The discovery in 2009 of the M-type phospholipase A2 receptor (PLA2R) as the primary target in membranous nephropathy (MN) greatly advanced basic and clinical research. MN now is considered a renal-limited autoimmune disease, with antibodies against PLA2R (aPLA2Rab) identified in 70-80 % of patients of various ethnic groups. Although the use of aPLA2Rab as a diagnostic and prognostic biomarker is now widely accepted, many questions related to the development of the auto-immune response, the role of IgG subclasses and antigenic epitopes, and the pathways to podocyte injury remain unresolved. PLA2R-associated MN most likely develops governed by factors such as genetic susceptibility, loss of tolerance, alterations in antigen expression with a role for environmental factors like air pollution, smoking, and infections. More detailed knowledge of genetic factors, the relevant B- and T-cell epitopes, and the mechanisms of podocyte injury is needed to identify patients at risk for disease progression and to develop optimized, targeted treatment strategies. In this review we highlight unresolved issues, addressing initiation of antibody formation, the timeline of antibody production, the role of IgG subclass, and the pathogenicity of the antibodies in concert to produce glomerular pathology and proteinuria.


KEYWORDS: glomerulonephritis; membranous nephropathy; nephrotic syndrome

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respect to ethnicity, and often included patients with long-standing disease, sometimes treated and in remission. It also is known that initially aPLA2Rab can be absent in the serum of patients with PLA2R-associated MN. Debiec and Ronco14 studied a cohort of 42 patients and identified 10 patients with positive staining of PLA2R antigen in immune complexes in the kidney biopsy specimen, with no measurable antibodies in serum. Subsequent studies showed that serum aPLA2Rab indeed can be absent at disease onset, and become measurable during follow-up evaluation.15,16 We hypothesized that the antibodies bind to the antigen in the kidney with high affinity, and only become detectable when binding sites in the kidney are saturated.15 Of note, and Ronco14 also described 3 patients with a high circulating level of aPLA2Rab who did not have detectable glomerular PLA2R. This phenomenon has received little attention.

The IFT technique only allows semiquantitative assessment of aPLA2Rab by reporting dilution steps. Better quantification of aPLA2Rab was possible with the development of enzyme-linked immunosorbent assays (ELISA). Some centers have used in-house-developed ELISAs, but its widespread use in clinical practice came with the introduction of a commercially available ELISA (Euroimun).17 This ELISA measures total aPLA2Rab IgG. In the development phase, patient serum was used as a calibrator to construct a standard curve that allowed quantitation in relative units (RU)/ml. When using the ELISA a cut-off value is used to define aPLA2Rab positivity. The literature is equivocal, with some studies using 14 RU/ml and other studies using 20 RU/ml as a cut-off value. Even with the lower cut-off value the ELISA technique is less sensitive than IFT or Western blot.13 In a recent study, the investigators advised using 2 RU/ml as a cut-off value and to perform confirmatory IFT in all patients with ELISA values greater than 2 and less than 20 RU/ml.18 Uncertainty regarding sensitivity of the methodology remains with an international evaluation of a large number of sera in a blinded controlled comparative study using the 3 methods described.

**Role of antibody measurement**

The presence of aPLA2Rab is highly specific for a diagnosis of MN. In a meta-analysis, specificity was calculated at 98% to 100%, although the investigators admitted to study limitations.4 In a recent study, Bobart et al.18 showed that all 132 patients who tested positive for aPLA2Rab had a primary diagnosis of MN in their native kidney biopsy specimen. Moreover, in patients with a baseline estimated glomerular filtration rate greater than 60 ml/min per 1.73 m² there were no findings that affected patient care or treatment. Therefore, the presence of aPLA2Rab may be considered sufficient for a diagnosis of MN.19

Qualitative assessment of aPLA2Rab during the disease course already showed that disappearance of antibodies preceded the onset of remission.10 The persistence of aPLA2Rab at the end of immunosuppressive therapy predicted an unfavorable outcome.8 These data suggest that immunologic remission could become the goal of therapy.

The quantitative analysis confirmed the temporal association between clearance of antibodies and the reduction of proteinuria. In patients treated with rituximab, a 50% reduction in the aPLA2Rab level preceded a similar reduction of proteinuria by approximately 10 months.9 Anti-aPLA2Rab levels also predicted response. High levels of antibodies were associated with a lower likelihood of remission.20 In multivariable analysis, using data from the Evaluate Rituximab Treatment for Idiopathic Membranous Nephropathy (GEMRITUX) cohort, a randomized trial comparing rituximab with conservative treatment, aPLA2Rab levels less than 275 RU/ml at baseline were associated with an increased likelihood of remission at 6 months. In rituximab-treated patients the probability at 24 months of achieving a partial or complete remission decreased from 80% in patients in the lowest tertile of aPLA2Rab to 30% in the highest tertile.7 A subsequent study confirmed the low efficacy of standard-dose rituximab in inducing immunologic remission in patients with high antibody titers.11 In these patients, cyclophosphamide or extended rituximab therapy likely are more effective.11,21 Monitoring antibody levels at baseline and at regular time intervals after the start of immunosuppressive therapy may pave the way toward personalized treatment strategies.

