Anti-Phospholipase A2 Receptor Antibodies in Membranous Nephropathy: from Bench to the Patient

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Abstract
Membranous Nephropathy (MN) is the leading cause of nephrotic syndrome in adults. Primary or idiopathic Membranous Nephropathy (iMN) has been considered as an organ-specific autoimmune disease with an unknown etiology. By contrast, secondary membranous nephropathy has been described as the expression of a systemic autoimmune response to malignancy or bacterial/viral infections. In the majority of iMN cases, glomerular lesions are determined by autoantibodies against a podocyte membrane protein, the M-type of phospholipase A2 receptor 1 (PLA2R1). Several studies have suggested that the detection of anti-PLA2R1 in patients sera with nephrotic syndrome may be pathognomonic of iMN, thus obviating the need for a diagnostic renal biopsy and an extensive workup for underlying causes. Many authors inferred that the measurement of anti-PLA2R1 may change the diagnostic algorithm in patients with nephrotic syndrome and guide treatment decisions in patients with iMN. Measurement of anti-PLA2R1 antibodies is now possible through an easy to use, commercially available assay. The aim of this review is to describe the clinical relevance of anti-PLA2R1 assessment in patients with MN, and to extensively discuss the biotechnological methods available to measure them.

Keywords. Membranous nephropathy; Autoimmune disease; Anti-PLA2R; Nephrotic syndrome

Introduction
Membranous nephropathy (MN) is a glomerular disease histologically characterized by an uniform thickening of glomerular basement membranes (GBM) caused by subepithelial electron-dense deposits of immune complexes, in which the antibody is an IgG. In about 2/3 of cases the etiology of MN is unknown and the disease is called primary or idiopathic. In the remaining cases MN may be secondary to systemic or autoimmune diseases, malignancies, infections, toxic agents or drugs.

Much of the current information about the pathogenesis of MN comes from the active and passive experimental models of Heymann nephritis (HN). Many attempts have been made to identify the antigen involved in HN. Eventually it has been identified in a large glycoprotein of 515 kd, called megalin, an endocytic receptor expressed on the luminal surface of the renal proximal tubules [1-4]. In small animals, megalin is also present in clathrin coated pits located along the base of podocyte foot processes, where it complexes with a Receptor-Associated Protein (RAP) that serves as a chaperon for megalin. In HN there are circulating antibodies directed against megalin that pass the GBM and bind to specific epitopes of megalin forming in situ immune complexes that attach to the GBM. The glomerular epithelial cells react by increasing the synthesis of megalin. Repeated cycles eventually lead to accumulation of immune complexes with consequent activation of reactive oxygen species, inflammation, and local activation of complement with formation of the cytolytic membrane-attack complex C5b-C9 [5].

However, the model of HN is not entirely applicable to iMN since megalin is not expressed on human podocytes. Thus, other antigens should trigger an antibody response and in situ deposition of immunocomplexes may also result from circulating unknown antigens.

The seminal study of Becker et al. has identified the M-type phospholipase A2 receptor 1 (PLA2R1) as the major target antigen in iMN [6]. These investigators found that about 70% of patients with iMN had circulating autoantibodies directed against PLA2R1.

This discovery soon confirmed by other investigators [7,8], led to hypothesize that iMN is an autoimmune disease in which circulating autoantibodies bind to PLA2R1 on podocytes leading to in situ formation of immune complexes, GBM inflammation, and massive proteinuria [9]. According to Salant [10], the development of idiopathic MN may be the result of three conditions: the presence of HLA-DQA1 alleles that confer susceptibility to autoimmunity, polymorphism of PLA2R1 that creates a unique conformation that can represent a target for autoantibodies, and production of hypogalactosylated IgG4 anti-PLA2R1 that activates the complement pathway. It is also possible that innate immunity is involved. Moreover, endogenous or external (i.e. virus or viral particles?) stimuli may increase the expression of PLA2R1 or shed some “cryptic” epitopes of PLA2R1.

A similar model is proposed for lupus glomerulonephritis. Accumulating data indicate that antiviral immunity has relevance in the pathogenesis of lupus nephritis. This idea is based on the existence of genetic variants that promote the persistence of nuclear particles in the extracellular space or inside lysosomes. Such nuclear particles mimic viral particles and their RNA or DNA components activate viral nucleic acid recognition receptors in antigen-presenting cells. These autoadjuvant effects of endogenous nucleic acids promote an inappropriate immune interpretation of the nuclear particles during antigen presentation [11].

