Clinical Relevance of Domain-Specific Phospholipase A2 Receptor 1 Antibody Levels in Patients with Membranous Nephropathy

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ABSTRACT

Background Antibodies against phospholipase A2 receptor 1 (PLA2R1) are found in 80% of patients with membranous nephropathy, and previous studies described three autoantibody-targeted PLA2R1 epitope regions. Although anti-PLA2R1 antibody levels are closely associated with treatment response and disease prognosis, the clinical role of epitope regions targeted by autoantibodies is unclear.

Methods In a prospective cohort of 150 patients with newly diagnosed PLA2R1-associated membranous nephropathy, we investigated the clinical role of epitope-recognition patterns and domain-specific PLA2R1 antibody levels by western blot and ELISA.

Results We identified a fourth epitope region in the CTLD8 domain of PLA2R1, which was recognized by anti-PLA2R1 antibodies in 24 (16.0%) patients. In all study patients, anti-PLA2R1 antibodies bound both the N-terminal (CysR-FnII-CTLD1) region and the C-terminal (CTLD7-CTLD8) region of PLA2R1 at study enrollment. The total anti-PLA2R1 antibody levels of patients determined detection of domain-specific PLA2R1 antibodies, and thereby epitope-recognition patterns. A remission of proteinuria occurred in 133 (89%) patients and was not dependent on the domain-recognition profiles. A newly developed ELISA showed that domain-specific PLA2R1 antibody levels targeting CysR, CTLD1, and CTLD7 strongly correlate with the total anti-PLA2R1 antibody level (Spearman’s rho, 0.95, 0.64, and 0.40; P<0.001, P<0.001, and P=0.002, respectively) but do not predict disease outcome independently of total anti-PLA2R1 antibody levels.

Conclusions All patients with PLA2R1-associated membranous nephropathy recognize at least two epitope regions in the N- and C-terminals of PLA2R1 at diagnosis, contradicting the hypothesis that PLA2R1 “epitope spreading” determines the prognosis of membranous nephropathy. Total anti-PLA2R1 antibody levels, but not the epitope-recognition profiles at the time of diagnosis, are relevant for the clinical outcome of patients with this disease.


Membranous nephropathy (MN) is an autoimmune disease caused by binding of circulating autoantibodies to antigens expressed on the podocyte surface. These autoantibodies are directed against phospholipase A2 receptor 1 (PLA2R1) or thrombospondin type 1 domain containing 7A (THSD7A) in 80% and 2%–3% of patients, respectively.1,2 Measurement of PLA2R1-antibody (PLA2R1-ab) and THSD7A-ab levels in patient serum have proven to
be a major advancement in the diagnosis and treatment of MN. Total PLA2R1-ab levels reflect disease activity and are associated with treatment response, remission, or relapse of proteinuria, and recurrence of disease after kidney transplantation.

The PLA2R1 consists of an N-terminal cysteine-rich domain (CysR), a fibronectin type II (FnII) domain, and eight consecutive C-type lectin-like domains (CTLD1–CTLD8). The immune dominant epitope region has previously been proposed to be located in the N-terminal CysR-FnII-CTLD1 region of the protein and was later suggested to be located in the CysR domain. Later, it was shown that in addition to the CysR domain, two additional epitope-containing regions are present in the CTLD1 and CTLD7 domains. On the basis of the latter study, it was suggested that the number of targeted PLA2R1 epitope regions may determine disease severity and prognosis. A follow-up study proposed that patients had a decreased remission rate if at the time of inclusion in the study PLA2R1-ab were directed against more than one PLA2R1 epitope region. This has led to the hypothesis of PLA2R1 “epitope spreading”; on the basis of this hypothesis, patients whose PLA2R1-ab at the time of study inclusion solely recognize the CysR domain have a better prognosis than patients whose PLA2R1-ab have “spread” toward recognition of the CTLD1 and/or CTLD7 domains.

Considering the potential clinical effect of the PLA2R1 “epitope spreading” hypothesis, we aimed to investigate the phenomenon using a well characterized prospective cohort of 150 patients with newly diagnosed PLA2R1-associated MN.

**METHODS**

**Study Cohort**

Sera from 150 consecutive patients with newly diagnosed PLA2R1-associated MN were studied. Some clinical characteristics of patients from this prospective cohort have been reported earlier. In this study we included the first 150 consecutive, PLA2R1-ab–positive patients recruited in the study (between 2010 and 2014), in order to have an appropriately long clinical follow-up observation period. All sera were collected within 6 months of renal biopsy and diagnosis of MN. Patients treated with immunosuppressive agents before study start were excluded from the study. At the time of study inclusion, 141 (94%) of the 150 patients were receiving a treatment with ACE-inhibitors or angiotensin receptor blockers; in eight of the remaining nine patients, treatment was initiated during the follow-up. After inclusion in the study, treatment decisions were made by the treating physicians on the basis of patient characteristics (i.e., proteinuria, nephrotic syndrome, renal function, etc.). The measurement of domain-specific PLA2R1-ab was not a prespecified analysis at the time of study start, because at the time of study initiation the respective pathogenic PLA2R1 epitopes were not known. All patient sera were characterized by ELISA (Euroimmun, Lübeck, Germany) to assign the total PLA2R1-ab level, as well as by an indirect immunofluorescence test (IFT; Euroimmun, Lübeck, Germany) and western blot. All 150 patients were positive for PLA2R1-ab in the IFT and western blot analysis, whereas 136 (90.7%) were positive in the ELISA (positivity defined as >20 U/ml, according to the manufacturer).