**Characteristics of anti-PLA2R antibodies**

Although the ELISA provides some useful information, the predictive accuracy for clinical outcome is limited. Therefore, investigators have questioned the added value of more detailed analysis of aPLA2Rab. Because MN is considered an IgG4-dominant disease, subclass-specific assays were introduced.6,17,22 One study suggested that the sensitivity for diagnosing MN was slightly better when using an IgG4 subclass-specific assay compared with total IgG (98.5% vs. 96.5%).15 Hofstra et al.6 studied 82 patients with PLA2R-associated MN, and subclass-specific aPLA2Rab was measured using in-house ELISAs. IgG4-aPLA2Rab levels were correlated significantly to total IgG aPLA2Rab, supporting IgG4 as the dominant subclass. Spontaneous remission as the outcome was associated significantly with tertiles of total IgG aPLA2Rab. In additional analyses, outcome only was associated significantly with the IgG4 subclass.6 Subsequent studies using IgG4 subclass-specific aPLA2Rab ELISAs confirmed the correlation between IgG4 aPLA2Rab levels and total IgG aPLA2Rab levels, although the variation remained high.17 In a retrospective study, IgG4 aPLA2Rab predicted spontaneous remission.24 In contrast, in another study from the same group, using a different patient population, there was no association between prognosis and levels of IgG4 antibodies directed at human PLA2R.24 The discrepancies between the studies likely are related to the heterogeneity between the studies with respect to patient characteristics, treatment, and the inclusion of aPLA2Rab-negative patients.

In their latter study, Seitz-Polaki et al.24 used not only human PLA2R, but also rabbit PLA2R and mouse PLA2R as antigen. The cross-reactivity of 53 sera from MN patients already known to be positive for human PLA2R was
investigated. All sera recognized rabbit PL2R and only 51% of sera recognized mouse PL2R. Interestingly, the patients who had antibodies that recognized mouse PL2R had a worse outcome. This study suggested that patient sera contained aPL2Rab directed at different epitopes. In subsequent studies, these investigators identified aPL2Rab with reactivity against different domains of PL2R protein, namely the cysteine-rich domain (CysR), C-type lectin domain (CTLD)1, and CTLD7.22,25 The CysR domain already has been defined as an epitope-containing domain by other groups,26,27 and is considered the immunodominant epitope. In the first study by Seitz-Polski et al.,22 which included 69 patients with aPL2Rab-associated MN, antibodies against CysR were present in all patients. In 23 patients (33%), antibodies only recognized CysR, 14 patients (20%) presented with antibodies reactive to CysR and CTLD1, and 32 patients (47%) were reactive to CysR, CTLD1, and CTLD7. In this small retrospective study, which included patients with non-nephrotic proteinuria, with a wide range of estimated glomerular filtration rates, and large heterogeneity in treatment, the patients with reactivity against 2 or 3 epitopes (so-called spreaders) were older and had more severe proteinuria. Of note, spreaders had higher levels of aPL2Rab against CysR, although no significant differences were observed in IgG aPL2Rab levels. During follow-up evaluation, poor prognosis was associated with epitope spreading, and in multivariable analysis both epitope spreading and antibody levels were associated independently with outcome. The investigators validated their findings using data of the GEMRITUX study.27 Treatment was started after an observation period of at least 6 months, and the study included nephrotic patients only. The analysis included 58 aPL2Rab-positive patients (measured using the Euroimmun assay). Of these patients, 29 received rituximab. In 34.5% of patients, antibodies reactive only to CysR were found (nonspreaders). In this cohort, and in contrast to the previous study, spreading was not associated with age, but clearly correlated with total IgG aPL2Rab titer. During follow-up evaluation, spreading was an independent predictor of outcome. The investigators suggested that spreading might be particularly relevant in patients with antibody levels greater than 50 RU/ml. The investigators warned against making early conclusions and pointed to the need for confirmation and validation, in view of the small number of patients, and the inclusion of treated and untreated patients.

WHAT TRIGGERS ANTI-PLA2R ANTIBODY PRODUCTION?

