

Anti-Phospholipase A₂ Receptor Antibodies Correlate with Clinical Status in Idiopathic Membranous Nephropathy

Julia M. Hofstra,* Laurence H. Beck, Jr.,[†] David M. Beck,[†] Jack F. Wetzels,* and David J. Salant[†]

Summary

Background and objectives Circulating autoantibodies against the M-type phospholipase A₂ receptor (anti-PLA₂R) were recently identified in the majority of patients in the United States with idiopathic membranous nephropathy (iMN). The objectives of this study were to assess the prevalence of anti-PLA₂R in a separate, European cohort of iMN patients and to correlate the presence of anti-PLA₂R with clinical parameters reflective of disease activity.

Design, setting, participants, & measurements Anti-PLA₂R levels were blindly assessed by a Western blot immunoassay in 54 serum samples from 18 patients with iMN collected in various stages of clinical disease. Anti-PLA₂R levels were correlated with other clinical parameters.

Results 77.8% of iMN patients in our cohort had antibodies reactive with human PLA₂R. The antibody levels in these patients correlated strongly with both clinical status and proteinuria ($r = 0.73$, $P < 0.01$).

Conclusions The role of PLA₂R as a major antigen in iMN was confirmed in an independent, European patient cohort, and levels of circulating anti-PLA₂R revealed a strong correlation with clinical disease activity. We propose that detection and measurement of these autoantibodies may provide a tool for monitoring of disease activity and treatment efficacy.

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Introduction

Idiopathic membranous nephropathy (iMN) is a single-organ autoimmune disease and a common cause of the nephrotic syndrome in adults. Accumulated evidence from the experimental rat model of Heymann nephritis and alloimmune membranous nephropathy (MN) from fetomaternal immunization to neutral endopeptidase has advanced the hypothesis that, in human MN, a circulating antibody reacts with and binds to a primary antigenic target on podocytes (1–4). Further insights into the pathogenesis of human disease were established in the past few years, as several other podocyte target antigens have been proposed on the basis of the presence of circulating antibodies in patients with MN (5,6).

Recently, we have identified circulating autoantibodies against the M-type phospholipase A₂ receptor (PLA₂R) in the majority of patients with iMN (5). Cumulative data from serum samples obtained in the Northeastern United States have shown that greater than 70% of patients with iMN have these autoantibodies, primarily of the IgG4 subclass, that are reactive with native and recombinant human glomerular PLA₂R. We have also documented a preliminary association of the presence of circulating anti-PLA₂R

with clinical disease activity (5). The goal of this study was therefore to assess the prevalence of anti-PLA₂R in a separate, European cohort of iMN patients and to correlate the presence of anti-PLA₂R with the clinical parameters reflective of disease activity.

Study Population and Methods

In the Nijmegen Medical Center, patients with recently diagnosed membranous nephropathy are evaluated using a standardized protocol as described (7). In brief, blood samples and timed urine samples are collected for the measurement of serum creatinine, albumin, cholesterol, β -2-microglobulin (β 2m), IgG, and transferrin, as well as urinary excretion of creatinine, β 2m, albumin, IgG, transferrin, and α -1-microglobulin. In addition, aliquots of serum and urine are centrifuged, and the supernatant is stored at -70°C . The patients are prospectively followed with repeated serum and urine collections, and additional data on treatment, remission, and survival are obtained. This protocol was approved by the ethics committee of our center. All of the patients gave written informed consent.

Serum samples of patients with a biopsy-proven iMN and a nephrotic syndrome were collected at

*Department of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands and [†]Department of Medicine, Nephrology Section, Boston University Medical Center, Boston, Massachusetts

Correspondence:

Dr. Julia M. Hofstra, Department of Nephrology 464, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: 31-24-3614761; Fax: 31-24-3540022; E-mail: J.Hofstra@nier.umcn.nl

several time points during their disease and treatment course. Membranous nephropathy was considered to be idiopathic when no secondary cause of MN was suspected on the basis of clinical and laboratory criteria. The samples were categorized by J.M.H. and J.F.W. as: nephrotic syndrome (serum albumin, <3.0 g/dl; proteinuria, >3.5 g/d), spontaneous remission (proteinuria reduction of >50% with proteinuria <3.5 g/d, no treatment with immunosuppressive drugs), treatment-induced remission (proteinuria reduction of >50% with proteinuria <3.5 g/d after treatment with immunosuppressive agents), persistent proteinuria (proteinuria >3.5 g/d or proteinuria reduction <50%), or relapse (proteinuria >3.5 g/d after a period of remission).