The aim of this review is to describe the clinical importance of measuring anti-PLA2R1 antibodies in patients with MN. An additional
lipolytic enzymes which hydrolyzes the change [17]. The pattern of antigen-peptide processing through conformational that are related to idiopathic membranous nephropathy could control idiopathic membranous nephropathy. Sequence variations in PLA2R1 present in normal podocytes and in immune deposits in patients with clearance of phospholipase A2, thereby inhibiting its action. PLA2R1 is present in presenting cells and both the alpha chain and the beta chain contain from extracellular antigenic proteins, which in idiopathic membranous nephropathy is higher with the HLA-DQA1 and PLA2R1 alleles. The description of these genetic associations has important implications. First, data point to the possibility that sequence variations within HLA and PLA2R1 are responsible for the development of idiopathic membranous nephropathy in whites. Second, the study provides support for the finding, reported by Beck and colleagues, that PLA2R1 is associated with idiopathic membranous nephropathy [6] and it underlines the importance of PLA2R1 antibodies detection in diagnostic chart of iMN. It clearly appears that the risk of idiopathic membranous nephropathy is higher with the HLA-DQA1 allele than with the PLA2R1 allele, suggesting that the HLA-DQA1 allele might facilitate autoantibody development targeting not only PLA2R1 but also other antigens. Literature strongly suggest that an interaction between genetic variants of immune-system proteins and glomerular components form the basis for the development of idiopathic membranous nephropathy, establishing a trigger (the immune system), a bullet (PLA2R1 autoantibodies), and a target (glomerular antigen). However, the causative link between the presence of the HLA–DQA1 risk allele and PLA2R1 autoantibodies remains unknown.

A report on a previous study, in which a traditional method was used (restriction-fragment–length polymorphism analysis), pointed out that HLA-DQA1 alleles could be involved in idiopathic membranous nephropathy [15]. HLA-DQA1 is part of a heterodimer consisting of an alpha chain (DQA) and a beta chain (DQB), both anchored in the membrane and forming the antigen-presenting groove. This heterodimer plays a central role in the immune system by presenting epitope peptides (9 to 15 amino acids in length) derived from extracellular antigenic proteins, which in idiopathic membranous nephropathy could, for example, be composed of extracellular PLA2R1 fragments [16]. Class II molecules are expressed in antigen-presenting cells and both the alpha chain and the beta chain contain polymorphisms that determine peptide binding specificities. PLA2R1 encodes a protein that probably exists in both a transmembrane form and a soluble form. The transmembrane receptor may increase in the clearance of phospholipase A2, thereby inhibiting its action. PLA2R1 is present in normal podocytes and in immune deposits in patients with idiopathic membranous nephropathy: Sequence variations in PLA2R1 that are related to idiopathic membranous nephropathy could control the pattern of antigen-peptide processing through conformational change [17].

Detection of Anti-PLA2-R Antibodies

Target antigen

Phospholipase A2 (PLA2) comprises a diverse family of lipolytic enzymes which hydrolyzes the sn-2 fatty acid ester bond of glycerophospholipids to produce free fatty acids and lysophospholipids [4]. Most of the biological activities of sPLA2s have been attributed to their enzymatic capacity to hydrolyze membrane phospholipids.

In 2009, Beck et al. described the M-type phospholipase A2 receptor as an autoantigen in MN based on immunoblot analysis and mass spectrometry [6]. PLA2R is a type I transmembrane glycoprotein with a molecular mass of 180 kDa and is composed of a large extracellular portion consisting of an N-terminal cysteine-rich region, a fibronectin–like type II domain, and a tandem repeat of eight carbohydrate-recognition domains (CRDs), as well as a short intracellular C-terminal region (Figure 1). Although PLA2R is expressed on alveolar type II epithelial cells and on neutrophils, data suggest that it is mainly restricted to kidney podocytes [5]. PLA2R has been found to promote senescence in human fibroblasts and is involved in both positive and negative regulation of secretory PLA2. Mammalian sPLA2 could attach to PLA2-R and induce pro-inflammatory signals by a receptor-mediated mechanism [5] (Table 1).

Antibody subclass

It is well know that iMN is an IgG4 dominant disease. Both Beck et al. and Qin et al. showed that antibodies against PLA2R were mainly of the IgG4 subclass, confirming the dominance of this immunoglobulin subclass [18,19]. Since IgG4 is not binding complement, this has sparked the debate on the pathogenic role of these antibodies. However, both Beck and Qin agree that other subclass specificities, particularly of the IgG1 and IgG3 subclass, can be found in most patients. The role of IgG4 is further questioned by the finding that IgG4 deposits in the biopsy do not always correlate with anti-PLA2R titre in serum [6,18].