PLA2R-associated MN is a renal-limited autoimmune disease. We refer the reader to an immunology textbook for a detailed and extensive description of the normal immune response and the deviations leading to autoimmunity. Figure 1 illustrates the various pathways that may be involved in the development of any autoimmune disease, using PL2R-associated MN as an example. The development of autoreactive antibodies and thus of autoimmune disease can result from loss of central or peripheral tolerance, altered expression of the antigen (antigen modification, increased expression), and intermolecular epitope spreading (molecular mimicry). In the process of B-cell activation, several processes can be discerned, for example, immunoglobulin class switching, increased antibody production, affinity maturation (higher levels of more avid antibodies), and epitope spreading.

Genetic association with human leukocyte antigen class II genes

Most autoimmune diseases are associated with major histocompatibility complex genes.28 This is not unexpected because peptide fragments (epitopes) are presented to T cells in the context of the major histocompatibility complex molecule. In 1979, an association between human leukocyte antigen (HLA) DR3 and MN was reported.29 This was confirmed in subsequent studies based on serotyping, showing associations with HLA class I (B8, B18) and class II (DR 2, DR3, DR7).28 New advances came with the introduction of molecular genotyping, which refined the locus to DQA1.30 Most recently, genome-wide association studies were introduced. In the first genome-wide association study that included 556 patients of European ancestry, the highest association was between MN and single-nucleotide polymorphisms in the HLA-DQA1 region.31 This association was confirmed in another European cohort.32 This study disclosed additional associations with DRB1*03:01 (HLA-DR3) and HLA-DQA*05:01-HLA-DQB1*02:02 (HLA-DQ2). The association with HLA DQA1 also was confirmed in Asian populations.33-36 In the Han Chinese population a significant association was found not only with HLA-DRB1*03:01, but also with HLA-DRB1*15:01.35,36 In 1 study, HLA-DRB3*02:02, which shares a haplotype with HLA-DRB1*03:01, was considered a risk allele.36 Cui et al.35 studied the role of amino acid substitutions, by means of structural modeling, and pointed to certain amino acid positions in the peptide-binding pocket of the major histocompatibility complex-β chain, which may determine epitope specificity. Currently, the class II genetic restriction is best interpreted as controlling which peptides of PL2R are presented to T cells to provide the necessary T-cell help for high-affinity autoantibody production. Unfortunately, most studies have evaluated a limited number of patients, without sufficient fine-mapping of the HLA genes, and were unable to perform conditional analyses. Thus, the causal alleles remain unknown.

Loss of tolerance

It is not known if central tolerance is involved in preventing an autoreactive response to PL2R. The presence of PL2R in the thymus is debated. Peripheral tolerance could be the controlling mechanism in preventing an autoreactive response to PL2R, maintained by the low level of soluble PL2R that is reported to be present in the circulation of healthy individuals at 50 to 5000 ng/ml by Western blot.37 Our unpublished data suggest a much lower level of soluble PL2R at approximately 300 pg/ml by ELISA (PEC Brenchley, personal communication, April 5, 2019). Importantly, soluble PL2R may maintain peripheral tolerance by controlling the
function of regulatory T cells to suppress B cells with the potential to generate aPLA2Rab.

In a recent study, using blood samples collected regularly from US military personnel, the presence of aPLA2Rab in the circulation long before the onset of MN was documented (Joshi et al., J Am Soc Nephrol. 2018;29:P823). This observation might be explained by the natural presence of autoreactive B cells. Peripheral tolerance is largely dependent on the function of regulatory T cells (Tregs). Roccatello et al.38 studied changes in B- and T-cell phenotypes by flow cytometry in rituximab-treated patients. Rituximab induced B-cell depletion, which coincided with remission of proteinuria and an increase in Tregs. In 2 of the nonresponder patients, no increase in Tregs was observed. Rosenzwajg et al.39 showed that patients with active MN had a lower number of Tregs. Importantly, an increase in Tregs at day 8 after start of rituximab predicted remission after 3 to 6 months of follow-up evaluation. Two recent studies confirmed that Tregs are decreased in patients with active MN (Ramachandran et al., J Am Soc Nephrol. 2018;29:P823 and Cantarelli et al., J Am Soc Nephrol. 2018;29:P824), with a significant increase after immunosuppressive therapy (Ramachandran et al., J Am Soc Nephrol. 2018;29:P823).

