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Detection and Monitoring PLA₂R Autoantibodies by LIPS in Membranous Nephropathy

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Abstract

Autoantibodies against the M-type phospholipase A₂ receptor (PLA₂R) are specific markers for primary membranous nephropathy (MN). Quantification of PLA₂R autoantibodies is an important, noninvasive tool that facilitates the diagnosis and monitoring of primary MN. In this report we describe a highly quantitative luciferase immunoprecipitation systems (LIPS) assay for detecting PLA₂R autoantibodies. For these studies, a cDNA fragment encoding the first 858 amino acids of PLA₂R protein was cloned to generate N-terminal antigen fusion constructs with *Gaussia* luciferase (Gluc) and Nano luciferase (NanoLuc) reporters. Following transfection, crude cell extracts containing the recombinant PLA₂R-luciferase fusion proteins were tested by LIPS on healthy controls, subjects with other kidney disease and subjects with MN. LIPS testing with both reporters detected robust PLA₂R autoantibody levels in a subset of patients with primary MN and demonstrated 100% sensitivity compared to ELISA and/or Western blotting. The PLA₂R-NanoLuc LIPS assay demonstrated 100% specificity matching the ELISA, but the specificity of the PLA₂R–Gluc LIPS assays was slightly lower (97%). Further analysis revealed that autoantibody levels determined by PLA₂R-NanoLuc LIPS correlated well with urinary protein excretion (R=0.79) and disease activity and was very sensitive for detecting clinical relapse. These results highlight the potential utility of the LIPS PLA₂R-NanoLuc assay for diagnosis and management of MN.

1. Introduction

Membranous nephropathy (MN) is an antibody-mediated autoimmune kidney disease that is a leading cause of nephrotic syndrome in adults (Glasscock, 2010). Patients with MN typically present with high grade proteinuria, edema, hypoalbuminemia and hyperlipidemia. Although some individuals with membranous nephropathy demonstrate spontaneous remission of the disease, up to 40% of untreated individuals suffer persistent high grade proteinuria and eventually progress to end stage kidney disease. MN is classified as primary or secondary depending on whether or not it is possible to find an etiology responsible for it.

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This distinction is important as it impacts treatment. In primary MN autoantibodies directed against intrinsic podocyte antigens produce immune deposits in the kidney (Glasscock, 2010). In 2009, a seminal study identified the M-type phospholipase A₂ receptor 1 (PLA₂R) as the target of circulating autoantibodies in the majority (70–80%) of cases of primary MN (Beck et al., 2009). Since this initial discovery, PLA₂R autoantibodies have proven to be a specific and sensitive biomarker of primary membranous nephropathy because they are rarely seen in secondary forms of MN and are not present in other kidney diseases or in sera of healthy controls. PLA₂R autoantibody titers also provide important information about the clinical activity of the disease, whereby changes in PLA₂R autoantibody levels precede changes in urinary protein excretion. Thus, measurement of PLA₂R autoantibody titers has emerged as an invaluable tool to facilitate the diagnosis of MN, to discriminate primary from secondary forms of MN and to monitor disease activity and treatment efficacy.

PLA₂R is a 180 kDa transmembrane glycoprotein expressed on the surface of podocytes and is composed of a very large extracellular region containing multiple cysteine disulfide bonds, a fibronectin-like type II domain and eight repeated C-type lectin-like domains (Beck et al., 2009). Although a variety of immunoassays have been developed to detect and quantify circulating PLA₂R autoantibodies (VanBeekHaas, 2015), they require the non-reduced form of the PLA₂R protein (Beck et al., 2009; Kao et al., 2015). Existing immunoassays including Western blotting (Beck et al., 2009), indirect immunofluorescence cell based assay (IIF-CBA)(Hoxha et al., 2011), enzyme-linked immunosorbent assay (ELISA) (Dahnrich et al., 2013; Timmermans et al., 2014) and addressable laser bead immunoassay (ALBIA) (Behnert et al., 2014), each have advantages and limitations. For example, Western blotting and IIF-CBA are both sensitive tests, but Western blotting is cumbersome and not suitable for routine clinical use, and IFA is subjective and only provides semi-quantitative results. ELISA is quantitative, but since the autoantigens are bound to the plastic surface, this detection method may miss important conformational epitopes. Currently, there is still a need for a sensitive, specific and high-throughput immunoassay to screen individuals and to monitor their PLA₂R autoantibody levels during treatment.