Coded samples were analyzed by L.H.B. and D.M.B. (Boston University) without knowledge of the clinical status for the presence of anti-PLA₂R by Western blotting against human glomerular proteins under nonreducing conditions. The sera were tested at 1:25, which has been shown to be both sensitive and specific for iMN (5), followed by detection of human IgG4. For those samples that were negative for IgG4-anti-PLA₂R, the immunoassay was repeated for total IgG anti-PLA₂R. The results were standardized with the use of a single known reactive MN sample, assayed under identical conditions for each experiment. The bands corresponding to PLA₂R were scanned on a Hewlett Packard flatbed scanner, and densitometry was performed using National Institutes of Health ImageJ. The 46 available positive samples were tested in two separate batches. Twenty-four samples were run in both batches to serve as internal controls. The correlation between anti-PLA₂R levels measured in the first and second runs was 0.97.

Statistical Analyses

For descriptive statistics, the data are presented as the means (\pm SD) or medians (range) when appropriate. The correlation between two parameters (nonparametric distributions) was analyzed by Spearman's rank coefficient of correlation. Mann-Whitney U and Kruskal-Wallis tests were used for comparison between groups. All of the statistics were performed using SPSS software, version 17.0 (Chicago, IL). The differences were considered significant with a *P* value < 0.05.

Results

We measured anti-PLA₂R levels by a Western blot immunoassay in coded serum samples from patients with iMN collected when they were nephrotic, in spontaneous or treatment-induced remission, or in relapse (see Study Population and Methods above).

Fourteen of 18 (77.8%) iMN patients had IgG4 autoantibodies to PLA₂R in their baseline sample (taken during a period of nephrotic syndrome). One of these patients did not show reactivity at presentation but showed reactivity in a serum sample taken 4 months later, while persistently nephrotic. The four patients who were nonreactive for IgG4 anti-PLA₂R were also negative when the immunoassay was repeated with an assay for total IgG anti-PLA₂R. Baseline characteristics of all patients are given in Table 1. In these small groups there were no significant differences

Table 1. Baseline characteristics (n = 18)

Gender (male/female)	14/4
Age (years)	51 \pm 10
sCreat (μ mol/l)	122 \pm 44
sAlbumin (g/dl)	2.6 \pm 0.7
Proteinuria (g/d)	10.2 \pm 4.7
β 2m excretion (ng/min)	1786 (99 to 62000)
IgG excretion (mg/d)	249 (21 to 3082)
eGFR (ml/min per 1.73 m ²)	50 (25 to 104)
s β 2m (mg/l)	3.66 (1.63 to 12.48)
sIgG (g/l)	4.34 (1.85–13.35)
Selectivity index anti-PLA ₂ R	0.24 \pm 0.12
(densitometric units)	10,042 (0–30115)

The data are given as the means \pm SD or median (range). Anti-PLA₂R, anti-PLA₂R antibodies; sCreat, serum creatinine; eGFR, estimated GFR calculated by extended Modification of Diet in Renal Disease formula (MDRD6). The selectivity index (SI) was calculated as IgG clearance/transferrin clearance. SI \leq 0.2 reflects selective proteinuria, whereas SI > 0.2 is considered nonselective proteinuria.

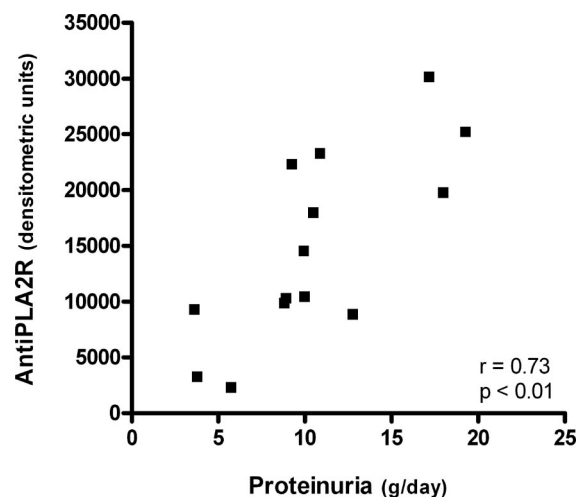


Figure 1. | Correlation between the anti-PLA₂R antibody level and proteinuria (baseline samples, n = 14).

between the anti-PLA₂R-positive and -negative patients with respect to age, gender, serum creatinine, serum albumin, or renal function.