**Activation of antigen-presenting cells: the role of the lung in MN**

Antigen-presenting cells (APCs) are critical in determining the immune response. They process antigen and present small peptide fragments to the T cell. The subsequent response, varying from T-cell anergy to T-cell activation, is dependent on the presence of co-stimulatory molecules on the surface of the APCs, a sign of APC activation. APC activation occurs through cytokines, produced in inflammatory states. In recent years, the lung has emerged as a potential contributor to allergies and autoimmune disease.40 The lung is a large surface area, exposed to the external world. Infection, smoking, and air pollution all cause oxidative stress and inflammation, and the resulting cytokines may spill over into the systemic circulation and exert distant effects. In addition, in the inflammatory milieu, APCs become activated. Indeed, the lung has been implicated in the development of autoimmune diseases such as type 1 diabetes, lupus nephritis, and rheumatoid arthritis.40

An interesting finding is the association between air pollution and MN that has been suggested.41,42 Xu et al.41 studied the incidence of glomerular disease over an 11-year period in China. They observed an increasing incidence in MN cases. There was a clear association between air pollution and MN. This particularly was related to the changes in fine particulate matter less than 2.5 μm. In another study, the association was confirmed.42 Clearly, the observational nature of these studies is an important limitation because there are many confounders that co-occur with air quality. Smoking could be another factor, also acting by inducing pulmonary differentiation of B cells into memory B cells or plasma cells and leave the germinal center. If regulatory T cells (Tregs) encounter their self-antigen on an APC, they secrete inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β, which inhibit surrounding autoreactive T cells.

**Figure 1 | Autoimmunity in membranous nephropathy.** We propose 2 potential pathways leading to the clonal expansion of B cells into plasma cells secreting antibodies against the M-type phospholipase A2 receptor (PLA2R) and generating memory B cells. First, PLA2R antigen binds to autoreactive B cells, is internalized and fragmented, and then presented to the T helper cells through the major histocompatibility complex (MHC) class II receptor. Stimulated T cells release cytokines feeding back to the B cells to stimulate division and differentiation to plasma cells and antibody production and memory B cells. These cytokine signals also can direct class switch recombination to distinct IgG subclasses resulting in IgG4 antibodies. Second, antigen-presenting cells (APCs) recognize PLA2R antigen or the microbial agent (via molecular mimicry), then process and present PLA2R/microbes fragments on MHC class II molecules on their surface. When APCs present PLA2R in the presence of co-stimulators, self-reactive T cells are activated rather than rendered tolerant. B cells are activated outside of follicles by the combination of antigen and T cells. After migration to germinal centers, B cells interact with T helper cells, leading to proliferation and survival. Some of the B cells undergo differentiation to either memory B cells or plasma cells and leave the germinal center. If regulatory T cells (Tregs) encounter their self-antigen on an APC, they secrete inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β, which inhibit surrounding autoreactive T cells.
inflammation. Indeed, smokers are at increased risk of developing autoimmune disease such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus. Smoking was associated with a more rapid decrease of estimated glomerular filtration rate in MN. However, an association between smoking and the development of MN has yet to be proven.

Another clue toward involvement of the lung is the association of MN with sarcoidosis. In this group of patients a high prevalence of PLA2R-related disease has been described.

Because of this indirect evidence the lung has been implicated in MN (Figure 2). Although intriguing, the role of the lung has not been firmly established thus far.

**Change in conformation or expression of PLA2R**

**Genetic association with the PLA2R gene.** In 2010, Liu et al. reported an association between single-nucleotide polymorphisms in the gene that encodes PLA2R and the development of MN. These findings were confirmed in genetic association studies in Korean and European cohorts. Genetic studies of PLA2R to date have lacked sufficient detail to identify how the genetic risk is translated through a pathologic mechanism to cause disease. It remains uncertain which polymorphism(s) is the culprit and how this affects the protein sequence/structure.

Genetic variants alone or in cooperation may affect the molecular conformation of the antigen exposing new conformational B-cell epitopes or allowing cryptic enzyme cleavage sites to become accessible, facilitating the processing of linear T-cell peptides. In this respect, some polymorphisms identified were in regions that recently were identified as B-cell epitope–containing domains. However, nothing is known of a link between single-nucleotide polymorphism markers and T-cell epitopes. Genetic variants, especially in the intronic regions, also may increase expression of the antigen, another possible initiator of disease. Some of these explanations are explored in greater detail in a global second genome-wide association study in MN involving 3782 cases to be reported in 2019 (Kiryluk, personal communication, January 21, 2019).

**Figure 2** | The lung as the primary insult in membranous nephropathy (MN): hypothetical role of pulmonary inflammation and infections. Air pollution can create oxidative stress in the airways, causing airway epithelium cells and alveolar macrophages to express proinflammatory cytokines. These cytokines can have local effects, including a specific stimulation of resting antigen-presenting cells (APCs). In the pulmonary tissue, the M-type phospholipase A2 receptor (PLA2R) or microbial agent become endocytosed and presented, triggering the maturation of airway-resident APCs. Activated APCs can, in turn, stimulate and activate T cells and, subsequently, B cells. In an individual in whom self-reactive lymphocytes have escaped mechanisms of central tolerance, these APCs initiate an autoimmune response. Plasma cells secrete and release autoantibodies in the circulation, which then can reach the kidney. Accumulation of anti-PLA2R antibodies in the glomerulus leads to kidney damage and the pathogenesis of MN.
All studies have confirmed the strong interaction and synergy between genetic variants in PLA2R and HLA class II genes. Thus far, no study has analyzed genetic associations separately in aPLA2Rab-positive versus aPLA2Rab-negative patients.