Whereas the data of the initial publication of Beck et al. [6] which suggested the unique association of anti-PLA2R1 IgG4 and iMN, other studies were performed to better understand the role of IgG subclasses.

Hofstra et al. first evaluated IgG subclasses. IgG4 was the dominant subclass in the majority of patients. Titers of IgG4, but not IgG1 or IgG3, significantly correlated with the occurrence of spontaneous remission [8]. To describe the natural pattern of the IgG subclass response to PLA2R, Kanigicherla et al. analyzed a subset of patients whose serum and biopsy samples were taken within 6 months and who were untreated by immunosuppressive agents before sampling. IgG4 and IgG2 subclasses, typically thought to be inefficient at complement fixation, were clearly dominant, constituting 50 and 37%, respectively, of the total subclass response [19].

Laboratory techniques

Direct immunofluorescence assay (DIA) was the first and pivotal technique to the detection of IgG4 deposit in glomeruli. PLA2R in immune deposits was initially assessed by confocal microscopy in paraffin blocks with affinity-purified specific anti-PLA2R antibodies (Figure 2, Panel A) [6]. The sensitivities of biopsy tests were 74% [6] but it has some practical limitations. It requires specialized equipment, including a confocal microscope, is not easy to automatize, is prone to human interpretation errors and delivers only semi-quantitative data, even when multiple incubations with different sample dilutions are performed.

In the light on these consideration Beck et al. employed a Western blot technique using glomerular extracts which were electrophoresed under nonreducing conditions [6]. Despite descriptions about an
improved variant of the method with a sensitivity of >90% [6], Western blotting analysis is difficult to use outside of an expert laboratory and the method is not suited for the evaluation of large sample numbers.

These limitations led to the development of a recombinant cell-based indirect immunofluorescence assay (RC-IFA) (Figure 2, Panel B) that uses the human cell line over-expressing the full-length human PLA2R1 as substrate [20] which is now commercially available (EUROIMMUN AG, Lubeck, Germany). For this assay slides are made that contain biochips containing HEK 293 cells transfected with cDNA coding for PLA2R and non-transfected cells as control. The bound antibodies are detected with FITC-labelled goat antihuman IgG.

However, even though RC-IFA can be considered as the reference method for the determination of several autoantibodies against integral membrane proteins of the outer cell surface, like NMDAR in autoimmune encephalitis [21], aquaporin 4 in neuromyelitis optica [22], CUZD1/GP2 in Crohn’s disease [23], it has some practical limitations. It requires specialized equipment, including a fluorescence microscope, is not easy to automatize, is prone to human interpretation.

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Patients (n)</th>
<th>PLA2R1+(n,%)</th>
<th>Assay</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hofstra et al. [8]</td>
<td>18</td>
<td>14.78</td>
<td>WB</td>
<td></td>
</tr>
<tr>
<td>Debiec and Ronco [7]</td>
<td>42</td>
<td>24.57</td>
<td>IF</td>
<td></td>
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<tr>
<td>Hoxha et al. [20]</td>
<td>100</td>
<td>52.52</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>Qin et al. [18]</td>
<td>60</td>
<td>49.82</td>
<td>WB</td>
<td>Cross-sectional study; 66% positive if limiting analysis to patients with proteinuria &gt;3.5 g/day</td>
</tr>
<tr>
<td>Hoxha et al. [20]</td>
<td>24</td>
<td>14.58</td>
<td>WB</td>
<td>Low titres of PLA2R present in 10/11 patients who were negative in standard assay Renal biopsies with mesangial or subendothelial deposits or glomerular infiltrating cells were excluded</td>
</tr>
<tr>
<td>Beck et al. (2011) [25]</td>
<td>14</td>
<td>12.86</td>
<td>WB</td>
<td>Patient characteristics not provided</td>
</tr>
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<td>Hoxha et al. [20]</td>
<td>81</td>
<td>53.65</td>
<td>IF</td>
<td>Prospective study</td>
</tr>
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<td>Schönemarck [9]</td>
<td>16</td>
<td>11.69</td>
<td>IF</td>
<td></td>
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<tr>
<td>Kanigicherla et al. [19]</td>
<td>40</td>
<td>29.73</td>
<td>ELISA</td>
<td></td>
</tr>
<tr>
<td>Hofstra et al. [8]</td>
<td>117</td>
<td>86.74</td>
<td>IF/ELISA</td>
<td>Cross-sectional study; table reflects only patients with active disease</td>
</tr>
<tr>
<td>Hofstra et al. [8]</td>
<td>88</td>
<td>61.69</td>
<td>IF/IF</td>
<td>Authors develop a standardized ELISA for the determination of autoantibodies against PLA2R1 that can be made widely available and used in a routine setting for clinical applications.</td>
</tr>
<tr>
<td>Salant [10]</td>
<td>200</td>
<td>196.98</td>
<td>ELISA</td>
<td>Prospective study</td>
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<tr>
<td>Oh et al. [26]</td>
<td>100</td>
<td>69.69</td>
<td>WB</td>
<td>Authors develop a standardized ELISA</td>
</tr>
<tr>
<td>Behnert et al. [24]</td>
<td>165</td>
<td>85.52</td>
<td>IF/WB/ALBIA</td>
<td>Authors explored the relationship between anti-PLA2R and disease activity and clinical outcomes. An Anti-Phospholipase A2 Receptor Quantitative Immunoassay</td>
</tr>
</tbody>
</table>