Luciferase immunoprecipitation systems (LIPS) is an immunoassay that employs light-emitting proteins to measure many different types of antibodies associated with infectious and autoimmune diseases (Burbelo et al., 2015). The LIPS immunoassay has several advantages over ELISA and other commonly used immunoassay methods. First, recombinant antigens used in LIPS are produced in mammalian cells allowing for the facile production of large recombinant proteins. In LIPS, soluble crude cell lysates of the directly-tagged luciferase recombinant antigens are simply extracted from transfected cells, which eliminates complicated purification schemes and allows for the antigens to be tested under native, non-reducing conditions. Second, LIPS as a fluid-phase immunoassay employing target antigens in their native conformation and is well suited to detect autoantibodies directed against linear and conformational epitopes associated with many autoimmune conditions (Burbelo et al., 2015; LiuEisenbarth, 2007). Quantitative antibody levels generated by LIPS assays typically span a large dynamic range and can monitor longitudinal changes in these levels over time in response to treatment (Burbelo et al., 2014; Ramanathan et al., 2008). To date, most LIPS studies have utilized *Renilla* luciferase (Ruc)-antigen fusion

proteins, but other luciferases, including *Gaussia* (Gluc) and Nano luciferase (NanoLuc), have also been employed (Burbelo et al., 2015; McLaughlin et al., 2016). Compared to Ruc, Gluc (Tannous et al., 2005) and NanoLuc (Hall et al., 2012) are smaller proteins and have higher light output. However, direct comparison of LIPS assays using these different reporters has not been reported.

The present study describes a novel LIPS immunoassay for detecting PLA₂R autoantibodies. We developed two different reporters based on chimeric fusion proteins corresponding to the N-terminus of PLA₂R fused to Gluc and NanoLuc. We compared the performance characteristics of the LIPS assays against a commercial ELISA to assess its utility for diagnosis of MN, for monitoring disease activity and for predicting relapse of the disease.

2. Material and methods

2.1 Patient sera

De-identified patient sera were collected at the Clinical Research Center at the National Institute of Health (Bethesda, MD) under National Institute of Diabetes and Digestive, and Kidney Diseases (NIDDK) institutional Review Board-approved protocols. The study included sera from eight healthy controls, 45 subjects with biopsy proven MN and 18 nephrotic disease controls including membranoproliferative glomerulonephritis, C3 glomerulopathy, minimal change disease, thrombotic microangiopathy, vasculitis, light chain deposition disease and class III/IV lupus nephritis. The MN cohort included a combination of treated and untreated, nephrotic and non-nephrotic patients with primary and secondary MN (lupus, Hepatitis B, malignancy associated, post stem-cell transplant). The diagnosis of primary or secondary MN was based on the combination of clinical and laboratory criteria. A subset of the MN cohort (n=13) were treatment-naïve patients with primary MN enrolled in an experimental clinical trial. They received immunosuppression with a combination of rituximab and cyclosporine (“treatment cohort”) as described in (Waldman et al., 2016) and were followed longitudinally. Serum and urine samples were collected at baseline (before initiating therapy) and at 3 month intervals over the 24 month trial period to evaluate changes in autoantibody levels and clinical parameters (e.g. protein excretion based on 24 hour urine collection) (Waldman et al., 2016). Partial and clinical remission were defined as urinary protein excretion of ≤ 3.5 g/24 h and <0.3 g/24 h, respectively. For the purpose of validating the LIPS assay, all baseline MN samples were tested by Western blotting (Beck et al., 2009) and/or a commercially available ELISA kit (EUROIMMUN US, Mountain Lakes, NJ) as previously described (Waldman et al., 2016) and classified as PLA₂R seropositive or seronegative. ELISA titer values higher than 20 RU/ml were considered positive per the manufacturer’s protocol. Serial quantification of PLA₂R autoantibody levels in the treatment cohort were determined by ELISA and compared to the LIPS assay. Testing of all samples was initially performed with the PLA₂R-Gluc LIPS assay using coded samples without knowledge of serological and clinical information.