**Altered or increased expression of PLA2R antigen.** In normal and non-MN diseased kidneys, PLA2R is expressed only faintly in a granular pattern on podocytes. Hoxha et al. observed increased staining of PLA2R in a coarse granular pattern lining the glomerular basement membrane (GBM) in patients with MN only, tightly related to the presence of aPLA2Rab in serum. The investigators evaluated mRNA expression in kidney biopsy specimens of patients and controls and found no evidence for increased transcription of the receptor in patients. Thus, there is no proof of increased production of PLA2R antigen owing to increased mRNA transcription. We hypothesize this increased staining for PLA2R antigen in the glomeruli in the absence of increased production might be caused by a process in which circulating aPLA2Rab captures PLA2R, normally shuttling between the endoplasmic reticulum and the membrane. Similar mechanisms have been described for other proteins. The resulting immune complexes will be shed from the membrane and form the larger subepithelial deposits.

Obviously, it is plausible that conformational changes or increased expression of PLA2R outside the kidney could trigger the autoimmune response. In this respect, we hypothesize that the lung might be relevant (Figure 2). Expression of PLA2R in human lung alveolar macrophages was described in 2005. More recently, PLA2R expression also was observed in bronchiolar tissue (von Haxthausen et al., J Am Soc Nephrol. 2018;29:P822).

The role of antigen presentation in pulmonary tissue in autoimmunity has been suggested in other autoimmune diseases. In anti-GBM disease 2 major epitopes of the \( \alpha3(IV)NC1 \) molecule have been identified: EA and EB. These antibodies are present at low levels and with low affinity in healthy individuals, but the epitope is hidden and conformational changes are necessary for antibodies to bind. The \( \alpha3(IV)NC1 \) molecule is present in both the lung and kidney. It has been suggested that these epitopes are exposed after a primary insult to the lung (as might occur as a consequence of smoking). Is MN another example of an autoimmune disease in the kidney in which processing of the antigen might start in the lung, as in anti-GBM disease? If this is the case, why do MN patients not present with symptoms of lung pathology? Although not firmly established, one explanation might be that the \( \alpha3(IV)NC1 \) molecule is present as a permanent structural component of the basement membrane in the lung contrary to PLA2R antigen. PLA2R is present on macrophages, which are mobile and designed to clear immune complexes efficiently. This might explain the renal-limited character of MN.

**Molecular mimicry**

Autoimmunity can be initiated when epitopes on microbes that share homologies with self-antigen (molecular mimicry) are presented to the immune system. Li et al. studied the occurrence of epitope mimicry by microbial peptides in the initiation of anti-GBM disease. These investigators defined a critical amino acid motif on human \( \alpha3(IV)NC1 \), based on known T- and B-cell epitopes. They searched the UniProt database for microbial peptides that mimic this critical motif. In total, 23,826 peptides were identified, and 7 of them were related to human infections. Subsequently, the frequency of serum antibodies recognizing these 7 microbial peptides were measured. Microbe-derived peptides were recognized in the majority of anti-GBM patients. An inhibition ELISA showed that these antibodies showed cross-reactivity with the B-cell epitope and \( \alpha3(IV)NC1 \). Of note, a low frequency of antibodies against 20 linear peptides unrelated to the critical amino acid motif were measured (3.9%).

Also in PLA2R-related MN, molecular mimicry may contribute to disease initiation. There is partial homology between a part of the major CysR epitope in PLA2R and an antigenic cell wall component of some Clostridium species. Natural exposure to such infections could increase the frequency of B cells with a B-cell immunoglobulin receptor able to bind PLA2R or PLA2R fragments containing this CysR epitope. However, other (epidemiologic and experimental) evidence for a role of molecular mimicry in MN is lacking.

**TIME COURSE OF ANTIBODY PRODUCTION AND SUBCLASS SWITCHING**

Beck et al. already suggested that aPLA2Rab were predominately of the IgG4 subclass. Indeed, MN is considered an IgG4-dominant disease. The mechanisms of antibody production of the IgG4 subclass only recently are better understood. It is suggested that there is a programmed order of the immune response, starting from IgM-producing B cells, followed by class switching to IgG-producing B cells, with production of IgG subclass antibodies in a fixed order of IgG3 > IgG1 > IgG2 > IgG4. However, IgG4 production also is considered the consequence of an allergic response, in which an allergenic peptide (which induced a T helper cell 2 cytokine profile) initially triggers the production of IgE antibodies. With prolonged exposure, and increased production of interleukin 10, the IgE response mitigates and is replaced by steadily increasing IgG4. In the allergic response, IgG4 blocks the action of IgE, thus limiting the allergic reaction. In clinical practice, this advantageous suppression of IgE action is used in the setting of desensitization therapy.

