of CNTN1 in human kidney and showed a low mRNA expression¹⁹.

As for PLA2R1, we demonstrated that CNTN1 is expressed in human glomeruli as an endogenous integral membrane protein of the podocyte but is not revealed in normal conditions as shown by the negative immunofluorescence and immunochemistry in our healthy donor. Reciprocally, PLA2R1 was detectable by immunofluorescence in the podocytes of PLA2R1 patients but not from healthy donors or CNTN1 patients. This suggests that *in situ* formation of immune complexes is initiated by the binding of circulating antibodies to the antigens, and that a common pathophysiology is induced by an immune response elicited after antigen unmasking. However, the unmasking mechanism remains to be determined. Alternatively, the antibody-mediated clustering of CNTN1 may favor its detection on the podocyte membrane or may increase CNTN1 surface expression. *In vivo* and *in vitro* models would be useful to further understand the pathophysiology of kidney involvement in the presence of anti-CNTN1 antibodies.

CNTN1 is a GPI-anchored cell membrane protein expressed on the extracellular side. Its 3D structure has been partially elucidated by both nuclear magnetic resonance and X-ray studies.

The secondary structure of CNTN1 has six disulfide bonds in its extracellular Ig-like domain. The antibody used for the western blot experiments was raised against the whole native protein expressed in the sf-21 cell line. As it has been suggested for the PLA2R1 protein⁴, structural modifications resulting from reduction of these disulfide bonds could explain the lower reactivity observed with the commercial antibody in western blots under reduced conditions. The strong expression of CNTN1 in the brain probably explains why the antibody gave a strong signal in human brain extracts despite reduction. As for PLA2R1, CNTN1 was not detected by immunoblot in rodent kidney (data not shown), limiting the use of these models to study the pathogenic function of anti-CNTN1 in kidney.

Anti-CNTN1 and anti-PLA2R1 antibodies predominantly comprise the IgG4 subclass and IgG4 deposits were colocalized with CNTN1 or PLA2R1, respectively, in kidney biopsy.

While IgG4 is the dominant subclass in patients with long-standing anti-PLA2R1-MN (electron microscopy showing stages III/IV), the predominant subclass in early MN (stages I/II by electron microscopy) is IgG1 (20). IgG4-PLA2R1-MN is considered as a renal autoimmune disease. After the formation of immune complexes, complement can be activated by three different pathways²¹. These processes trigger cell damage which is responsible for proteinuria and end-stage renal failure. In our study, the main subclass of anti-CNTN1 was IgG4, but the IgG4-CNTN1 complex was associated with early-stage MN. This suggests that the onset of the neurological disease precedes MN. The kidney-renal function was normal in all patients on diagnosis of MN/CIDP while in other types of MN, about 30% of the patients have chronic renal failure at time of the diagnosis²².

CNTN1 is the first common antigen target involved in MN associated with CIDP as we demonstrated that the eluted IgG from the kidney bind CNTN1 at the paranode. As mentioned above, the prevalence of CNTN1-reactive CIDP patients is low in Europe and Asia^{13,15}. Interestingly though, the kidney target is common: half (5/11) of the characterized anti-CNTN1 positive CIDP patients had MN, in contrast with the general CIDP population. Importantly, the CNTN1-reactive patients with MN and CIDP had a higher rate of mortality: three out of the five patients with MN and CIDP died, as opposed to none of those affected with CIDP only. Furthermore, the neurological score of severity was similar for all patients whereas three patients affected by both CIDP and MN had an uncontrolled neurological disease and two of them died of swallowing disorders. This suggests that kidney involvement is more predictive of mortality than the neurological severity score. However, treatment was

less intense in the MN group, which could also explain the difference in the mortality rates. Thus, we recommend that CIDP patients be systematically screened for proteinuria, especially those with CNTN1 related CIDP.

The incidence of anti-CNTN1 IgG4 has not been evaluated in isolated MN to date. We did not find anti-CNTN1 antibodies in the nine patients with anti-PLA2R1 and anti-TSHD7A negative isolated MN. This deserves to be explored in larger MN cohorts in the future.

To conclude, CNTN1 is the first discovered target involved in combined MN and anti-CNTN1-related CIDP. It appears to be a common association in CNTN1 positive patients and a relevant marker of disease severity. Further studies are needed to elucidate the pathological significance of these autoantibodies and their role in MN.