Table 1: Prevalence of anti-PLA2R in idiopathic membranous nephropathy.
errors and delivers only semi-quantitative data, even when multiple incubations with different sample dilutions are performed.

More recently, an ELISA protocol was published that uses a recombinantly produced extracellular domain of PLA2R1 to form the solid-phase [8].

The authors measured PLA2R1 antibody titer and subclass in a well defined cohort of 117 Caucasian patients with idiopathic membranous nephropathy and nephrotic-range proteinuria [8]. In this cohort, PLA2R1 antibodies were positive in 74% and 72% of patients using IIFT and ELISA, respectively. Concordance between both tests was excellent.

Sera from healthy individuals were used to define the normal range. Using mean + 3 Standard Deviations (SD), they proposed a threshold above 40 U/ml as positive [8]. However the study did neither evaluate the robustness of the method nor its specificity on a large number of disease controls.

More recently Behnert et al. developed a quantitative, high throughput capture immunoassay that has at least comparable sensitivity and specificity of the RC-IFA and avoids the subjective assessment of RC-IFA especially in the presence of other autoantibodies that may obscure a true positive result. They tested recombinant overexpressed PLA2R as a potential target on an addressable laser bead immunoassay (ALBIA) diagnostic platform [24]. This work indicates that in addition to conformational epitopes, human anti-PLA2R reactivity in a commercially available CBA and an addressable laser bead immunoassay is significantly absorbed by peptides representing epitopes of PLA2R [24].

Relationship between Serum anti-PLA2R and Disease Activity

A number of studies have been performed to better define the correlation between serum anti-PLA2R levels and iMN activity.

Beck et al. serially measured anti-PLA2R antibodies in 18 iMN patients in serum collected at baseline, during remission and at relapse of nephrotic proteinuria. Anti-PLA2R1 antibodies disappeared during remission and reappeared in all cases of relapse [6]. Hofstra et al. found that spontaneous remission occurred significantly less frequently in patients with high antibody titer [8]. In another study, a reduction of serum titer of anti-PLA2R1 antibodies preceded the decrease of proteinuria in iMN patients treated with Rituximab, suggesting that the antibody titer may predict patient’s outcome [25].

Qin et al. found a shorter time-remission in patients with lower titer of antibody and suggested that the anti-PLA2R1 titer might have a prognostic significance [18]. However, Oh et al. did not find that the antibody measurement at the onset of nephrotic syndrome could predict the clinical outcome of disease [26].

Taken together these results seem to indicate that changes of serum anti-PLA2R antibody titer may be an useful tool to assess the disease activity and might also predict the outcome.

Future studies in larger patient cohorts are necessary to analyze if autoantibody reactivity to specific regions of the receptor is of prognostic relevance for the affected patients.

Diagnostic Values of Serum Antibodies versus Biopsy

Hoxha et al. investigating in parallel serum and kidney biopsies of patients with iMN found a strong expression of PLA2R1 in the glomeruli of 61/88 patients, 60 of them showed also serum anti-PLA2R1 positivity. The remaining 27 serum negative patients showed a faintly positivity for PLA2R1 staining at glomerular level, whereas in 15 patients a secondary cause of MN was found [20]. Svobodova et al. tested paraffin-embedded kidney biopsies of 84 patients with MN for the presence of PLA2R in glomerular immune deposits and measured circulating anti-PLA2R antibodies using the indirect immunofluorescence test. In 45 of 65 (69%) patients with iMN, PLA2R was detected in sub-epithelial deposits along GBM. Circulating anti-PLA2R antibodies were detected in 20 of 31 (65%) sera from patients sampled during active disease. Six patients with active disease were negative for circulating anti-PLA2R antibodies despite PLA2R1 antigen positivity in the biopsies. Only 8 of 37 (22%) sera sampled at the time of remission were PLA2R positive while PLA2R1 antigen was found in 22 of the 37 (59%) corresponding biopsies [27].