**Cloning and Expression of PLA2R1 Deletion Constructs**

PLA2R1 deletion constructs were designed as previously described. As a first step, the wild-type human PLA2R1 cDNA (GenBank NM007366) was cloned into the pGEM-Teasy vector (Promega, Madison, WI). The PLA2R1 deletion constructs, to which an HA-tag was fused to the C terminus of the coding sequence, were then generated by PCR and cloned into the pLPCX expression vector (#631511; Clontech Laboratories Inc.). Details of the PLA2R1 deletion constructs are presented in Supplemental Table 1. Details of the recombinant protein expression in HEK293 cells are given in the Supplemental Material.

**Cloning and Expression of Soluble PLA2R1 Domains**

Three additional soluble PLA2R1 domain constructs (sCysR-CTLD1, sCTLD1–CTLD2, and sCTLD2–CTLD6) were generated (Supplemental Table 2). The soluble constructs were amplified by PCR, further adding an N-terminal CD8 leader sequence (MALPVTALLLPLALLLHAARP) and a C-terminal HA-tag. The constructs were then directly cloned into the pcDNA3.1(+) mammalian expression vector (#V79020; Thermo Fisher Scientific). Recombinant soluble proteins were expressed in HEK293 cells as described in the Supplemental Material. Cell supernatants, containing the secreted soluble proteins, were collected and stored at –20°C.

**Western Blot Analysis**

Successful expression of the full-length PLA2R1, and deletion and soluble constructs, in transfected HEK293 cells was confirmed by western blot analysis detecting the HA-tag, with subsequent optimization of the loading amounts. Details are
provided in the Supplemental Material. All western blot analyses using human sera as primary antibodies were performed as previously reported under nonreducing conditions, unless otherwise stated.\(^5\) Patient sera were diluted 1:100 in 0.5% (wt/vol) milk in PBS-T and HRP-conjugated mouse anti-human IgG (#9040–05, 1:24,000 dilution in 3% [wt/vol] milk in PBS-T [blocking buffer]; Southern Biotech) was used as a detection antibody. A control cohort of 35 individuals was analyzed at a 1:100 serum dilution on the deletion and soluble constructs (six healthy individuals, six patients with FSGS, seven patients with minimal change disease, seven patients with membranoproliferative glomerulonephritis [MPGN], and nine patients with IgA nephropathy). A selected subgroup of 27 individuals from the control cohort was further analyzed at a 1:25 serum dilution on the soluble constructs (six healthy donors, five patients with FSGS, six patients with IgA nephritis, five patients with minimal change disease, and five patients with MPGN). In order to exclude unspecific binding of human IgG to the constructs, serum from one healthy donor was analyzed undiluted on the soluble constructs.

### Cloning, Expression, and Purification of PLA\(_2\)R1 Domain Fc Fusion Proteins

To generate stable, high-quality PLA\(_2\)R1 protein domains for use in ELISA,\(^4\)–\(^6\) the PLA\(_2\)R1 domains containing epitopes recognized by human PLA\(_2\)R1-ab (CysR/FnII, CTLD1, CTLD7, and CTLD8) were fused N-terminal to the rabbit antibody Fc (rFc) unit (Supplemental Material). The CysR domain was joined with the FnII domain taking the known crystal structure of the PLA\(_2\)R1 homolog uPARAP into consideration,\(^17\) which revealed a close association of these two domains. With the CysR/FnII construct we aimed to enhance protein folding and avoid the presence of a potential hydrophobic patch on the isolated CysR domain. Codon-optimized synthetic DNA (Twist Biosciences; Supplemental Table 3) was cloned into the pcSE2.5 vector (kindly provided by Dr. Thomas Schirrmann, Braunschweig),\(^14\) such that the PLA\(_2\)R1 domains were followed by an rFc tag. As a negative control, a llama nanobody rFc fusion protein was used (control rFc). Recombinant fusion proteins were expressed in HEK-6e cells and the secreted proteins were purified over protein A sepharose (#17096303; GE Life Sciences) according to the manufacturer’s protocol, concentrated to 1–2 mg/ml by ultrafiltration (MWCO: 10 kD), buffer exchanged to Dulbecco’s phosphate-buffered saline (DPBS; Gibco by Life Technologies), and stored at 4°C. The fusion proteins were stable for several months at 4°C. Successful expression and purification of the rFc fusion proteins was confirmed by Coomassie-stained SDS-PAGE (Supplemental Material).