METHODS

Serum and kidney-tissue samples

Sera from patients with CNTN1-positive CIDP patients were collected and analyzed. We also collected and analyzed sera from patients with anti-PLAR1 and anti-TSHD71 negative MN. Written informed consent was obtained from each participant. The study was approved by the Ethics Committee of Montpellier University Hospital (IRB-MTP-2020-01-20200339) and of the Assistance Publique des Hôpitaux de Marseille (PADS19-365). Paraffin-embedded kidney tissue sections and frozen tissue sections were collected from the same patients.

MN was diagnosed by renal biopsy in patients who lacked the features suggestive of secondary MN, such as antinuclear antibodies or hepatitis B antibodies in the serum.

Normal glomeruli were obtained from a kidney that was unsuitable for transplantation because of vascular issues. This sample was exempt from the requirement of informed consent according to the institutional review board's approved use of organs and tissues from deceased donors for research.

Serum anti-CNTN1 screening

Reactivity to CNTN1 and determination of the isotype was performed by flow cytometry as described previously¹⁴ and confirm by ELISA (see below).

Brain protein extraction

The whole procedure of brain protein extraction was performed on ice using two surgical pieces of peri-tumoral brain white matter provided by the pathology department. Briefly, the pieces of brain were rinsed several times in cold phosphate buffered saline (PBS) to remove surface contaminants. They were then cut into small pieces with a sterile razor blade and shredded with sterile pliers. The resulting paste was homogenized in an RIPA buffer (Tris 50mM, pH 7.4, NaCl 150mM, NP-40 1%, sodium deoxycholate 0.5%, SDS 0.1%) containing protease inhibitors (ThermoFisher Scientific) in a glass Potter-Elvehjem homogenizer. After a 30-minute incubation on ice, insoluble materials were removed by centrifugation at 10 000 g for 10 min.

Human glomerular extract preparation

Pieces of renal cortex were excised after decapsulation. The glomeruli were prepared according to the method described by ²³. Briefly, cortical tissue was cut into small pieces with a sterile razor blade and shredded with sterile pliers. The resulting paste was rinsed in cold PBS at pH 7.4 and crushed through a 300 µm-inox sieve. At this step, the glomeruli and tubular fragments were not retained. The glomeruli were then isolated and washed twice with cold PBS on a 100 µm-nylon filter (Millipore). The purity of the preparation was checked under a phase-contrast microscope. After centrifugation at 300 g for 5 min, the pellet was resuspended in an equal volume of RIPA buffer containing protease inhibitors (ThermoFisher Scientific). The glomeruli were then manually ground ten times in a glass Potter-Elvehjem homogenizer. Glomerular proteins were extracted on ice for 20 min, with intermittent shaking. The insoluble fraction was removed by centrifugation at 14 000 g for 10 min at 4°C.

Immunoblotting

Protein concentration of both human glomerular extracts and brain extracts were determined using the bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific). For western blot analysis, 20 µg of total proteins in Laemmli buffer (Tris 70mM, pH 6.8, glycerol 10%,

SDS 1%, bromophenol blue 0.005%, Bio-Rad) with or without 2-mercaptoethanol (355mM, Sigma) as a reducing agent were loaded per lane, separated on a 4-15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes using the Transblot Turbo system (Bio-Rad). The membranes were saturated with tris-buffered saline-tween (TBS-T) (Tris 20mM, pH 7.2, NaCl 150mM, Tween 20 0.05%) containing bovine serum albumin (BSA) 2%, at room temperature (RT) for 1 hr to block unspecific binding, and were then incubated with primary antibodies in blocking buffer at 4°C overnight. The primary antibodies used were goat anti-CNTN1 antibody (R&D systems; AF904, 1:400), rabbit anti-PLA2R1 polyclonal IgG (Sigma-Aldrich; HPA012657, 1:1000), anti-NF155 (Eurogenetec; generated against peptide SQKRQQASFPGDRPR; 1:500), anti-NF186 (Cell Signaling Technology; 15034S; 1:500) and anti-Caspr1 (Gift from Dr. Laurence Goutebroze; 1:500). After three washes with TBS-T for 15 min, the membranes were incubated for 1 hr at RT with the proper horseradish peroxidase (HRP)-conjugated secondary antibody (anti-goat 1:10000; Sigma-Aldrich and anti-rabbit 1:2000; Abcam) in blocking buffer. After another three washes with TBS-T for 15 min, the protein bands were detected using HRP substrate (Millipore) emitting chemiluminescence recorded with a ChemiDoc Imaging system (Bio-Rad).