In those patients positive for anti-PLA₂R, the level of autoantibodies as established by densitometry correlated well with several baseline characteristics that are related to the severity of disease. The anti-PLA₂R level correlated positively with proteinuria ($r = 0.73$, $P < 0.01$, Figure 1), serum β 2m ($r = 0.72$, $P < 0.01$), urinary IgG excretion ($r = 0.70$, $P = 0.03$), urinary β 2m excretion ($r = 0.67$, $P < 0.01$), and serum creatinine ($r = 0.57$, $P = 0.03$). Anti-PLA₂R level was negatively correlated with estimated GFR ($r = -0.53$, $P = 0.049$).

Densitometry of the anti-PLA₂R Western blot signal also correlated well with clinical status during follow-up (Table 2). In 12 patients positive for anti-PLA₂R autoantibodies at baseline, a subsequent sample obtained during complete or partial (urine protein <3.5 g/d) remission was available. Remissions could be both spontaneous or treatment-induced. However, when proteinuria at baseline was >10 g/d, no spontaneous remissions occurred. Median time from presentation to onset of remission was 18 months (range, 11 to 45). In seven of the patients, there was also a third sample obtained after a relapse of proteinuria. In one patient (Table 2, Patient 6), a spontaneous remission occurred 16 months after the initial presentation. For this remission there was no sample available, but subsequent samples were obtained during a relapse of proteinuria and a second spontaneous remission after this relapse.

Overall, anti-PLA₂R levels were high in the initial phase of nephrotic syndrome, decreased significantly during remission, and increased again during relapse ($P < 0.01$ Kruskal-Wallis test; Figure 2). In these small groups there were no differences between spontaneous or treatment-induced remissions. Of note, an abnormal pattern was seen in one patient in whom the anti-PLA₂R level did not decrease during partial remission of proteinuria (see Figure 2 and Table 2, Patient 7). In this patient, a relapse of nephrotic range proteinuria occurred during subsequent follow-up and was accompanied by progressive loss of renal function.

We have recently re-evaluated the eight patients who had a relapse of proteinuria after a period of remission. Median follow-up from relapse to final evaluation was 26 months (range, 12 to 96). In three patients, severe proteinuria persisted (median proteinuria, 9.3 g/d; range, 9.3 to 14.2), and in these individuals, the levels of anti-PLA₂R were stable or increased (see supplemental Figure e1 available at the website). In five patients, proteinuria decreased from median 8.3 (range, 6.6 to 10.0) to 4.0 g/d (range, 1.2 to 4.8). In these patients, anti-PLA₂R-autoantibody levels decreased or even disappeared (Figure e1). Of note, when plotting all of the available data of anti-PLA₂R levels against proteinuria, a significant correlation was again observed ($r = 0.75$; Figure 3).

Discussion

We found that 77.8% of iMN patients in our European cohort had antibodies reactive with human PLA₂R, similar to the proportion of anti-PLA₂R-positive primary MN patients in the United States (5) and other international iMN-patient cohorts (L.H.B., unpublished observations). Although the exact mechanisms and the role of PLA₂R in pathogenesis of iMN are currently still unknown, these data strengthen the role of PLA₂R as the major target antigen in iMN. Other recently described antigens may play a role in our anti-PLA₂R-negative patients (6).

Our blinded analysis of samples collected at various stages of clinical disease activity revealed a clear association of anti-PLA₂R antibodies with clinical status as defined by proteinuria. Anti-PLA₂R levels were high when patients had nephrotic levels of proteinuria, were substantially decreased or absent in the setting of spontaneous or treatment-induced remission, and were again increased in

association with disease recurrence. Remarkably, we observed that the level of anti-PLA₂R as semiquantitatively assessed by immunoblotting was positively correlated with the amount of proteinuria. This was not only true for baseline samples but also when combining all data obtained during follow-up. Although a clear causal relationship cannot be established by these data, it is tempting to speculate that higher titers of anti-PLA₂R autoantibodies may lead to more subepithelial antibody-antigen immune deposits, greater podocyte damage, and thus increased levels of urinary protein. This hypothesis is further strengthened by the observation that anti-PLA₂R-autoantibody levels at baseline also strongly correlated with other markers of renal damage such as urinary excretion of β_2m and IgG and serum-creatinine levels. Future work is needed to determine whether these antibodies are directly pathogenic to the human podocyte.