### PLA\(_2\)R1 Domain-Specific ELISA

Purified PLA\(_2\)R1 domain rFc fusion proteins were used to establish novel ELISAs. rFc fusion proteins (PLA\(_2\)R1 domains as well as the negative control) were used to coat microtiter plates (#82.1581; Sarstedt) overnight at 4°C. All subsequent incubation steps were performed at room temperature. The plates were blocked using blocking buffer 2 (4% [wt/vol] milk in DPBS supplemented with 0.05% Tween-20) for 1.5 hours. Human sera were diluted in blocking buffer 2 (Supplemental Material) and applied for 2 hours. The amount of bound human IgG was detected by incubation with HRP-conjugated mouse anti-human IgG (#9040–05, 1:10,000 dilution in blocking buffer 2; Southern Biotech) or a mixture of mouse anti-human IgG3 Hinge-HRP and mouse anti-human IgG4 Fc-HRP (#9210–05 and #9200–05, 1:2500 for IgG3 and 1:5000 for IgG4; SouthernBiotech) for 1 hour. For the individual PLA\(_2\)R1 domain-specific ELISAs, a specific set of highly positive index sera was used to generate calibration curves. Linearity was demonstrated by serial dilution experiments. The coefficients of variation of each PLA\(_2\)R1 domain-specific ELISA were determined using four samples per PLA\(_2\)R1 domain-specific ELISA. The samples were measured 16-fold in one set (intra-assay) or three-fold in ten sets (interassay) (Supplemental Table 4). Further experimental details are given in the Supplemental Material. One patient in the cohort could not be analyzed by ELISA due to lack of sufficient amounts of serum. Also, a control cohort of 50 individuals was used in the analysis (nine healthy individuals, ten patients with FSGS, ten patients with minimal change disease, ten patients with MPGN, and 11 patients with IgA nephropathy).

### Statistical Analyses

Descriptive analyses of continuous data are presented as median and first to third quartile unless stated otherwise. For categoric data, absolute counts and percentages are reported. Mann–Whitney \(U\) and Kruskal–Wallis tests were used for group-wise comparisons of continuous and ordinal variables, whereas Fisher’s exact tests were used for group-wise comparisons of categoric variables. Remission of proteinuria was defined as proteinuria of <3.5 g/24 hours and at least 50% reduction from the time of study inclusion. Complete remission of proteinuria was defined as proteinuria <0.5 g/24 hours. Cox regression analyses were applied to assess the effects of total PLA\(_2\)R1-ab levels and domain-specific antibody levels on the clinical outcome defined as remission of proteinuria. The effect of domain-specific antibody levels was first investigated in a univariate model and, in the case of a statistically significant result, the analysis was performed after adjustment for total PLA\(_2\)R1-ab levels. The effect of total PLA\(_2\)R1-ab levels was adjusted for sex, proteinuria, serum creatinine, and immunosuppressive treatment. Results of Cox regression analyses are presented as hazard ratios (HRs) with corresponding 95% confidence intervals (95% CIs) and \(P\) values. Statistical significance was defined as \(P<0.05\). All statistical analyses were performed using SPSS software, version 21.

**RESULTS**

### Expression of Membrane-Bound PLA\(_2\)R1 Deletion Constructs

We first characterized the PLA\(_2\)R1 domain regions recognized by PLA\(_2\)R1-ab in patients with MN. For this, PLA\(_2\)R1 deletion...
constructs were generated, in which progressively complete structural domains were deleted, starting from the most N-terminal CysR domain (Figure 1A). In order to maximize the comparability to previous studies, the construct design was identical to previously published constructs. The constructs were shown to be glycosylated and epitope recognition was conformationally sensitive and specific to patients with PLA2R1-associated MN (Supplemental Figure 1). Sera from the control cohort \( n = 35 \) failed to recognize any of the constructs under nonreducing conditions.

### Epitope Regions Targeted by PLA2R1-Ab from Patient Sera

The initial western blot analysis of sera from 150 patients with newly diagnosed PLA2R1-associated MN was performed at a serum dilution of 1:100, identical to the previously published approach, but using anti-human total IgG instead of anti-human IgG4 as detection antibody. This led to the identification of four distinct PLA2R1-ab epitope binding patterns (Table 1): (1) epitope only in the CysR domain (Figure 1B); (2) CTLD1 was the most C-terminal recognized domain (Figure 1C); (3) CTLD7 was the most C-terminal recognized domain (Figure 1D); and (4) CTLD8 was the most C-terminal recognized domain (Figure 1E). Noteworthily, the number of patients recognizing the CTLD7 domain was significantly higher than the previously reported 46%, because in our analysis 83% of patients had PLA2R1-ab recognizing the C-terminal region (CTLD7 and/or CTLD8 domains) of human PLA2R1.