Immunofluorescence studies

The biopsy sections were deparaffinized by three successive baths in Neo-Clear® (Sigma) for 5 min each. The tissue was then rehydrated by submerging the slides for 10 min twice in 100%, 90%, 70%, 50% ethanol successively, and finally in water (5 min). To retrieve the antigen, the sections were immersed in an EDTA buffer at pH 8.5 (Sigma-Aldrich) at 95°C twice for 30 min. After cooling, the sections were rinsed in water, equilibrated in PBS then saturated in PBS-BSA 1mg/ml-Triton X100 0.1% at RT for 1 hr to block unspecific binding. The tissues were then incubated at 4°C with primary antibodies in blocking buffer overnight. The primary antibodies used were goat anti-CNTN1 (1:500), Alexa Fluor® 488-conjugated mouse anti-PLA2R1 (clone 12-6-5, 1:250, Novus Biologicals), mouse anti-THSD7A (clone CL3778, 1:200, Sigma-Aldrich) and rabbit anti-human IgG4 (clone RM120, 1:500, Novus Biologicals). Following three washes in PBS-Triton X100 0.1% for 5 min, the slides were incubated for 1 hr at RT with proper Alexa Fluor®-conjugated affinity purified donkey secondary antibodies from Jackson ImmunoResearch (1:500) in blocking buffer. The sections were washed once with PBS-Triton X100 0.1% for 5 min, twice in PBS for 5 min and briefly

in water before being dried and mounted in Mowiol 4–88 (Tris 0.1 M, pH 8.5, Mowiol 4–88 10%, glycerol 25%). The prepared slides were analyzed using an inverted microscope Axiovert 200 M driven by Axiovision software (Carl Zeiss). Colocalization of staining was checked using an inverted confocal laser scanning microscope Leica SP8. Images were processed using ImageJ software.

Immunohistochemistry

High sensitivity and specificity immunohistochemistry was performed using a 3rd generation polymer detection system – Polink-2 Plus HRP Anti-GOAT DAB Detection Kit (GBI Labs) – according to the manufacturer's instructions. Briefly, the sections were deparaffinized, rehydrated and subjected to antigen retrieval as described previously after blocking endogenous peroxidases. The sections were then incubated at 4°C with the primary antibody in blocking buffer overnight. The primary antibody used was goat anti-CNTN1 (1:500). Following three washes in PBS-Triton X100 0.1% for 2 min, the slides were incubated for 30 min at RT with goat antibody enhancer. After another three washes in PBS-Triton X100 0.1% for 2 min, the slides were incubated for 30 min at RT with polymer-HRP for goat. Diaminobenzidine was used as the chromogen. Finally, the sections were mounted in Mowiol as described previously. The prepared slides were analyzed using an upright microscope (Olympus BX43).

qRT-PCR analysis

Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions and summarized in Supplementary methods.

Mass spectrometry analysis:

Briefly, proteins from SDS-PAGE gel electrophoresis were digested with trypsin. The recovered peptides were separated onto a reverse phase column, detected on a Qexactive HFX, Thermofisher mass spectrometer and identified using the Maxquant software (version 1.6.10.43).

IgG elution from kidney-tissue specimens

IgG was eluted from a pool of frozen remnant cores of human anti-PLA2R1 positive MN samples (n=5); and from three independent concomitant MN + CIDP samples. Briefly, frozen remnant cores of human non-fixed biopsy specimens were sliced into 20- μ m sections and pooled in a 1.5 mL microtube (according to pathological and clinical diagnosis) to obtain sufficient eluted IgG for analysis. After three washes in PBS, the remaining paste was centrifugated for 2 min at 12500 rpm to eliminate residual OCT compounds, blood and external contaminants. The last wash was kept as a negative control.

50 μ l of elution buffer, pH 2.8 (Thermofisher Scientific), was incubated with each sample for 5 min and collected after centrifugation. The eluate was neutralized with 5 μ L of neutralization buffer (Tris 1M pH 8.5). A second and a third eluate resulting from the same procedure was obtained and then neutralized.