Thus, in most patients with iMN the serum anti-PLA2-R1 antibodies positivity is of heavy diagnostic value whereas its negativity may require a histological confirmation or additional clinical research to exclude a secondary cause of MN.

Literature review provided evidence that binding of autoantibodies to PLA2R domains is likely more complex than previously thought.
It is important to appreciate that even when proteins are “denatured” using various chemical modalities (i.e. mercaptoethanol, dithiothreitol, or SDS) that transfer from SDS polyacrylamide gels to solid phase matrices through an alcohol interphase can result in significant protein refolding. These findings supporting reports that human IMN autoantibody binding to PLA2R depends on conformational epitopes.

It is well known that iMN is an IgG4 dominant disease, with IgG4 being the dominant IgG subclass in renal biopsies of patients with iMN. Both Beck et al. and Qin et al. showed that antibodies against PLA2R were mainly of the IgG4 subclass, confirming the dominance of IgG4 [6,18]. However, the same authors agree that other subclass specificities, particularly of the IgG1 and IgG3 subclass, can be found in most patients [6,18]. The role of IgG4 is further questioned by the finding that IgG4 deposits in the biopsy do not always correlate with anti-PLA2R titre in serum. Qu et al. did not observe IgG4 in 6/42 patients with iMN [28]. Hoxha reported three patients with anti-PLA2R in serum and PLA2R in the biopsy with negative IgG4 staining [20].

Conclusions
Membranous nephropathy is an autoimmune disease usually associated with a nephrotic syndrome. Although the pathogenesis of iMN remains incompletely defined, a strong association with the HLA-DQA1 allele has been found in patients with iMN. Ponticelli and Glassok in a more recent review highlighted that endogenous or exogenous injuries, such viral attack, initiated tissue damage and caused structural alterations of PLA2R. Several studies and animal models suggested that viruses and other infectious insults are implicated in the aetiology of many human autoimmune diseases [28]. In these studies, regardless of the initial antigenic specificity of the immune response spreads to include self-epitopes other than that which initiated the inflammatory process. The resulting inflammation and tissue damage then primes a hierarchical cascade of auto reactive T-cell specificities, even allowing cryptic or sequestered epitopes to be processed and presented [28].

Apart from other clinical and histological parameters, the positivity of anti-PLA2R antibody may represent an useful and specific marker to discriminate between idiopathic and secondary MN. The accuracy of the test to identify iMN and to exclude secondary causes of MN awaits well-designed prospective studies, and it is expected that quantitation of the anti-PLA2R antibodies will be important for prediction of prognosis as well as guidance of therapy. However a few exceptions may occur.

Although a relevant percentage of patients with iMN present high anti PLA2R1 titer, antibodies may be undetectable in few cases of histological confirmed iMN. This may be related to low sensitivity of the method employed, an early spontaneous remission of MN or finally a wrong diagnosis of iMN. Also when proteins are “denatured” using various chemical modalities can result in significant protein refolding. Behnert et al. also discovered that human autoantibodies to PLA2R appear to target multiple domains of the receptor, a finding that supports the phenomenon of inter-molecular epitope spreading of B cell responses [24].

It’s nevertheless true that the variability of ligand-autoantibodies interaction could be a critical step that affecting laboratory methods and could be explain some exceptions and incongruences presenting in the literature.

Finally, anti-PLA2R antibody serum measurement could be an alternative diagnostic tool to confirm the presence of iMN so allowing to avoid percutaneous renal biopsy in difficult cases. Although renal biopsy is extensively used for establishing the diagnosis, the prognosis, and the management of renal disease, it may be rarely responsible of minor or severe complications. Moreover there are some cases in whom percutaneous renal biopsy is contraindicated such as bleeding diathesis, anatomic or functional unique kidney, small kidneys with cortical sclerosis or atrophy, uncooperative behaviour and pregnancy. In these conditions the serum anti PLA2R measurement has an irreplaceable role to assess the diagnosis of iMN.

References