In two patients, findings were seemingly inconsistent. In one patient, anti-PLA₂R autoantibodies were absent at first presentation but clearly positive 4 months later. In the second patient, anti-PLA₂R autoantibodies remained present during remission and were absent with increasing proteinuria during follow-up.

We have no biologically plausible explanation for the findings in the first patient. Autoantibodies were absent in the sample taken at presentation, shortly after a renal biopsy that exhibited all of the features typical of an idiopathic membranous nephropathy and at a time when the patient was severely nephrotic (proteinuria, 12.5 g/d; serum albumin, 2.4 g/dl). Because the repeated sample was strongly positive, we have to acknowledge the possibility of an inadvertent mislabeling of the first specimen.

We have proposed that the immunologically active phase of MN (defined by the presence of circulating anti-PLA₂R antibodies) must end before a corresponding improvement is seen in proteinuria or other clinical features (8). This makes sense conceptually, because some degree of recovery of both the glomerular basement membrane and podocyte structural damage need to occur before the proteinuria can completely resolve. Residual proteinuria may at times remain indefinitely in the absence of immunologic activity, because of tubulointerstitial damage or secondary glomerular changes from prolonged disease. For this reason, a definition of partial remission based only on urinary protein may be flawed, because similar levels of proteinuria can occur with or without the presence of immunological activity. It is thus possible that our patient, in whom there was no anti-PLA₂R detectable in the sample at the end of follow-up, although there was still a persistent proteinuria of 4.8 g/d, had actually already remitted immunologically but had residual proteinuria on the basis of secondary focal segmental glomerulosclerosis lesions. The serum-albumin level at that moment had slightly increased to 3.3 g/dl, which in our opinion supports the diagnosis of secondary focal segmental glomerulosclerosis rather than active membranous nephropathy.

Variability in proteinuria caused by dosing and compliance with inhibitors of the renin-angiotensin system and/or declining renal function may also lead to spurious conclusions about remission status that are based only on amounts of urinary protein. Thus, in the patient in whom

Table 2. Clinical course in anti-PLA₂R positive patients with remission of proteinuria during follow-up (n = 13)

Patient	Presentation			Remission				Relapse					
	Proteinuria (g/d)	eGFR (ml/min per 1.73 m ²)	aPLA ₂ R Level (dens. units)	Time to Remission (months)	Proteinuria (g/d)	eGFR (ml/min per 1.73 m ²)	aPLA ₂ R Level (dens. units)	Type of Remission	Treatment	Time to Relapse (months)	Proteinuria (g/d)	eGFR (ml/min per 1.73 m ²)	aPLA ₂ R Level (dens. units)
1	3.8	71	3203	24	2.0	77	438	Spont.					
2	3.6	62	9251	15	0.2	62	0	Spont.					
3	5.8	83	2236	18	2.1	90	877	Spont.					
4	10.5	25	17927	13	0.4	48	0	Ther.	MMF + P 12 months				
5	12.8	51	8802	19	0.4	96	135	Ther.	MMF + P 12 months				
6	8.8	95	9823	16 ^b	1.6	93	ND ^b	Spont.		67	8.3	95	3780
7	8.9	104	10260	45	1.5	86	8555	Spont.		51	6.6	99	7416
8	12.5	66	25116 ^a	20	0.3	83	0	Ther.	CP + P 12 months	75	20.2	71	19757
9	10.0	44	14510	18	0.7	67	399	Ther.	CP + P 12 months	104	7.1	50	9491
10	18.0	64	19721	28	1.8	57	341	Ther.	CP + P 12 months	69	9.7	50	18373
11	10.0	34	10379	11	0.1	61	0	Ther.	CP + P 12 months	44	7.9	64	9491
12	17.2	31	30115	14	0.8	86	0	Ther.	CP + P 12 months	19	10.0	55	8769
13	10.9	27	23235	23	0.4	35	0	Ther.	CP + P 12 months	65	4.9	31	6934

eGFR, estimated GFR by MDRD6 formula; aPLA₂R, anti-PLA₂R; dens. units, densitometric units; Time to Remission, time between presentation and onset of remission; Time to Relapse, time between onset of remission and onset of relapse; Spont., spontaneous remission; Ther., therapy-induced remission; MMF, myco-phenolate mofetil; P, prednisone; CP, cyclophosphamide.

^aSample 4 months after presentation: in the initial sample anti-PLA₂R was not detected (see text).

^bThere is no sample available for this remission (see text).

