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TITLE PAGE**Letter to the Editor****Improvement of Idiopathic Membranous Nephropathy diagnosis with ultrasensitive quantitative detection of Anti-Phospholipase A2 Receptor**

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Capsule Summary

The work focused on development of a sensitive, quantitative assay for serum PLA2R-IgG with wide dynamic working range, as an aid to differential diagnosis of IMN and to exclude secondary Membranous Nephropathy with slight elevation of anti-PLA2R-IgG from IMN.

Key words

Membranous nephropathy, idiopathic membranous nephropathy, phospholipase A2 Receptor; time-resolved fluoroimmunoassay

To the Editor:

The diagnosis of glomerulonephritis is generally based on kidney biopsy, which few diagnostic serologic markers have been described. M-type phospholipase A2 receptor (PLA2R) is a type I transmembrane protein abundantly expressed on glomerular podocytes. Antibodies against PLA2R were described by Beck et al¹ as serum markers for idiopathic membranous nephritis (IMN). Recently, anti-PLA2R antibodies were found to correlate with disease activity and proteinuria in IMN.² Anti-PLA2R antibody levels decreased or disappeared upon treatment induced or spontaneous remission in patients with IMN. In serial investigations, anti-PLA2R antibodies were found in 70% of IMN and a small percentage (5–25%) were positive in sera from patients with secondary membranous nephritis (MN) by qualitative analysis, such as Western immunoblot, IIF-CBA, and ELISA,^{3,4,5} which negatively impacted the differential diagnosis of IMN. A quantitative assay is needed to distinguish IMN from secondary MN. Taking advantage of the highly sensitive time-resolved fluoroimmunoassay (TRFIA) format, we developed a quantitative method for detection of anti-PLA2R antibody in serum, and utilized it in the evaluation of renal patients. In this study, recombinant PLA2R was generated by cloning into 293T cells. Goat anti-human IgG antibodies (Jackson Immuno Research, USA) were labeled with Eu³⁺. The optimal coating concentration of PLA2R antigen was approximately 5 µg/mL. The addition of a fluorescence intensifier enhanced the original fluorescence by 1 million times and improved the sensitivity and range in the anti-PLA2R-IgG-TRFIA. AutoDELFI₁₂₃₅ (Perkin Elmer, USA) was used to read Eu³⁺ fluorescence in microtiter wells.

Serum samples from 286 healthy volunteers without evidence of nephropathy, gastroduodenal disorder, or liver diseases were collected from Jiangyuan Hospital (China). Serum samples collected from 161 kidney disease patients were from the Affiliated Wuxi People's Hospital of Nanjing Medical University (China). Each patient had renal biopsy and pathological examination at the time of blood sampling. Of these cases, 77 were IMN, 53 were IgA nephropathy, 14 were lupus erythematosus nephropathy, 2 were Henoch Schonlein purpura nephritis, 5 were diabetic nephropathy, 2 were malignant hypertension arteriolar nephrosclerosis and 8 were hepatitis B virus-associated glomerulonephritis. The institutional review boards of Jiangyuan Hospital and the Affiliated Wuxi People's Hospital of Nanjing Medical University approved the study, respectively. The written informed consents were all obtained from the subjects.

IMN was diagnosed by renal biopsy in patients who lacked features suggestive of secondary membranous nephropathy. Moreover, the IMN biopsy was scored according to the severity of the deposit in a subepithelial location (II>I). Serum anti-PLA2R-IgG concentrations were detected by anti-PLA2R-IgG-TRFIA. Statistical analyses were performed with Prism 5.0 software (GraphPad Software, USA).

The detection range of anti-PLA2R-IgG-TRFIA was 0.02-340 mg/L. The intra- and inter-assay coefficients of variation (CVs) were 3.2% and 5.6%, respectively. Based on the disease pathology in kidney tissue samples, the 77 cases of IMN were divided into IMN I, which was 44 cases characterized by the presence of scattered or more regularly distributed small immune-complex-type electron-dense deposits in the subepithelial zone, and IMN II, which was 33 cases characterized by projections of basement membrane material around the

subepithelial deposits. Serum from healthy volunteers and patients with IgA nephropathy, lupus nephropathy and other kidney disease were analyzed (see Fig. 1 and Table 1).

Using a cut-off of <0.9 mg/L (based on the mean of the healthy volunteers $+2.58SD$), the positive rate for the IMN was 88.3%. However, 35.8% IgA nephropathy cases, and 50.0% lupus nephropathy cases would also be positive. To improve the clinical utility, we adjusted the cut-off value to 1.99mg/L based on the ROC curves (see Fig. E1),so that all cases of IgA nephropathy, lupus erythematosus, and other kidney diseases were below the cut-off, while 57/77(74.0%) of IMN were positive. Thus, the positive rate (sensitivity) was 74.0% for INM group and the specificity was 100%. These suggested that IMN could be distinguished from IgA nephropathy, lupus nephropathy and other renal disease using appropriate thresholds of serum anti-PLA2R-IgG levels for different clinical purposes.