The reactivity of the eluted IgG was tested by ELISA as described in (24). Briefly, polystyrene microwells were coated with 25 ng of human recombinant CNTN1 (Sino Biological Inc) in PBS at 4°C overnight. The plates were then blocked with 0.5% casein sodium in PBS and 0.05% Tween-20 at RT for 1 hr and incubated with biopsy eluate diluted 1:10, 1:20, 1:50, 1:100, 1:500, 1:1000 and 1:5000 in blocking solution, at RT for 1 hr. HRP-conjugated donkey antibody to human IgG (dilution, 1:2000; Jackson ImmunoResearch), or IgG subclasses (dilution, 1:1000; Thermofisher Scientific) was added, and the plates were developed with SIGMAFAST OPD tablets (Sigma-Aldrich). The plates were washed between each incubation with PBS, and subsequently read at 495 nm (ELx800, Biotek). Positive results were defined as those showing optical density (OD) values above the OD mean values of healthy controls plus three standard deviations.

The specificity of eluted IgG from anti-PLA2R1 positive MN biopsy specimens was confirmed by indirect immunofluorescence on PLA2R1-expressing cells (Euroimmun). 20 μ L of 1:10 or 1:100 diluted eluate was incubated with slide-adhesive PLA2R1-expressing or non-expressing cells for 30 min at RT. The slides were then washed in PBS-Tween 1% for 5 min. Then, 25 μ L of fluorescein-labelled anti-human immunoglobulin was incubated with each reaction field for 30 min in the dark. After incubation, the slides were washed in PBS-Tween

for 5 min. 10 μ L of glycerol/PBS was applied on each reaction field and a cover glass was added. The prepared slides were analyzed using an inverted microscope Axiovert 200 M driven by Axiovision software (Carl Zeiss).

Eluted IgG were also tested on teased sciatic nerve fibers from male C57BL/6J mice as previously described (20). Briefly, the teased fibers were permeabilized by immersion in -20°C acetone for 10 min, blocked at RT for 1 hr with PBS containing 5% fish skin gelatin and 0.1% Triton X-100 and incubated overnight at 4°C with eluted IgG (1:200) and a goat antiserum against CNTN1 (1:2000). The slides were then washed several times and revealed with Alexa-conjugated secondary antibodies (1:500; Jackson ImmunoResearch).

DISCLOSURE

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AUTHOR CONTRIBUTION

MLQ, NB, CL, AB and JD designed the study, NB, CL, JD, AB did the experiments, AB , MLQ, JD, NB, CL, , MT wrote the manuscript, ED, VP, AC, IS, MCM, HP, NM, GT, LF, CR participated to the study, collected data from patients and biopsies and revised the manuscript.

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Figures, Legends:

Figure 1: Flow chart

Figure 2: Diagnosis of anti-CNTN1 and IgG subclasses by flow cytometry using HEK transfected with human CNTN1

Reactivity to CNTN1 and antibody isotype were determined by flow cytometry using HEK transfected with human CNTN1 and represented as mean fluorescence intensity (MFI).

Figure 3: CNTN1 is expressed in human glomeruli detected by Immunoblot

Immunoblot of reduced (+) or non-reduced (-) human brain extract or normal human glomerular extract with an anti-CNTN1 or an anti-PLA2R1 antibody. Molecular weights are shown on the left in kDa. The arrow shows the band of interest.

Ab: antibody; CNTN1: contactin 1; 2-ME: b-mercaptoethanol; HBE: human brain extract; MW: molecular weight; PLA2R1: phospholipase A2 receptor

Fig 4: Panel A Immunofluorescence staining for IgG4, CNTN1, PLA2R1 and TSHD7A on deparaffinized kidney biopsy sections from patients presenting both MN and CIDP, from a patient with anti-PLA2R1-MN, from a patient with membranous lupus nephritis (LN class V), and from a healthy donor. No significant signal was detected using the secondary antibody alone.

Figure 4: Panel B: CNTN1 and IgG4 deposits are colocalized in glomeruli from four patients with MN associated with CIDP.

Each column represents a single patient. A: Immunofluorescence staining for IgG4, CNTN1, PLA2R1 and TSHD7A on deparaffinized kidney biopsy sections from patients presenting both MN and CIDP, from a patient with anti-PLA2R1-MN, from a patient with membranous lupus nephritis (LN class V), and from a healthy donor

B: CNTN1 (green) and IgG4 (red) staining and their colocalization (merge) were examined by confocal microscopy. Scale bar: 20 μ m.