2.2 Generation of mammalian expression vectors for PLA₂R-Gaussia and PLA₂R-Nano luciferase

A commercially available cDNA clone for full-length human PLA₂R was obtained (Origene). PCR was used to amplify fragments of the coding sequence of PLA₂R cDNA. Several PLA₂R DNA fragments were initially subcloned in-frame into the pREN2 vector by C-terminal fusion down-stream of Ruc as previously described (Burbelo et al., 2005). Two additional PLA₂R luciferase fusion constructs, containing amino acid residues 1 to 858 of PLA₂R, were also generated as N-terminal fusion proteins and the primer adapter sequences used to amplify and clone the PLA₂R fragment are available upon request. One of the N-terminal PLA₂R constructs was built in the mammalian expression vector pGAUS3 vector expressing Gluc (Ferre et al., 2016; Lee et al., 2016) and contained the endogenous start methionine of PLA₂R along with its signal peptide. Another construct using the same N-terminal PLA₂R fragment was generated as a chimeric fusion protein with nanoluciferase (NanoLuc, Promega). In order to build this construct, a new vector derived from pGAUS3, called pNANO3, replaced the Gluc fragment with NanoLuc. The pNANO3 vector was then used to subclone the N-terminal DNA fragment of PLA₂R in front of NanoLuc (Fig. 1). Of note, additional C-terminal PLA₂R fusions with NanoLuc were not tested because NanoLuc-C-terminal fusion proteins show high non-specific binding to the protein A/G beads (Burbelo, unpublished). Plasmids for these different mammalian expression vectors containing the correct PLA₂R inserts were grown up and plasmid DNA was prepared using a Qiagen Midi kit. DNA sequencing confirmed construct integrity.

2.3 LIPS autoantibody analysis

The LIPS technology, employing light-emitting luciferase fusion proteins, was performed as previously described (Burbelo et al., 2009). The different PLA₂R-light emitting constructs were transfected into Cos1 cells with Fugene-6 and cell lysates harvested 48 hours later to obtain crude cell extracts. For autoantibody testing, serum samples were diluted 1:10 in assay buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) and diluted aliquots (10 µl) were then evaluated using a 96-well plate format as described (Burbelo et al., 2009). For these tests, serum (equivalent to 1 µl of serum), 40 µl of buffer A and 50 µl of Cos1 cell extract containing 10⁷ light units (LU) of a particular luciferase-antigen extract were used. After incubation at room temperature for one hour, a microtiter filter plate containing protein A/G beads captured the IgG antibody-antigen complexes for one hour incubation. The antibody-antigen-bead complexes were then washed eight times with buffer A and twice with PBS on a microtiter filter plate to remove unbound antigens. After the final wash, light units (LU) were measured in a Berthold LB 960 Centro microplate luminometer (Berthold Technologies, Bad Wildbad). While injection of coelenterazine substrate (Promega) was used for detection of Ruc and Gluc reporter activity, the Nano-Glo® substrate (Promega) was used for NanoLuc LU measurements.

2.4 Statistical analysis

GraphPad Prism software (San Diego, CA) was used for analyzing the antibody levels in this study. Geometric mean antibody levels, expressed as mean log (10) LU and 95% confidence intervals (CI), were calculated and presented as antilog values. The non-parametric Mann-

Whitney *U* statistical test was used for comparison of antibody levels in the different subject groups. Calculations of sensitivity and specificity for the two PLA₂R LIPS assays involved cutoff limits derived from the mean LU value plus five standard deviations based on results from the nephrotic disease controls. Test performance of the PLA₂R-Gluc and PLA₂R-NanoLuc LIPS tests were also evaluated using the area under the curve (AUC) from receiver operator characteristic (ROC) analysis. The ELISA data in RU/ml were plotted using log 10 transformed values.