On the basis of the results from the initial western blot analysis, we investigated the association of clinical characteristics of patients with the observed epitope-recognition patterns. The comparison of patients exhibiting PLA2R1-ab directed exclusively against the N-terminal region with patients showing N- and C-terminal recognition revealed striking differences in the PLA2R1-ab levels. Patient sera with exclusive N-terminal PLA2R1 recognition (patterns 1 and 2) had a median PLA2R1-ab level of 19 U/ml (first to third quartile, 9–67 U/ml) as measured by ELISA, whereas sera with additional C-terminal epitope recognition (patterns 3 and 4) showed a median PLA2R1-ab level of 175 U/ml (first to third quartile, 86–325 U/ml) \( (P < 0.001; \text{Supplemental Table 5, Table 1}) \). In addition, patients with C-terminal epitope recognition had higher levels of proteinuria at baseline marked with an asterisk. (B) Only the CysR-CTLD8 full-length PLA2R1 protein is recognized (pattern 1). (C) CTLD1 was the most C-terminal recognized domain resulting in positivity for the CysR-CTLD8, FnII-CTLD8, and CTLD1-CTLD8 deletion constructs (pattern 2). (D) CTLD7 was the most C-terminal recognized domain giving a signal in all constructs down to CTLD7-CTLD8, but failing to react with CTLD8 (pattern 3). (E) CTLD8 was the most C-terminal recognized domain such that all PLA2R1 deletion constructs were detectable (pattern 4).
(P<0.001), received significantly more often immunosuppressive treatment (P<0.001), and had less frequent spontaneous remission of proteinuria (P<0.001). The same findings were made when patients were grouped according to the four epitope-recognition patterns (Supplemental Tables 6 and 7), resulting in a progressive increase of PLA2R1-ab levels, proteinuria, more frequent use of immunosuppression, and less frequent spontaneous remission from patients with CysR-epitope (pattern 1), to patients with additional CTLD1-epitope (pattern 2), CTLD7-epitope (pattern 3), and CTLD8-epitope (pattern 4) recognition. Patients with spontaneous remission had significantly lower total PLA2R1-ab levels at baseline, compared with patients with no spontaneous remission of proteinuria (median 46 U/ml versus 185 U/ml, P<0.001). In all groups, the most common first line immunosuppressive treatments used were calcineurin inhibitors and alkylating agents; however, in 42% of patients a second line immunosuppressive treatment was used during follow-up.

Characterization of N-Terminal Domains by Western Blot
The experimental setup of the initial western blot screening did not allow us to characterize the epitope patterns in detail. Specifically, it was unclear whether patients with recognition patterns 3 and 4 were able to recognize the N-terminally located CysR and/or CTLD1 domains. In order to better characterize the N-terminal epitope region, the soluble PLA2R1 domain constructs sCysR-CTLD1, sCTLD1-2, and sCTLD2-6 were generated. The expression of the soluble constructs was confirmed using anti-HA antibodies, and sera from the control cohort (n=35) did not show any specific binding to the constructs (Supplemental Figure 2).

Notably, all 150 sera (100%) recognized an epitope in the sCysR-CTLD1 construct, confirming that PLA2R1-abs from all patients exhibit epitope recognition in the N-terminal region of PLA2R1 (Figure 2). Moreover, 78 (52.0%) sera recognized an additional epitope within the sCTLD1-CTLD2 construct (Figure 2A). Considering that none of the sera showed a detectable signal for the CTLD2-CTLD6 construct, these 78 sera most probably recognized an epitope in the CTLD1 domain. Noteworthy, the presence of a CTLD1 epitope was not correlated to the presence or absence of epitopes in CTLD7 and/or CTLD8 (P=0.59 and P=0.65, respectively; Supplemental Table 8).

PLA2R1 Domain-Recognition Profiles Are Influenced by the PLA2R1-Ab Level
As presented above, patient sera with exclusive N-terminal PLA2R1 recognition had an almost ten-fold lower PLA2R1-ab level compared with patient sera with additional C-terminal epitope recognition (Table 1). Therefore, we next investigated whether the initial western blot results on the epitope-recognition patterns might be related to the total PLA2R1-ab level. For this purpose, serial dilutions of 1:100, 1:1000, and 1:10,000 of four CTLD8-ab–positive sera were used as primary antibodies in western blot analysis. All remaining experimental conditions

Table 1. Summary of the initial western blot screening on deletion constructs

<table>
<thead>
<tr>
<th>Recognition Pattern</th>
<th>Most C-Terminal Recognized Construct</th>
<th>Domains Recognized by Patient Seraa</th>
<th>Number of Antibody-Positive Patients</th>
<th>Total PLA2R1-Ab Level (Median, First to Third Quartile, U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CysR-CTLD8</td>
<td>CysR</td>
<td>19 (12.7%)</td>
<td>19 (9–67)</td>
</tr>
<tr>
<td>2</td>
<td>CTLD1–8</td>
<td>CTLD1 (possibly CysR)</td>
<td>7 (4.7%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CTLD7–8</td>
<td>CTLD7 (possibly CysR and/or CTLD1)</td>
<td>100 (66.7%)</td>
<td>175 (86–325)</td>
</tr>
<tr>
<td>4</td>
<td>CTLD8</td>
<td>CTLD8 (possibly CysR, CTLD1, and/or CTLD7)</td>
<td>24 (16.0%)</td>
<td></td>
</tr>
</tbody>
</table>

*The experimental setup of the initial western blot screening revealed the most C-terminal domain recognized by each patient serum (i.e., CTLD8 in patients with recognition pattern 4). However, in an individual patient it did not allow the identification of additionally targeted domains located more N-terminally to the recognized C-terminal domain (i.e., patients with recognition pattern 4 might express antibodies to CysR, CTLD1, and/or CTLD7, in addition to the CTLD8 antibody).
were identical, including the exposure time during chemiluminescent development. Interestingly, when sera were diluted 1:1000, recognition up to the CTLD7–8 construct was obtained; however, none of the sera showed reactivity with CTLD8 (Figure 3A). At 1:10,000 dilution, only the binding to full-length CysR-CTLD8 construct could be detected, whereas reactivity for the truncated constructs was lost. The median PLA2R1-ab level of these four sera was 378 U/ml when measured by ELISA. In order to reflect the increased dilutions of the western blot analyses (from 1:100 to 1:1000 and 1:10,000, respectively), the sera were diluted 1:10 and 1:100 and PLA2R1-ab levels measured by ELISA, resulting in median PLA2R1-ab levels of 32 and 3 U/ml, respectively. Serial serum dilution experiments of patient sera with domain-recognition pattern 2 led to a weaker CTLD1 recognition at 1:10,000 dilution, whereas in patient sera with domain-recognition pattern 1 positivity was completely lost (Supplemental Figure 3).