Autoantibodies of the IgG4 subclass predominantly are present in diseases such as pemphigus vulgaris or some forms of myasthenia gravis. In these diseases, the IgG4 antibodies were pathogenic, as proven by passive transfer experiments or the induction of disease by using Fab fragments. In anti-GBM disease, most patients have anti-GBM antibodies of the IgG1 or IgG3 subclass. In few patients, antibodies of the IgG4 subclass dominated, and these patients presented with an unusually mild disease course. Thus, in anti-GBM disease the IgG4 antibodies are likely less pathogenic.
Although MN is considered an IgG4-dominant disease, and some investigators focus on measurement of IgG4 subclass aPLA2Rab, there is no proof that IgG4 aPLA2Rab are responsible for disease. Indeed, aPLA2Rab of other IgG subclasses are present in the majority of patients. Seventy-six percent of patients in a European cohort6 and 92% of patients in a Chinese cohort had aPLA2Rab of IgG1 subclass.68 The studies did not mention the percentage of patients with either IgG1 or IgG3 aPLA2Rab. Still, in 5% to 10% of patients, antibodies of IgG4 subclass were not detectable, and in these patients IgG1 or IgG3 antibodies always were present.6 Although both IgG4 aPLA2Rab and IgG1 aPLA2Rab correlated with total IgG aPLA2Rab levels, we re-analyzed the data and the relationship between IgG4 aPLA2Rab and IgG1 aPLA2Rab in this study was less tight (Pearson r = 0.451). No study closely evaluated the time course of subclass-specific aPLA2Rab. Histologic studies have suggested that although IgG4 is the dominant subclass in patients with long-standing MN (electron microscopy stages III/IV), IgG1 is the predominant subclass in early MN (electron microscopy stages I/II).69,70 These findings suggest that the onset of MN starts with the formation of aPLA2Rab of the IgG1 (or IgG2/3) subclass, with increased formation of IgG4 during prolonged disease activity. No study provided information on the presence of IgE aPLA2Rab.

In clinical practice, disease onset is not easily recognized by patients with MN. Thus, referral to nephrology care and kidney biopsy are performed many months after the onset of edema. At the time of first presentation at nephrology care, aPLA2Rab are of high affinity,27 which is compatible with maturation of the immune response.

The clinical course of MN is quite variable, some patients develop spontaneous remission and other patients have disease that progresses to end-stage renal disease. The immunologic response patterns mimic the clinical course: aPLA2Rab disappear spontaneously in patients with remission, and persist, often at high levels, in patients with progressive disease. The reasons for these differences have not been elucidated.

**PATHOGENICITY of aPLA2R ANTIBODIES**

Although there is a close temporal relationship between aPLA2Rab and clinical parameters (proteinuria), formal proof for the pathogenicity of the antibodies is lacking. Proof of the pathogenicity would need studies that show that the disease/podocyte injury and proteinuria can be induced after passive transfer of the aPLA2Rab. Animal models are not yet available because wild-type mice and rats lack podocytic PLA2R. Mice with a podocyte knock-in of the N-terminal domains of human PLA2R-CysR-CTL3, which contains the major B-cell epitope (P28mer) have been constructed (Rhoden et al., J Am Soc Nephrol. 2018;29:P823) for passive transfer experiments of human IgG1, IgG3, and IgG4 subclass-specific aPLA2Rab and mouse anti-human aPLA2Rab to test for induction of proteinuria.

**Complement-mediated podocyte injury**

The almost universal presence of C3 in the subepithelial deposits in membranous nephropathy21,22 and the strong association between the intensity of glomerular C3 staining and disease severity22 supports a role for complement in proteinuria development. However, the role of complement in MN is undetermined. IgG4, the dominant IgG subclass present in MN, does not bind complement and is unable to activate the classic complement pathway. It was suggested that complement may be activated through the mannose binding lectin (MBL) pathway.73–76 Indeed, the presence of complement components such as C4d, factor B, and properdin in the glomerular deposits is compatible with activation of the MBL pathway.76 In 1 study, IgG4 binding to MBL was suggested (Ma et al., J Am Soc Nephrol. 2011;22:P62A). However, in an interesting case study, Bally et al.77 showed the development of MN in a patient with proven MBL deficiency, but showed complement activation via the alternative pathway, indicating that the MBL pathway is not required for membranous nephropathy to develop. Some investigators have suggested that IgG4 is not the pathogenic subclass. Indeed, in most patients with MN, antibodies of the complement fixing IgG1 and IgG3 subclasses have been found.50 Still, the regular absence of C1q deposits71 argues against activation or involvement of the classic complement pathway. A recent study pointed to another mechanism of complement activation, independent of immune complex formation. In a mouse model of MN, investigators showed loss of glomerular heparan sulfate proteoglycans from the GBM, which bind complement factor H, causing local dysregulation of the alternative pathway.78 Seikrit et al.79 described patients with MN and an unexplained and fast deterioration of kidney function in whom antibodies against complement factor H were present.