3. Results

3.1 Development of recombinant luciferase fusion proteins for detecting PLA₂R autoantibodies

The PLA₂R protein is encoded by 1463 amino acid residues and contains a signal peptide (amino acids 1–20) along with a large extracellular domain (amino acids 21–1397) (Ancian et al., 1995). We initially tested for autoantibodies in seropositive MN patients by measuring two Ruc-tagged C terminal PLA₂R antigen fusion proteins with LIPS. However, these recombinant fusion proteins showed low luciferase activity and testing revealed that they were not useful for detecting PLA₂R autoantibodies (data not shown). We speculated that the problem might be due to improper folding of the PLA₂R protein as C-terminal fusion proteins. As an alternative approach, we generated a large portion of the extracellular region of PLA₂R (amino acids 1–858) as N-terminal chimeric fusion proteins with Gluc and NanoLuc, two luciferases, which have higher luciferase activity than Ruc luciferase. Transfection of each of these constructs into 100 mm² dishes of Cos1 cells produced high amounts of PLA₂R-Gluc and PLA₂R-NanoLuc luciferase activity in the soluble cell extract yielding enough lysate for 200 and 800 clinical LIPS tests, respectively. Below we describe the performance of these recombinant proteins in LIPS.

3.2 Detection of PLA₂R autoantibodies by LIPS for diagnosis of MN

A total of 71 subjects (with and without MN) were tested for PLA₂R autoantibodies using the recombinant PLA₂R-Gluc and PLA₂R-Nano proteins. Among the 45 subjects with MN, 21 were PLA₂R seronegative and 24 were PLA₂R seropositive based on Western blot (albeit three were very weak positives). Both LIPS assays discriminated between the 4 different groups (healthy controls, nephrotic disease controls, PLA₂R seropositive and PLA₂R seronegative MN). Strikingly, the LIPS assays detected much higher autoantibody levels in the seropositive MN subjects compared to the seronegative MN subjects, healthy controls and disease controls (Fig. 1). In the case of the PLA₂R Nano LIPS assay, the geometric mean level (GML) of autoantibodies in the seropositive MN subjects was 1,035,000 LU (95% CI; 935,700–1,375,000) versus 21,050 LU (95% CI; 6,880–55,327) in the seronegative MN subjects, 16,190 LU (95% CI; 14,860–18,190) in nephrotic disease controls and 15,880 LU (95% CI; 14,180–17,850) in healthy controls (Fig. 1B). Further analysis by Mann Whitney *U* tests of both LIPS assays showed highly statistically significant PLA₂R autoantibody levels in the seropositive MN samples compared to the other groups (Fig. 1).

ROC analysis showed that the NanoLuc LIPS assay had perfect diagnostic performance with AUC values of 1.0, while the Gluc assay had a value of 0.998. However, visual inspection of

the scatter plots revealed that the NanoLuc LIPS assay more clearly differentiated the seropositive from seronegative samples than the Gluc assay (Fig. 1). This observation was further substantiated when diagnostic cut-off values were assigned, (derived from the mean plus five standard deviations of the LU values obtained from the nephrotic disease control samples) yielding values of 47,268 LU for Gluc and 33,242 LU for NanoLuc LIPS assay. Interestingly, both LIPS assays detected antibody in an apparently seronegative MN patient (Fig. 1, denoted by the star). Detailed analysis of this patient's serum revealed that it was not immunoreactive with other unrelated Nano-Luc and Gluc antigen reporters (data not shown). This patient was also tested for the presence of antibodies to thrombospondin type 1 domain-containing 7A (THSD7A) which are found in a small subset of patients with primary MN and he was found to be seronegative (data not shown). This patient (Patient 4) was part of the treatment cohort and serial PLA₂R antibody testing was performed (Fig. 2C). LIPS analysis confirmed that the PLA₂R autoantibody levels decreased in response to therapy and correlated with changes in proteinuria (Fig. 3C). Based on these findings, we conclude that this patient was likely seropositive for anti-PLA₂R but was below the level of detection of ELISA or Western blotting. We took these results into consideration when calculating sensitivity and specificity.