CIDP: chronic inflammatory demyelinating polyneuropathy; CNTN1: contactin 1; IgG4: Immunoglobulin G of subclass 4; MN: primary membranous nephropathy

Figure 5: Immunochemistry staining for CNTN1 on deparaffinized sections. Panels A and B: kidney biopsy sections from patients presenting both MN and CIDP (patient 1). Panel C: kidney biopsy section from a patient with anti-PLA2R1-MN. Panel D: kidney biopsy section from a patient with membranous lupus nephritis (LN class V). Panel E: kidney biopsy section from a healthy donor. Panel F: Brain section.

Figure 6: IgG Eluted from kidney biopsies are raised against CNTN1.

A: Serial dilutions of eluted IgG from kidney biopsies of CNTN1 positive patients (Patients 2, 3 and 5) or PLA2R1 positive patients (PLA2R1) were tested by ELISA against CNTN1. As control, the last wash before elution was tested (wash). Only the eluate from CNTN1 positive biopsies reacted toward CNTN1.

B: The subclass of eluted IgG was determined by ELISA against CNTN1. IgG4 appeared as the predominant isotype. Asterisks denotes the significant reactivities.

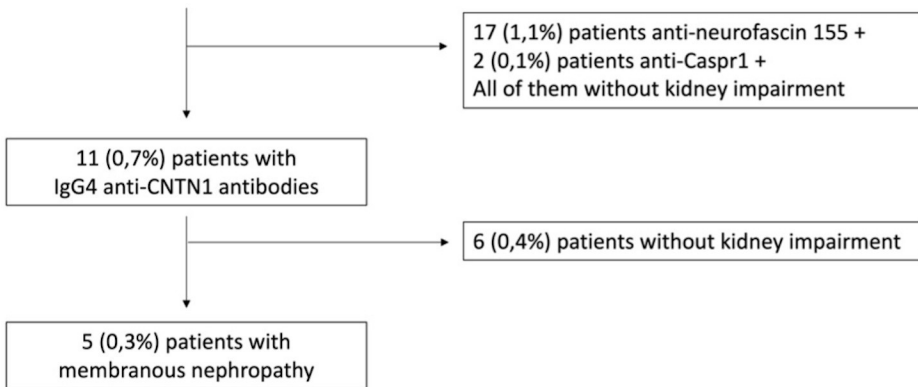
C: Mouse teased sciatic nerve fibers were incubated with eluted IgG from CNTN1 or PLA2R patients' biopsies (green) and immunostained with antibodies against CNTN1 (red). Eluted IgG from CNTN1 positive biopsies deposited at paranodes. Scale bar: 10 μ m.

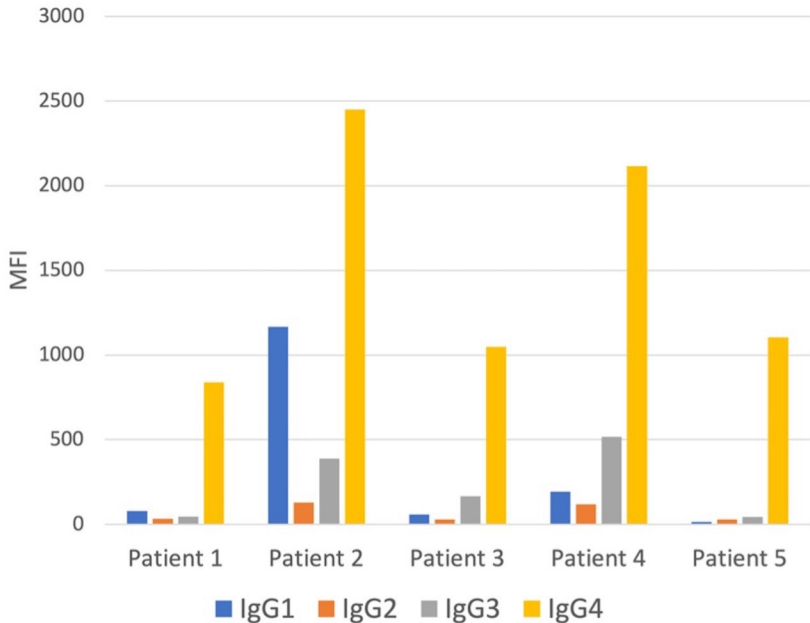
CNTN1: contactin 1. OD: optic density. Pat: patient. PLA2R : phospholipase A2 receptor.