Figure 3. Influence of serum dilution on the recognition pattern of PLA2R1 epitope regions. (A) Representative western blots of serum dilution experiments (total n=4). When a lower serum concentration was used (serum dilutions 1:1000 and 1:10,000, instead of 1:100), the number of recognized PLA2R1 deletion constructs was reduced. An asterisk marks the last recognized deletion construct. (B–D) Vice versa, when a higher serum concentration was used (1:25 instead of 1:100), CTLD7–8 and occasionally the CTLD8 deletion constructs were detected (bottom), which were not detected in the initial western blot screening (top). Three representative examples with different recognition patterns are shown: (B and C) Recognition of sCTLD1-CTLD2 and CTLD7-CTLD8. (D) Recognition of CTLD7-CTLD8 and CTLD8.
Next, we re-evaluated the binding characteristics of the 26 sera with exclusive N-terminal recognition profile (patterns 1 and 2; Figure 1, Supplemental Table 5, Table 1) on the CTLD7–8 and CTLD8 deletion constructs under nonreducing conditions, with increased detection sensitivity. For this, sera were used at dilutions of 1:100 and 1:25. Moreover, the experimental approach was chosen such that each epitope did not occur more often than in two lanes on the western blot membrane, instead of in up to ten lanes in the initial western blot screening. Under these conditions, all 26 analyzed sera recognized the CTLD7–8 deletion construct (Figure 3, B–D), and six sera also gave a positive signal for the CTLD8 deletion construct (Figure 3D).

Taken together, the complete cohort of 150 PLA2R1-ab–positive patients with MN already exhibit autoantibodies directed against the N-terminal and C-terminal regions of PLA2R1 at the time of renal biopsy and diagnosis of MN. As a consequence, at diagnosis the recognition of PLA2R1 by patient antibodies always targets at least two protein domains, which clearly contradicts the predicted phenomenon of PLA2R1 "epitope spreading" during the clinical follow-up of patients.11,12

Measurement of N-Terminal–Specific PLA2R1-Ab Level
In order to investigate the levels of PLA2R1-ab targeting specific PLA2R1 domains and to analyze their clinical role for treatment response and prognosis, domain-specific ELISAs were developed (Supplemental Figure 4). The CysR/FnII-specific ELISA identified 145 (97.3%) of 149 analyzed patients with MN to have CysR/FnII-specific antibodies (Figure 4A, Supplemental Figure 5, Supplemental Table 4). For one patient no serum was available for ELISA measurements. All four patients for whom the CysR/FnII-specific ELISA level did not pass the cut-off value were tested negative for total PLA2R1-abs by ELISA (median total PLA2R1-ab level, 4 U/ml). In those patients, PLA2R1-abs were only detectable by IFT and western blot. Hence, the CysR/FnII-ab level in those four patients might be below the detection limit of the CysR/FnII-specific ELISA. Noteworthily, in ten additional patients in our study cohort, who had a total PLA2R1-ab level <20 U/ml (Supplemental Material), the CysR/FnII-specific ELISA gave a clearly positive signal. Overall, the CysR/FnII-specific antibody level was highly correlated with the total PLA2R1-ab level (Spearman’s rho, 0.95; P<0.001; Table 2).
The CTLD1-specific ELISA revealed that the CTLD1 domain was recognized by 78 (52.3%) PLA2R1-ab–positive patients (Figure 4B). CTLD1-ab–positive patients had significantly higher total PLA2R1-ab levels compared with CTLD1-ab–negative patients (Table 2) and their CTLD1-ab level significantly correlated with the total PLA2R1-ab level (Spearman’s rho, 0.64; P<0.001). The results of the CTLD1-specific ELISA are in accordance with the western blot results with eight outliers (5.3%); of these outliers, four were positive in ELISA and negative in western blot, whereas four were negative in ELISA but positive in western blot. CTLD1-abs were undetectable in two of 14 patients, who were tested negative for total PLA2R1-ab by commercial ELISA. In both patients, CTLD1-abs were also detectable by western blot.

**Measurement of C-Terminal–Specific PLA2R1-Ab Level**

The CTLD7- and CTLD8-specific ELISAs appeared to be less sensitive, which resulted in considerably lower numbers of 29 (19.5%) and four (2.7%) CTLD7-ab– and CTLD8-ab–positive patients, respectively (Supplemental Figure 6). We therefore chose to reanalyze these domain-specific antibodies using a mixture of IgG3- and IgG4-specific secondary antibodies, as previous studies showed that these two subclasses are present in 99% and 100% of PLA2R1-ab–positive patients with MN, respectively.18 Thereby, in our approach the bias induced by not detecting all IgG subclasses was reduced compared with ELISA setups where only IgG4 antibodies are measured.