Using cut-off values, the PLA₂R-Gluc and PLA₂R-NanoLuc LIPS tests showed 100% (23/23 samples) sensitivity in detecting anti-PLA₂R-positive MN sera (Fig. 1). However, the specificity was greater with the PLA₂R-NanoLuc LIPS test compared to the PLA₂R-Gluc test (100% vs. 95%, respectively). This was due to discordant results in one patient who was negative for anti-PLA₂R by both ELISA and NanoLuc LIPS (Fig. 1B), but demonstrated low positive PLA₂R immunoreactivity with the Gluc reporter (Fig 1A denoted by triangle). Additional testing of this MN sample showed immunoreactivity with several other unrelated, recombinant Gluc-proteins confirming false positive immunoreactivity with the Gluc backbone reporter (data not shown). Together, these results support the better performance of the PLA₂R-NanoLuc compared to the PLA₂R-Gluc LIPS assay for diagnostic screening of MN.

3.3 Measuring serial anti-PLA₂R antibodies by LIPS for MN treatment monitoring

We next compared the efficacy of the PLA₂R-NanoLuc LIPS test against ELISA for longitudinal autoantibody monitoring in the treatment cohort (Patients 1–13) (Waldman et al., 2016). In the pre-treatment samples, eleven of 13 (85%) patients had detectable circulating autoantibodies against PLA₂R based on the LIPS assay (Fig. 2 and data not shown). Nine of these patients were seropositive by ELISA and one additional patient (Patient 5) was seronegative by ELISA, but had very low antibody titer based on Western blot. As shown in Fig. 3D, the LIPS assay was more sensitive than the ELISA for detection of autoantibody in this patient.

After initiation of immunosuppressive treatment, the PLA₂R-NanoLuc LIPS assay detected significant reduction in antibody levels in the anti-PLA₂R seropositive MN patients. These results were concordant with the ELISA (Fig. 2). However, the timing of antibody depletion (defined as immunologic remission) differed between the two assays, likely reflecting the detection thresholds. Based on ELISA, 75% of the anti-PLA₂R seropositive patients

achieved antibody depletion by 3 months, whereas only 33% of patients showed antibody depletion by this time point based on the LIPS assay (Fig. 2, panel A–F). In seven patients, the LIPS assay continued to detect low concentration of autoantibody for up to six months after it was undetectable by ELISA. An increase in antibody levels as detected by LIPS and ELISA was associated with subsequent clinical relapse in three patients (described below; Fig. 2, Panel D–F). With the exception of one false positive sample, as described above, the results generated with PLA₂R-Gluc LIPS assay were in concordance with the PLA₂R-NanoLuc data (**data not shown**).