1500 patients with chronic
inflammatory demyelinating
polyradiculoneuropathy

from 08/2016 to 03/2020

Tested for the anti-neurofascin 155, anti-neurofascin 186 and the anti-contactin 1 antibodies





H Brain Extract

Glomerular extract

Glomerular extract

2-ME

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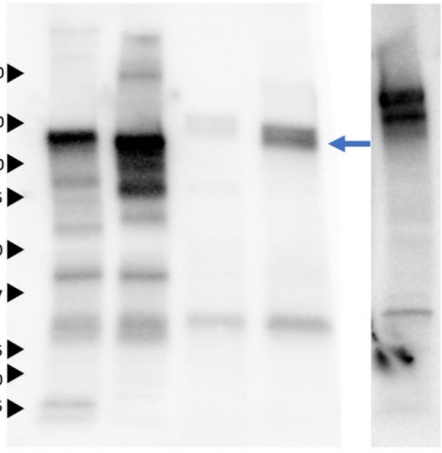
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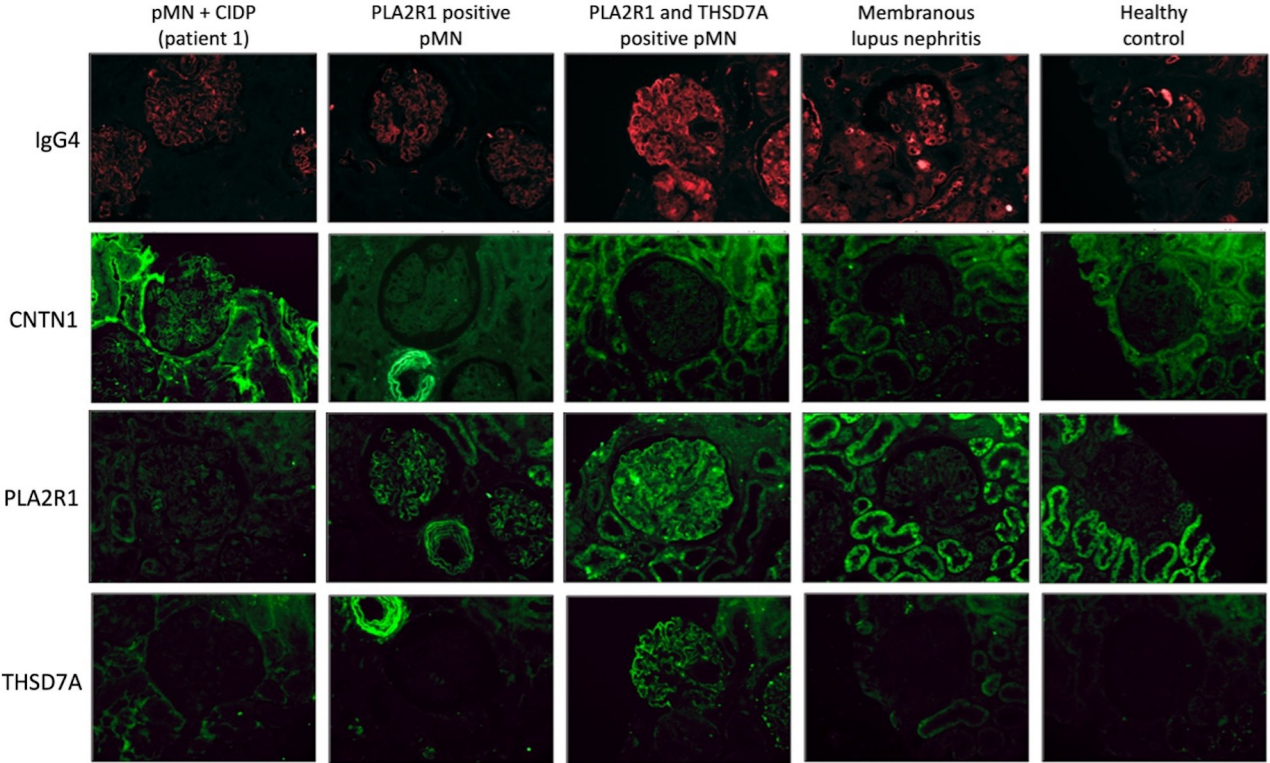