This approach identified 57 (38.3%) and 15 (10.1%) CTLD7-ab– and CTLD8-ab–positive patients, respectively (Figure 4, C and D, Supplemental Figure 5, Supplemental Table 4, Table 2). Similar to the observation for CTLD1-ab–positive patients, the total PLA2R1-ab levels were significantly higher in the CTLD7-ab– and CTLD8-ab–positive patients compared with their negative counterparts (Table 2). In CTLD7-ab–positive patients the CTLD7-ab level significantly correlated with the total PLA2R1-ab levels (Spearman’s rho, 0.40; P=0.002). In CTLD8-ab–positive patients the correlation was not statistically significant; however, only 15 (10.1%) patients had detectable CTLD8-abs by ELISA. None of the 14 patients, who were tested negative for total PLA2R1-ab by commercial ELISA, gave a positive signal in the CTLD7- or CTLD8-specific ELISA. Compared with our western blot analysis, which gave a positive signal for the CTLD7–8 deletion construct for all 150 analyzed sera, the ELISA showed a significantly lower sensitivity for detection of domain-specific antibodies, as previously also shown for total PLA2R1-abs.5 For CTLD8, 12 of 15 CTLD8-ab–positive patients were also positive in our western blot analysis. Twelve patients were tested positive for CTLD8-ab by western blot, but were negative in the ELISA.

Noteworthily, as shown in the western blot analyses (Supplemental Table 6), in the ELISA analyses we also found a statistically significant progressive increase of total PLA2R1-ab levels from patients with Cys-R-ab positivity, to patients with CTLD1-ab, CTLD7-ab, and CTLD8-ab positivity (Table 2, P<0.001). Moreover, we found that the ELISA antibody standard that is used to calibrate the commercial PLA2R1-ab ELISA recognizes both the N- and C-terminal domains of PLA2R1 (Supplemental Figure 7, Supplemental Material).

**Clinical Role of Domain-Specific PLA2R1-Ab Level**

PLA2R1-ab levels at diagnosis have been shown to predict remission of proteinuria in patients with MN.6 We first confirmed these findings in our cohort of 150 patients. Total PLA2R1-ab level was predictive for the clinical end point (defined as remission of proteinuria) with an HR of 0.87 per two-fold increase of PLA2R1-ab levels (95% CI, 0.79 to 0.95; P=0.003). After adjusting for the clinically relevant parameters of age, sex, proteinuria, serum creatinine, and immunosuppressive treatment, the total PLA2R1-ab level again was predictive for the clinical end point (HR, 0.87; 95% CI, 0.79 to 0.97; P<0.01). Next, we analyzed whether domain-specific antibody levels might also predict the clinical outcome in our cohort, defined as remission of proteinuria during a median prospective follow-up time of 54 months (first to third quartile, 36–66 months). In the univariate analysis only the

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**Table 2. Summary of the PLA2R1 domain-specific ELISA**

<table>
<thead>
<tr>
<th>PLA2R1 Domain</th>
<th>Number</th>
<th>Spearman’s Rho (P Value)</th>
<th>Total PLA2R1-Ab Level (Median, First to Third Quartile, U/ml)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CysR/FnII</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>145 (97.3%)</td>
<td>0.95 (P&lt;0.001)</td>
<td>158 (66–309)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>4 (2.7%)</td>
<td>—</td>
<td>4 (3–6)</td>
<td></td>
</tr>
<tr>
<td>CTLD1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>78 (52.3%)</td>
<td>0.64 (P&lt;0.001)</td>
<td>195 (104–351)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>71 (47.7%)</td>
<td>—</td>
<td>89 (43–189)</td>
<td></td>
</tr>
<tr>
<td>CTLD7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>57 (38.3%)</td>
<td>0.40 (P=0.002)</td>
<td>254 (175–454)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>92 (61.7%)</td>
<td>—</td>
<td>88 (41–184)</td>
<td></td>
</tr>
<tr>
<td>CTLD8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>15 (10.1%)</td>
<td>0.08 (P=0.79)</td>
<td>700 (263–969)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>134 (89.9%)</td>
<td>—</td>
<td>132 (58–260)</td>
<td></td>
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</table>

—, not applicable.
CysR-ab level showed a statistically significant association with the clinical outcome (HR, 1.00; 95% CI, 1.00 to 1.00; *P* = 0.04). After adjustment for total PLA2R1-ab level, this association was no longer statistically significant (HR, 1.00; 95% CI, 1.00 to 1.00; *P* = 0.64). No statistically significant association was found between CTLD1-ab, CTLD7-ab, or CTLD8-ab level and clinical outcome in the univariate analyses (Table 3). We also analyzed whether positivity for CTLD1-ab, CTLD7-ab, or CTLD8-ab was associated with the clinical outcome and again found no statistically significant correlation (Table 3).