3.4 Relationship of PLA₂R-NanoLuc LIPS antibody levels to disease activity

Since many studies have shown that the concentrations of anti-PLA₂R antibodies correlate with urinary protein excretion and disease activity (Beck et al., 2011; Hoxha et al., 2012; Kanigicherla et al., 2013; Radice et al., 2016; Timmermans et al., 2015; Waldman et al., 2016), the relationship between PLA₂R autoantibody levels determined by NanoLuc LIPS and changes in proteinuria were evaluated in the treatment cohort. As shown in Fig. 3, immunosuppressive treatment led to reduction in antibody levels in all patients which was then followed by a more gradual decline in proteinuria. In patients who experienced a relapse of the nephrotic syndrome (Patients 5, 6 and 9) an increase or reemergence of antibody preceded the increase in proteinuria (Fig. 3, D–F). In patient 9, both the LIPS and ELISA assays showed that the antibody was fully depleted after three months of treatment, but then reemerged in association with relapse. However, the PLA₂R autoantibody was detected as seropositive by the LIPS assay six months earlier than it was detected by ELISA (18 months vs 24 months respectively) (Fig. 2F). The increase in proteinuria in this patient was apparent by 19 months (Fig. 3F). Thus, the timing of immunologic relapse based on the LIPS assay fit the clinical observations better than the results from the ELISA. Patient 6 became seronegative for anti-PLA₂R by 3 months based on the ELISA and then re-emergence of antibody was detected at 12 months (Fig. 2E). Based on the LIPS assay, this patient never achieved seronegative status before relapse. Clinically, this patient only transiently achieved partial remission of proteinuria (at 9 months). Again, the antibody profile provided by LIPS correlated better with the clinical observations than did the ELISA results. Patient 5 did not have detectable PLA₂R antibody at baseline based on ELISA (as described above) but then had appearance of anti PLA₂R antibody at 24 months which coincided with clinical relapse whereas she was seropositive by LIPS at baseline, became seronegative by 3 months and then had reappearance of antibody by 24 months. These results support that the LIPS assay is highly sensitive for detecting clinically important changes in autoantibody levels.

Overall comparison of the LIPS autoantibody levels obtained with the PLA₂R-NanoLuc assay in the 11 PLA₂R seropositive-treated MN correlated well with the level of proteinuria over the different time points ($R=0.79$, $p<0.001$). In contrast, the level of PLA₂R autoantibodies at baseline as determined by LIPS did not correlate well with the corresponding level of proteinuria as reflected by the poor correlation coefficient ($R=0.05$). Other studies have found varying associations between level of proteinuria and antibody levels at time of disease onset (Hofstra et al., 2011; Hofstra et al., 2012; Li et al., 2016) and

our low correlation coefficient may be due to our patient cohort or differences in autoantibody detection.

4. Discussion

The M type phospholipase A₂ receptor represents a major target of pathogenic autoantibodies in primary MN. Its large size and the conformational epitopes associated with its extracellular domain structure make PLA₂R a challenging target for the development of serological immunoassays. All published clinical tests employ mammalian-cell-derived PLA₂R recombinant protein as the target autoantigen. Two of the immunoassays, IIF-CBA (Hoxha et al., 2011) and Western blotting (Beck et al., 2009), overexpress PLA₂R in mammalian cells and use the overexpressing cells or cell extracts, respectively, to produce qualitative results. The ELISA (Dahnrich et al., 2013; Timmermans et al., 2014) and ALBIA immunoassays generate quantitative results from solid-phase immunoassays that employ recombinant PLA₂R protein purified from mammalian cells. Here we demonstrate that LIPS can accurately measure PLA₂R autoantibody levels with high diagnostic performance that matches the ELISA and Western blotting in specificity, but has higher sensitivity for detecting seropositivity in MN samples. The LIPS format showed robust detection for diagnosis, reliable differentiation of primary and secondary forms of MN and may provide a better reflection of disease activity than ELISA because it detected circulating antibody when it was below the detection threshold for the ELISA. The PLA₂R target used in LIPS corresponds to a protein fragment containing only the first 858 of 1359 amino acid residues of the extracellular domain and represents a smaller size PLA₂R recombinant protein than used by Western blotting, ELISA and other immunoassays (Beck et al., 2009; Behnert et al., 2014; Dahnrich et al., 2013; Hoxha et al., 2011; Timmermans et al., 2014). It is important to highlight that this N-terminal protein fragment used for LIPS testing contains two of the three known immunodominant PLA₂R epitopes, the cysteine-rich and C-type lectin domain 1 (CTLD1) domains, which are both located within the N-terminal 350 amino acid residues of the molecule. At the time we initiated this project, the third immunodominant region, the CTLD7 domain located between amino acid residues 1116–1233 involved in epitope spreading (Fresquet et al., 2015; Kao et al., 2015; Seitz-Polski et al., 2016), was not known and was not included for LIPS testing due to technical reasons related to the recombinant molecule construction. Since peptide mapping studies analyzing autoantibody immunoreactivity have also found that many of the antigenic epitopes of PLA₂R are conformational in nature (Behnert et al., 2013; Kao et al., 2015), these results obtained by LIPS also likely implicate the superiority of fluid-phase immunoassays over solid-phase immunoassays in detecting conformational epitopes (LiuEisenbarth, 2007). Our current work along with other published and unpublished studies highlight the advantages and disadvantages of performing LIPS with different luciferase reporters. As described here for PLA₂R, NanoLuc produces excellent N-terminal antigen fusion proteins for LIPS. Moreover, the high luciferase activity levels of N-terminal fusion proteins with Gluc also renders it a better option for studying antibody responses against secreted and membrane proteins than Ruc. However, our studies have also found that some Gluc constructs can occasionally show false positive immunoreactivity with selected sera. Based on these