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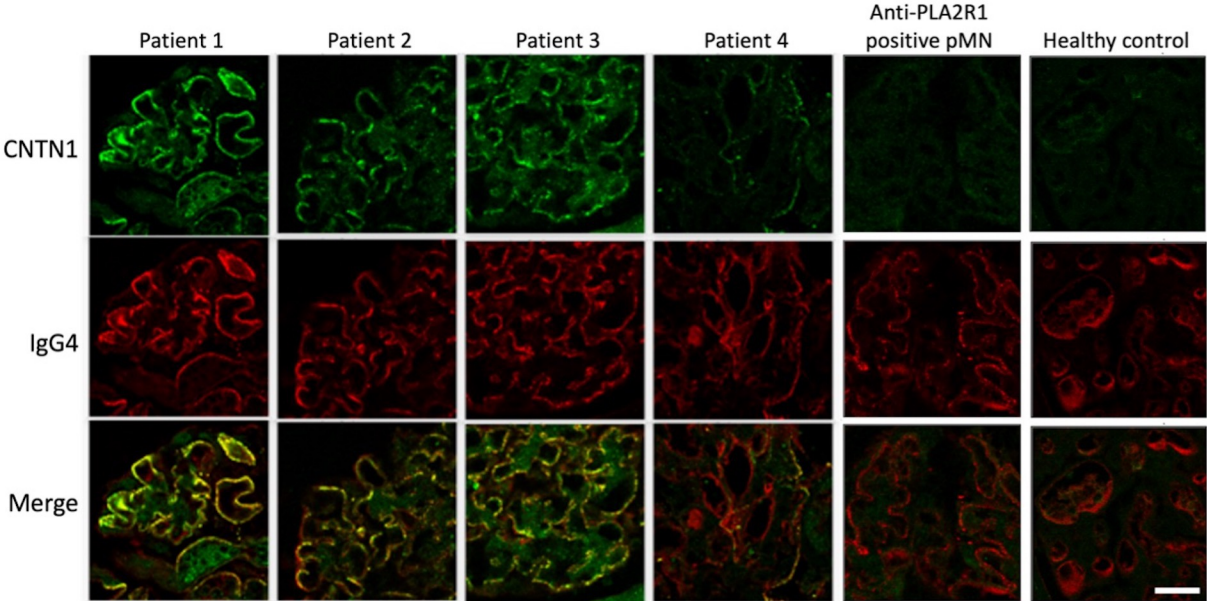
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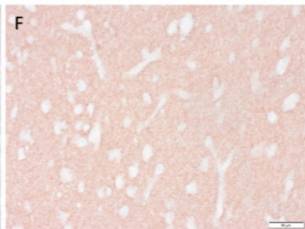
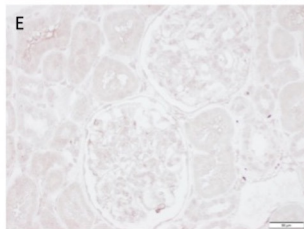
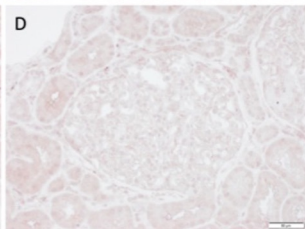
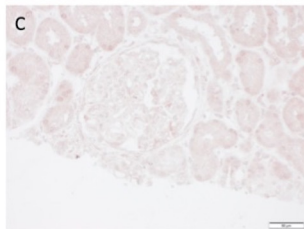
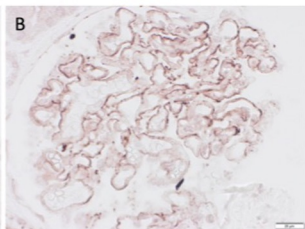
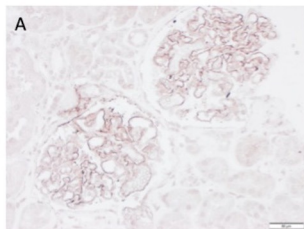