**DISCUSSION**

MN is a very good example of how a better understanding of disease pathophysiology may lead to improved diagnosis and treatment of patients. The identification of two target antigens in MN was essential in order to adapt treatment more closely to the immunologic disease activity and individual patient needs. In this context, one important aspect relates to the question of whether autoantibodies in all patients with PLA2R1-associated MN target the same molecule region, or whether differences between patients exist, which might explain the diverse disease outcomes. Three important studies have shown that at least three PLA2R1 epitope regions are targeted by autoantibodies in MN, Furthermore, the hypothesis of PLA2R1 “epitope spreading” was proposed, suggesting two groups of patients: those with PLA2R1-ab recognizing only the CysR domain; and patients in whom the immune response “spreads” during clinical follow-up toward the C-terminal end of PLA2R1, and in whom PLA2R1-ab also recognize CTLD1 and CTLD7 epitopes. Patients in whom “epitope spreading” takes place are suggested to have a more severe form of disease and a worse prognosis.

The aim of our study was to characterize the clinical role of PLA2R1 epitope regions, recognized by PLA2R1-ab from patient sera. We investigated a very well characterized, prospectively observed patient cohort of 150 patients with newly diagnosed PLA2R1-associated MN. Moreover, in this prospective approach we chose to include only patients who had received no immunosuppressive therapy before study inclusion. In order to allow comparability to previously published studies, an identical construct design was used in the initial western blot analysis. In our cohort of 150 patients with newly diagnosed PLA2R1-associated MN, we found epitopes in the CysR, CTLD1, and CTLD7 domains, as previously described, but additionally identified the most C-terminal CTLD8 domain as a novel, fourth epitope region.

The results of our western blot analysis clearly demonstrate that the detected epitope-recognition profiles are dependent on the total PLA2R1-ab levels and the sensitivity of the chosen detection method. Serum dilution experiments showed that an increased serum dilution—which automatically corresponds to a lower titer in a patient—in the western blot analysis resulted in a decreased number of PLA2R1 domains being recognized. When higher serum concentration and a more sensitive methodologic approach were used, we were able to identify recognition of domains that were not recognized in the first screening round. As a consequence, the exact experimental conditions, especially the applied serum dilution, and also careful experimental performance, incubation times, and the type of secondary antibody used, strongly influence the results. We identified in every patient included in the study at least two epitope regions targeted by PLA2R1-ab at the time of renal biopsy. Moreover, taking into consideration the limitations in the sensitivity of techniques, we cannot rule out that patients who were negative in our western blot assay still exhibit very low PLA2R1-ab levels against other protein domains, which, however, are below the detection limit of the approach. It is important to note that in this study the epitope profiles were characterized on the domain level. Therefore, it is possible that an individual domain contains multiple epitopes, which can be differentially recognized in individual patients.

Our data led to the conclusion that in PLA2R1-associated MN either the phenomenon of “epitope spreading” can take place at the very early stages of disease development, long before the diagnosis of MN is made, or alternatively no “epitope spreading” occurs and a multispecific immune response is raised from the start. In order to answer this question, the analysis of biomaterial and clinical data acquired at the time of the actual disease onset, when the autoimmune process is triggered, would be needed. However, in both cases “epitope spreading” would not play a role in the treatment response or prognosis of disease, because at the time a treatment decision or prognosis prediction is made, the immune response in the patient already targets numerous epitopes. These findings are clinically highly relevant, because they demonstrate that targeting only one domain-specific antibody might not be the preferred treatment approach in these patients. Rather, a specific treatment considering all pathogenic PLA2R1 epitopes would be needed. Moreover, the domain-specific antibody levels did not provide additional prognostic value compared with total PLA2R1-ab levels, which were predictive for the clinical end point in our cohort, as has been shown before. Interestingly, 31 (20.7%) patients in our cohort had a spontaneous remission of proteinuria during follow-up, although they had antibodies directed against both the N- and

<table>
<thead>
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<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P Value</th>
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<tr>
<td>CysR-ab level</td>
<td>1.00</td>
<td>1.00 to 1.00</td>
<td>0.04</td>
</tr>
<tr>
<td>CTLD1-ab level</td>
<td>1.00</td>
<td>0.99 to 1.00</td>
<td>0.11</td>
</tr>
<tr>
<td>CTLD7-ab level</td>
<td>1.01</td>
<td>1.00 to 1.03</td>
<td>0.05</td>
</tr>
<tr>
<td>CTLD8-ab level</td>
<td>0.97</td>
<td>0.92 to 1.02</td>
<td>0.18</td>
</tr>
<tr>
<td>CTLD1-ab positivity</td>
<td>1.26</td>
<td>0.89 to 1.79</td>
<td>0.19</td>
</tr>
<tr>
<td>CTLD7-ab positivity</td>
<td>0.78</td>
<td>0.55 to 1.12</td>
<td>0.18</td>
</tr>
<tr>
<td>CTLD8-ab positivity</td>
<td>0.89</td>
<td>0.48 to 1.66</td>
<td>0.72</td>
</tr>
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</table>
C-terminal domains of PLA2R1. These 31 patients had significantly lower total PLA2R1-ab levels at baseline, again showing the relevance of total PLA2R1-ab levels in contrast to recognition of multiple epitopes on PLA2R1 concerning the clinical outcome of disease.