**Non-complement-mediated podocyte injury**

PLA2R is thought to mediate some of the effects of the soluble phospholipases. These molecules play a role in cell proliferation, airway constriction, smooth muscle contraction, and inflammatory responses.80 mRNA expression studies have documented the presence of mRNA of PLA2R most abundantly in the kidney; other tissues with mRNA expression were muscle, lung, and placenta. In the kidney, PLA2R is observed in the podocyte. The function of PLA2R in the podocyte is unknown. PLA2R antibodies bind to PLA2R with high affinity.77 Thus, it is possible that upon binding of the aPLA2Rab, the function of the podocyte changes. Indeed, aPLA2Rab (in the absence of complement activation) was shown to modulate podocyte cell biology. Anti-PLA2R antibodies from 5 MN patients induced oxidative stress in differentiated human podocytes, increased cell apoptosis, and compromised the permeability of the cell monolayer (Fresquet et al., J Am Soc Nephrol. 2015;26:P354). How aPLA2Rab induces these changes and disrupts PLA2R function, however, remains unknown.

In kidney tissue 2 different mRNA transcripts are present, one encoding the membrane-bound PLA2R, the other

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A-E van de Logt et al.: Anti-PLA2R antibody in membranous nephropathy

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**Title:**

Anti-PLA2R antibody in membranous nephropathy

**Abstract:**

Anti-PLA2R antibodies are detectable in the sera of patients with membranous nephropathy (MN). The pathogenic role of these antibodies is currently debated.

**Introduction:**

MN is a common cause of nephrotic syndrome and renal failure. The pathogenesis of MN is complex and involves both intrinsic and extrinsic factors.

**Methodology:**

Sera from MN patients and healthy controls were tested for the presence of anti-PLA2R antibodies by immunofluorescence and western blotting.

**Results:**

Anti-PLA2R antibodies were detected in the sera of MN patients, but not in healthy controls.

**Conclusion:**

Anti-PLA2R antibodies in MN patients may play a role in the pathogenesis of the disease, but further studies are needed to elucidate their exact mechanism of action.
encoding a soluble form of the receptor. Indeed, Watanabe et al. recently showed that soluble PL2R can be found in normal healthy volunteers. PL2R binds to collagen type I and type IV through the fibronectin type II domain (FNII). The membrane-bound receptor mediated the interaction between collagen I and β1 integrin, affecting podocyte mobility. Interestingly, the soluble receptor blocked the interaction between collagen and integrins, and thus suppressed a migratory response of the podocyte. Theoretically, antibodies against PL2R1 on the cell membrane could affect podocyte function. The annexin 2/S100A10 complex was identified as a novel binding partner of PL2R in podocytes. This PL2R/annexin 2/S100A10 complex was found on the podocyte plasma membrane and in secreted extracellular vesicles. This implies that PL2R may be at the heart of actin cytoskeleton reorganization and tight junction assembly, 2 functions known to be modulated early in proteinuric MCD.

The possibility that antipodocyte antibodies induce podocyte injury or alter podocyte function independent from complement has been shown by studies that evaluated THSD7A. Tomas et al. studied the pathogenicity of THSD7A autoantibodies. Injection of serum and purified IgG in mice induced proteinuria within 3 days. Proteinuria increased further, which was paralleled by the development of mouse anti-human IgG antibodies and the development of immune complexes containing human and mouse IgG, characteristic of the autologous phase. Interestingly, in this model, C3 deposits were seen in this later phase, but not in the initial heterologous phase. In a subsequent study, they developed rabbit anti-THSD7A antibodies. Again, injection of rabbit serum induced proteinuria that was complement-independent. In vitro, incubation of podocytes with anti-THSD7A antibodies caused cytoskeleton rearrangement and activation of focal adhesion signaling. Of note, when using zebra fish larvae, knockdown of THSD7A caused podocyte changes with injury of the glomerular filtration barrier and development of edema, suggesting an important role of THSD7A in glomerular filtration barrier integrity. These findings clearly show that proteinuria in models of MN may not be dependent on complement. In a recent study, evidence was found that THSD7A functions as a foot process protein and stabilizes the membrane of the podocytes.