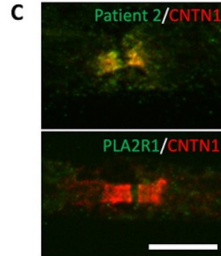
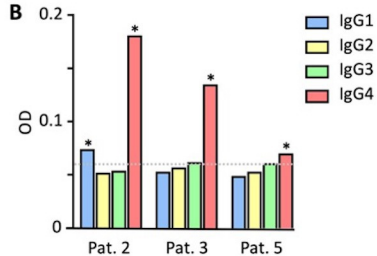
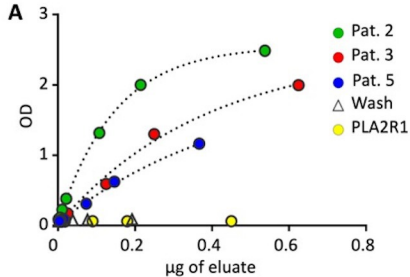
CNTN1

PLA2R1







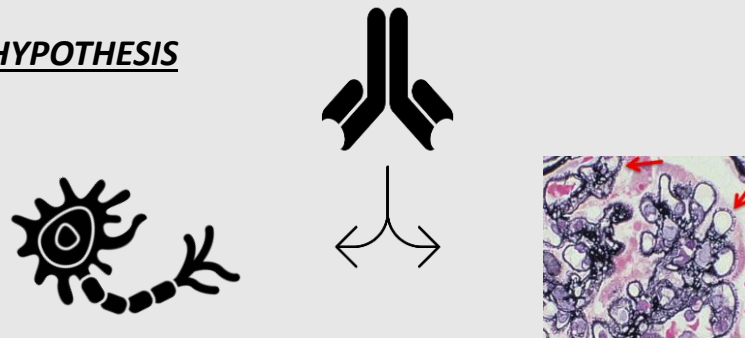


Contactin-1 is a novel target antigen in membranous nephropathy associated with chronic inflammatory demyelinating polyneuropathy.

MN can be associated with chronic inflammatory demyelinating polyneuropathy (CIDP). A common antigenic target has yet to be identified

CNTN1 as a shared antigen in both diseases?

HYPOTHESIS

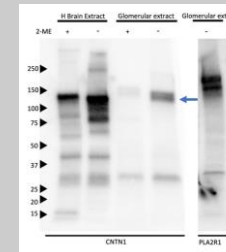


We investigated kidney biopsies from five patients positive for anti-contactin 1 (CNTN1) IgG4 antibodies and presenting combined MN and CIDP and use control MN biopsies.

We screened the presence of contactin in normal glomerulus, eluted IgG from biopsy sections against CNTN1 and nerve tissue.

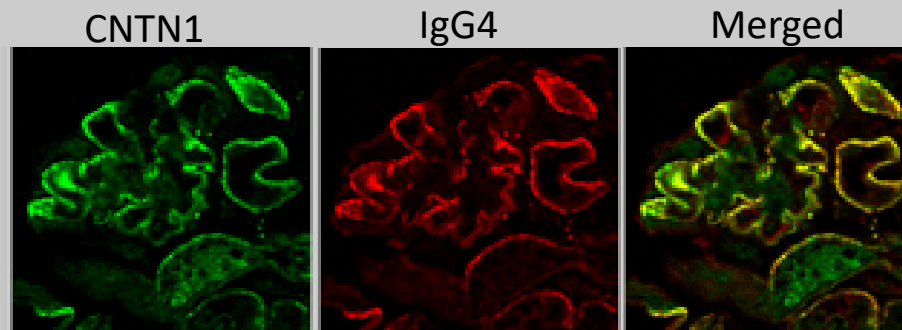
RESULTS

Western blot revealed CNTN1 expression in normal kidney glomeruli.

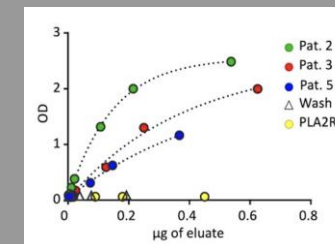


50% (5/11) of patients with anti-CNTN1 positive CIDP have a MN.

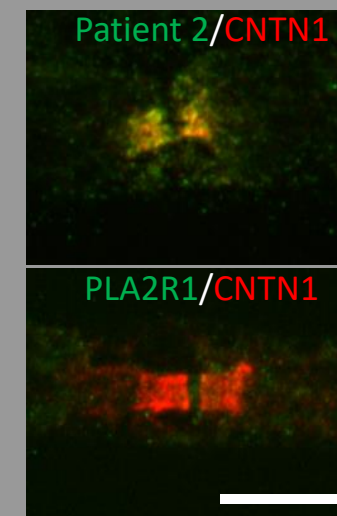
Confocal microscopic analysis showed the presence and colocalization of CNTN1 and IgG4 on the glomerular basement membrane of our patients.



CNTN1 colocalizes with IgG4 in kidney biopsies of those patients



The eluted IgG from CNTN1 positive biopsy sections bind the CNTN1



Eluted IgG from kidney biopsies bind to Ranvier's node

CONCLUSION

Contactin-1 = 1st discovered target involved in combined MN and anti-CNTN1-related CIDP