findings, potential immunoreactivity should be independently validated with independent immunoassays and/or tested by LIPS using other luciferase fusions.

The percentage of anti-PLA₂R seropositivity in the MN treatment cohort was higher based on the LIPS assay as compared to the ELISA and Western Blot assays (85% vs. 69% vs. 77%, respectively). The LIPS assay detected antibody in two additional samples compared to ELISA and one additional sample compared to Western Blot. These differences in seropositivity reflect differences in the sensitivity of assays. Work by Qin et al also highlighted how changes in the detection assay result in greater prevalence of anti-PLA₂R positivity in a given cohort. In a Chinese cohort with primary MN, 82% of patients were anti-PLA₂R positive based on standard Western blot but use of a more sensitive assay found low titer anti-PLA₂R antibody in 10 of 11 apparently negative patients which increased the prevalence of PLA₂R seropositivity to 98% (Qin et al., 2011). Importantly, despite the increased sensitivity of the LIPS assay, the specificity of the LIPS assay remained high and antibody seropositivity was not found in the nephrotic disease controls, in patients with secondary causes of MN or healthy controls.

Assessment of the longitudinal performance of the NanoLuc LIPS assay in the treatment cohort showed excellent correlation between antibody levels and clinical parameters. In accordance with published studies, changes in antibody levels detected by LIPS preceded changes in urinary protein excretion and a decrease in anti PLA₂R antibody level was associated with improvement, whereas reappearance heralded a relapse of the disease. The LIPS had greater sensitivity than the ELISA such that the LIPS continued to detect low levels of antibody after initiation of treatment when it was below the detection threshold of ELISA; conversely, the LIPS assay detected antibody earlier than the ELISA in the setting of relapse. This greater sensitivity of the LIPS assay is significant for several reasons: anti-PLA₂R levels at the completion of therapy may predict long term outcome. In a study by Bech et al, almost 60% of patients who were antibody negative at the end of therapy remained in remission whereas all patients who had persistent antibody experienced clinical relapse (Bech et al., 2014). Second, it is anticipated that immunosuppressive treatment of MN will be tailored based on the individual's antibody profiles such that treatment may be discontinued when antibody is depleted in order to minimize toxicity. This underscores the importance of being able to detect low levels of autoantibody to avoid premature discontinuation of therapy which can lead to relapse.

In conclusion, the PLA₂R-Nano LIPS assay represents a simple and accurate immunoassay for the detection and monitoring of PLA₂R autoantibodies in primary membranous nephropathy. The ease and simplicity of the high-throughput LIPS format, requiring only a few hours of hands-on work without autoantigen purification is highly advantageous for testing large numbers of human samples. Incorporation of additional autoantigen targets such as thrombospondin type 1 domain-containing 7A (THSD7A) (Tomas et al., 2014), found in a smaller subset of PLA₂R seronegative MN patients might further increase the clinical applicability of this format for diagnosing and monitoring MN patients. Lastly, applying LIPS and other immunoassays to study the autoantibody profile of MN patients who show spontaneous remission, might yield further insights into disease mechanisms and novel pathways for therapeutics.