### Table 1 | Unresolved questions on antibodies against PL2R in membranous nephropathy

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<td>Is kidney biopsy PL2R antigen staining or anti-PL2R serology the gold standard for diagnosing PL2R-associated MN?</td>
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<td>Is aPL2Rab serology robust enough to avoid renal biopsy?</td>
<td>→ define optimal cut-off values for the ELISA assay and evaluate sensitivity and specificity of the various assays (ELISA/IFT/Western blot) for diagnosing PL2R-related MN</td>
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<tr>
<th>aPL2Rab titer</th>
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<tr>
<td>Evaluate accuracy of aPL2Rab ELISA titer at baseline and change of titer during follow-up evaluation for predicting natural course of disease (spontaneous remission, progression)</td>
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<tr>
<td>Evaluate accuracy of aPL2Rab ELISA titer (at start of therapy and changes during therapy) for predicting response to therapy. Can change in titer guide treatment withdrawal?</td>
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<td>Evaluate accuracy of more specific assays (IgG subclass-specific, epitope-specific)</td>
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<th>Characteristics of aPL2R</th>
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<td>Evaluate the time course of IgG subclass-specific aPL2Rab</td>
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<td>Evaluate time course of epitope-specific aPL2Rab</td>
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<tr>
<td>Evaluate the presence of non-IgG aPL2Rab (e.g., IgE, IgM, IgA)</td>
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<tr>
<td>Evaluate presence of aPL2Rab in healthy controls and in family members of patients with MN (HLA-typed)</td>
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<th>Prediction</th>
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<tr>
<td>Is there added value of measuring antigen-specific B- and T-cell subsets in diagnosis, predicting prognosis, monitoring of therapy, and relapse after therapy?</td>
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<tr>
<td>Investigate the frequency of serum antibodies against molecular peptides that mimic the critical motif (after characterization of B-cell and T-cell epitopes)</td>
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<tr>
<td>Does HLA or anti-PL2R predict the chance of recurrence after kidney transplantation?</td>
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<th>Effect of aPL2R on kidney</th>
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<td>What are the effects of aPL2Rab on the podocyte (in vitro studies) in the kidney using animal models or human kidney transplant?</td>
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<tr>
<td>How does aPL2Rab eluted from kidney biopsy specimens compare with serum aPL2Rab?</td>
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patients with high PLA2Rab have the most severe disease and are at increased risk of progression, it can be questioned if removal of antibodies by immunoadsorption might be beneficial. Currently, a small pilot study of aPLA2Rab immunoadsorption is being performed to establish the feasibility of rendering patients seronegative for aPLA2Rab in the absence of immunosuppression.

Recognition of the B-cell epitopes would enable the development of specific targeted therapies as reviewed for pemphigus vulgaris. Removal of pathogenic aPLA2Rab by immunoadsorption may reduce disease activity, but it will not cure the disease. It will remain necessary to block antibody production. Identification of the pathogenic B-cell epitopes may enable development of treatment strategies that specifically target the autoreactive B cells, causing antigen-specific B-cell depletion. One option is the targeted cell death of autoreactive B cells by infusion of a toxin-conjugated, epitope-containing fragment of the PLA2R antigen that binds to the anti-PLA2R B-cell receptor. A more durable elimination of aPLA2R-reactive B cells is expected from cellular immunotherapy, also known as chimeric autoantibody receptor T-cell therapy. In this approach, the patient's T cells are modified ex vivo, with incorporation of CD28 and/or CD137 co-stimulatory domains and the pathogenic epitope of PLA2R in the T-cell receptor. These T cells will specifically kill PLA2R-specific B cells, and their persistence as memory chimeric autoantibody receptor T cells will provide continuous surveillance against re-emergence of PLA2R-positive B cells.

On the other hand, identification of T-cell epitopes might allow development of immune vaccination, which should enable the development of antigen-specific immune tolerance. In this situation, the goal is immunization of MN patients using a pool of T-cell peptides to restore specific Tregs, allowing suppression of B-cell production of anti-PLA2R.

It remains important to decipher the mechanisms of pathogenicity of the aPLA2Rab. If IgG4 antibodies cause podocyte injury by binding to PLA2R, independent from complement or Fc receptor–mediated neutrophil activation, it would provide opportunities to develop treatment strategies using small peptides that would interfere with the binding of the antibodies to the PLA2R antigen. On the other hand, if podocyte injury is dependent on complement activation, we must consider the use of anticomplement therapies.

In conclusion, in the 10 years after the discovery of PLA2R as the major autoantigen in MN, great progress has been made. Many questions still remain unsolved (Table 1), and we expect the answers in the next decade.

DISCLOSURE
All the authors declared no competing interests.

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