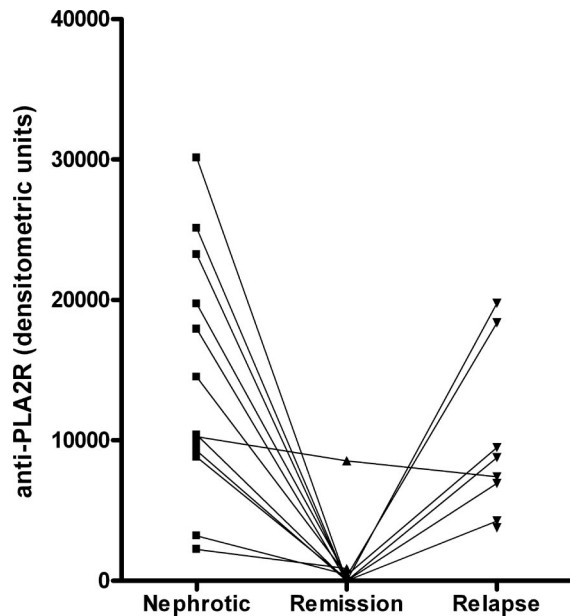


Figure 2. | Anti-PLA₂R-autoantibody levels during the clinical course of 13 anti-PLA₂R-positive patients with remission of proteinuria during follow-up. Nephrotic, sample taken during period of proteinuria >3.5 g/d with serum albumin <3.0 g/dl; Remission, sample taken during clinical remission with proteinuria <3.5 g/d; Relapse, sample taken during clinical relapse of proteinuria >3.5 g/d after a period of remission.

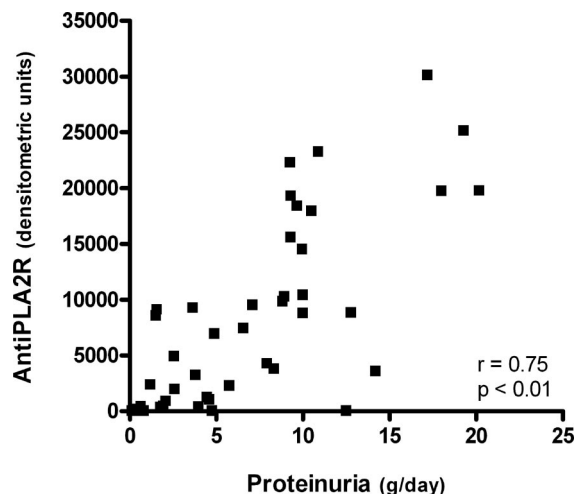


Figure 3. | Correlation between the anti-PLA₂R antibody level and proteinuria. All of the samples were taken during the clinical course ($n = 46$) from 14 patients with positive aPLA₂R antibodies at baseline.

anti-PLA₂R antibody levels were detected despite a clinically-defined remission, the lower level of urinary protein may not have been reflective of a true immunological remission. Indeed, the patient's eventual progressive loss of renal function is consistent with persistent immunological disease activity throughout this apparent remission. This point may become relevant in patients with advanced renal failure from iMN who are eligible for transplantation,

because the presence or absence of anti-PLA₂R auto antibodies may influence the potential for recurrent disease in the allograft (9). However, further studies are warranted to establish the risk of recurrent iMN in anti-PLA₂R-positive patients.

In conclusion, the role of PLA₂R as a major antigen in iMN was confirmed in an independent, European patient cohort, and levels of circulating anti-PLA₂R revealed a strong correlation with clinical disease activity. Furthermore, although anti-PLA₂R-autoantibody levels correlated strongly with proteinuria, we propose that both can also provide independent information, especially when they are discordant. In those occasions when low or absent anti-PLA₂R levels indicate the remission of immunologic activity, "residual" proteinuria may reflect as-yet unresolved structural changes in the glomerulus or interstitial/tubular damage.

We propose that detection and measurement of these autoantibodies may play an important role in the monitoring of disease activity and treatment efficacy. Larger studies are clearly needed to confirm our data and to evaluate the sensitivity and specificity of change in anti-PLA₂R-autoantibody level for predicting a change in disease activity in the individual patient.

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L.H.B. and D.J.S. report having a patent pending for a diagnostic immunoassay to detect anti-PLA₂R autoantibodies in membranous nephropathy.

J.M.H. and L.H.B. contributed equally to this work. D.M.B. is currently a medical student at New York University School of Medicine, New York, New York.

Disclosures

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