Another open question is whether the level of antibodies specific for a certain PLA2R1 epitope region, or the epitope-recognition patterns of the serum, could explain the diversity of clinical appearance observed in MN. To address this question, we developed a PLA2R1 domain-specific ELISA, which allowed us to quantify the titer of the individual PLA2R1 domain-specific antibody. The CysR- and CTLD1-specific ELISAs showed very good signal-to-noise ratios, in contrast to the CTLD7- and CTLD8-specific ELISAs, suggesting that the CysR and CTLD1 domains are preferentially recognized by the antibodies. This discrepancy might indicate a strong difference in the domain-specific antibody level, or affinity, or both, and is in line with the presented western blot serum dilution experiments, in which an increased serum dilution leads to a declining recognition of the C-terminal domains. Considering the recently published low-resolution Cryo-EM structure of soluble PLA2R1,19 it appears that the N-terminal region of PLA2R1 is significantly more accessible for antibody binding. This might reflect the increased immunogenicity compared with the more hidden C-terminal CTLD7–8 region.

In conclusion, we confirmed the finding that different PLA2R1 epitopes are targeted by autoantibodies in patients with MN and define a novel target domain in CTLD8. The PLA2R1 autoimmune response is polyclonal with a prevalence for recognition of the CysR domain, which might be explained by the better accessibility of the domain. Further, PLA2R1-1bs in all patients already recognize at least two epitope regions of the protein at the time of diagnosis. Both the epitope-recognition patterns and domain-specific antibody levels strongly correlated to total PLA2R1-ab levels. The information delivered by PLA2R1-ab levels was the key feature for prediction of clinical outcome, whereas the clinical informational value of domain-specific PLA2R1-1bs was limited. Overall, our data do not support the hypothesis of PLA2R1 “epitope spreading” as a prognostic and treatment marker in patients with MN.

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Dr. Hoxha and Dr. Stahl were responsible for the conception and design of the study. Dr. Zahner performed the cloning and expression of the PLA2R1 deletion and soluble constructs. Dr. Hoxha, Dr. Zahner, and Dr. Reinhard analyzed and interpreted the western blot data. Dr. Reinhard, Dr. Menzel, and Dr. Koch-Nolte designed the rFc fusion proteins, which were cloned, expressed, and purified by Dr. Menzel. Dr. Reinhard developed and performed the PLA2R1 domain-specific ELISA. Dr. Hoxha and Dr. Reinhard analyzed and interpreted the ELISA data. Drafting and revising of the article were performed by Dr. Reinhard, Dr. Hoxha, and Dr. Stahl. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work.

DISCLOSURES

Dr. Hoxha reports grants from Deutsche Forschungsgemeinschaft, and grants from Else Kröner-Fresenius Stiftung, during the conduct of the study. Dr. Koch-Nolte reports grants from Deutsche Forschungsgemeinschaft, during the conduct of the study; and grants from Deutsche Forschungsgemeinschaft, and personal fees from MediGate, outside the submitted work; in addition, Dr. Koch-Nolte has a patent VHHC-containing heavy chain antibody pending. Dr. Stahl reports grants from Deutsche Forschungsgemeinschaft, during the conduct of the study. Dr. Zahner reports grants from Deutsche Forschungsgemeinschaft, during the conduct of the study.

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SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2019030273/-/DCSupplemental.

Supplemental Methods.
Supplemental Results.
Supplemental References.
Supplemental Figure 1. Quality control of recombinant PLA2R1 deletion constructs by western blot.
Supplemental Figure 2. Quality control of recombinant soluble PLA2R1 constructs by western blot.
Supplemental Figure 3. Influence of serum dilution on the recognition of N-terminal PLA2R1 domains.
Supplemental Figure 4. PLA2R1 domain rFc fusion proteins.
Supplemental Figure 5. Linearity of PLA2R1 domain-specific ELISA.
Supplemental Figure 6. Results of the ELISA screening for CTLD7-ab and CTLD8-ab detected with anti-human total IgG.
Supplemental Figure 7. Western blot using the Euroimmun standard as primary antibody.
Supplemental Table 1. Protein sequence information of the PLAb1 deletion constructs.
Supplemental Table 2. Protein sequence information of the soluble PLAb1 domain constructs.
Supplemental Table 3. Regions of the PLAb1 protein sequence fused to the rabbit Fc.
Supplemental Table 4. Intra- and interassay coefficients of variation for the total-IgG- and IgG3/IgG4-based ELISAs.

Supplemental Table 5. Clinical characteristics and follow-up of patients with exclusive N-terminal PLAb1 domain recognition (CysR+CTLD1) and patients with N- and C-terminal PLAb1 domain recognition (CTLD7+CTLD8) from the initial western blot screening.

Supplemental Table 6. Clinical characteristics and follow-up of patients depending on the epitope-recognition pattern in the initial western blot screening on the PLAb1 deletion constructs.

Supplemental Table 7. P values for all differences of clinical characteristics of patients depending on the epitope-recognition profile shown in Supplemental Table 6.

Supplemental Table 8. Correlation of CTLD1-ab positivity to the presence of epitopes in CTLD7 and CTLD8.

REFERENCES


See related editorial, “Refining Our Understanding of the PLA2R-Antibody Response in Primary Membranous Nephropathy: Looking Forward, Looking Back,” and article, “Detection of PLA2R Autoantibodies before the Diagnosis of Membranous Nephropathy” on pages 8–11 and 208–217, respectively.