To clarify the association of the quantity of anti-PLA2R in serum with the fluorescence intensity of PLA2R antigen in tissue biopsy, a histological examination was performed with the biopsy sample from each patient. Staining with unrelated primary antibody plus secondary antibody was negative in all biopsy samples. Staining with anti-PLA2R antibody resulted in a range of intensities. Subsequently, the agreement of these two methods was analyzed (see Fig. E2). The serum anti-PLA2R level was not directly comparable to the indirect immunofluorescence results.

The discovery and determination of anti-PLA2R-IgG in serum provides an ideal marker to aid in diagnosis, assess activity and treatment timing, select drugs, and evaluate curative effects in IMN. To our best knowledge, this is the first time that a sensitive, quantitative analysis of serum anti-PLA2R-IgG-TRFIA has been established. The observation that some

patients with IMN do not have PLA2R autoantibodies could be explained by limitations of current immunoassays. The analytical sensitivity of our method is 0.02mg/L and 88.3% IMN could be detected, which was better than other reports. Why were anti-PLA2R antibodies not detected in all patients with IMN irrespective of the test used? There are some possible explanations for this discrepancy. First, there are two alleles that account for the genetic risk to develop IMN, HLA-DQA1 and PLA2R1.⁶ Interestingly, the association with HLA-DQA1 was stronger than with PLA2R1, which suggests that HLA-DQA1 allele might facilitate autoantibody development targeting not only PLA2R but also other antigens. Thus, existence of anti-PLA2R antibodies could provide further classification for IMN and enable prognostic analysis. Second, there are previous reports that a small percentage(5–25%) of samples were positive in sera from patients with secondary MN detected by qualitative methods, such as Western immunoblot, IIF-CBA, and ELISA,^{3,4,5} which could impact the differential diagnosis of IMN. In our study, it is possible to distinguish weakly positive IMN from normal control or secondary MN patients through setting suitable thresholds with sensitivity over 74% and specificity up to 100%.

In conclusion, quantitative analysis with high sensitivity could distinguish idiopathic and secondary membranous nephropathy, and anti-PLA2R-IgG-TRFIA offers the opportunity to measure a marker to help diagnose, classify, and eventually monitor the course of patients with IMN.

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Table 1. The serum anti-PLA2R-IgG levels in different test groups

	Healthy Volunteer (negative control) (n=286)	IgA nephropathy (n=53)	lupus nephropathy (n=14)	other kidney disease (n=17)	IMN (n=77)
range of concentrations (mg/ L)	0.07-0.88	0.14-1.78	0.22-1.99	0.15-0.84	0.34-90.89
mean±SD (mg/ L)	0.50±0.16	0.76±0.36	1.17±0.61	0.47±0.28	9.28±15.25
positive rates (anti-PLA2R-IgG>0.91mg/L)	0	35%	50%	4.3%	88.3%
positive rates (anti-PLA2R-IgG>1.99mg/L)	0	0	0	0	74.0%

Figure Legends

Fig 1. Serum anti-PLA2R-IgG levels for TRFIA results by disease state. The determination accuracy of serum anti-PLA2R-IgG, from high to low, is IMN, lupus erythematosus nephropathy, IgA nephropathy, and other kidney disease. IMN I, IMN II, lupus erythematosus nephropathy, IgA nephropathy, and other kidney disease vs. Healthy volunteers was $p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$, $p=0.0037$, respectively. IMN I vs. IMN II was $p=0.0355$.

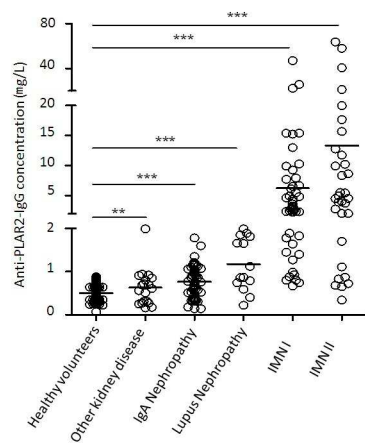


Figure E Legends

Fig E1. Serum PLA2R levels and receiver operating characteristic (ROC) curves for TRFIA results by type of nephropathy. Area-under-the-curve (AUC) of ROC for sensitivity and specificity of anti-PLA2R-IgG in IgA nephropathy, lupus kidney disease, and IMN were >0.500 (0.7285, 0.8472, 0.9798, respectively). The determination accuracy of serum anti-PLA2R-IgG, from high to low, is IMN, lupus erythematosus nephropathy, and IgA nephropathy.

Fig E2. Comparison of serum anti-PLA2R-IgG to PLA2R antigen in situ for IMN.

M-type phospholipase A2 receptor (PLA2R) was detected by indirect immunofluorescence in tissue biopsy samples. Anti-PLA2R-IgG was used to detect PLA2R levels in biopsy samples from each patient to compare with TRFIA results. Fluorescence intensity increased in samples A-D. E. Comparison of detection by immunofluorescence and TRFIA.