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Highlights

- We describe LIPS assays for measuring PLA₂R autoantibodies for diagnosis of primary MN using N-terminal PLA₂R-Gluc and PLA₂R-NanoLuc chimeric proteins.
- We compared the performance of the LIPS assays to the ELISA for assessing longitudinal changes in PLA₂R autoantibody levels and examined the relationship between changes in antibody level and clinical disease activity.
- The PLA₂R-NanoLuc LIPS immunoassay demonstrated 100% sensitivity and specificity, reliably detected important changes in antibody levels better than the ELISA method, and antibody levels correlated well ($R=0.78$) with disease activity as defined by urinary protein excretion.

Fig. 1.

LIPS detection of PLA₂R autoantibodies in healthy normal controls, nephrotic disease controls and MN patients. Classification of serologic status of MN patients as seronegative (Sero neg) or seropositive (Sero pos) was based on PLA₂R antibody testing by ELISA and/or Western blot. Autoantibodies against PLA₂R were measured by LIPS using chimeric fusion proteins with (A) Gluc and (B) NanoLuc. Each circle represents antibody levels in light units (LU) derived from individual subjects plotted on the Y-axis using a log₁₀ scale. The cut-off value for the two different reporters is shown by the dotted line and was based on the mean plus 5 SD of the nephrotic disease control group. Low levels of PLA₂R antibody was detected by both LIPS assays in one seronegative MN patient (denoted by star) and was considered to be a “true positive” (see text for details). One other seronegative MN patient (denoted by the triangle) had antibody detected by the Gluc LIPS assay (A) but not with the NanoLuc LIPS (B). Further testing supported that the result obtained with the Gluc LIPS assay was a false positive. Statistical analysis by the Mann Whitney *U* test only evaluated the differences in the antibody levels between the MN seropositive group with the disease controls and MN seronegative group.

Fig. 2. PLA₂R autoantibodies for monitoring response to immunosuppressive treatment of MN. Representative plots comparing serial anti-PLA₂R titers detected by LIPS assay and ELISA in PLA₂R autoantibody positive MN patients enrolled in a clinical trial and followed for 24 months. The X-axis denotes the time in months after initiation of immunosuppressive treatment. The left Y axis represents the scale of the PLA₂R autoantibody levels in LU determined by LIPS and the values are plotted in blue. The ELISA values in RU/ml were log 10 transformed and are plotted on the right Y axis in black. There was a significant reduction in the PLA₂R autoantibody levels after treatment as determined by LIPS and ELISA. Antibody remained detectable above the cutoff (blue dotted line) for longer duration by the LIPS assay compared to the ELISA (black dotted line). In three patients (P5, P6 and P9), an increase in antibody levels detected by LIPS and ELISA, denoted by the arrows, preceded clinical relapse as defined by an increase in proteinuria. The pre-treatment (baseline) serum sample from patient P4 showed autoantibody levels in a range that was detectable by LIPS, but undetectable by ELISA or Western blotting.

Fig. 3.

Association between anti-PLA₂R autoantibodies and clinical disease activity. Representative plots of PLA₂R autoantibody levels by the PLA₂R-NanoLuc LIPS (blue circles) and proteinuria (black squares) over time in seropositive patients in the treatment cohort are shown. The X-axis denotes the time in months after initiation of immunosuppressive treatment. The left Y-axis represents the scale of the PLA₂R autoantibody levels in LU determined by LIPS. The right Y-axis denotes the level of urinary protein excretion expressed as grams per day. The blue dotted line represents the detection threshold for LIPS; the black dotted line represents complete remission status (proteinuria <300 mg/day). PLA₂R autoantibody levels dropped substantially after immunosuppression initiation (**Panel A–F**). There is a lag time between the immunologic remission and clinical remission. In Patients 5, 6 and 9, PLA₂R autoantibody level reemerged, which was associated with clinical relapse of disease (denoted by arrows) and an increase in proteinuria (**Panel D, E and F**). Patient 4 was seronegative by ELISA and western blot but low level of antibody was detected by LIPS (**Panel C**). During treatment the antibody dropped further to undetectable levels and was followed by complete remission of proteinuria