CLINICAL RESEARCH

Anti-Phospholipase A_2 Receptor Antibody Titer Predicts Post-Rituximab Outcome of Membranous Nephropathy

Piero Ruggenenti,*† Hanna Debiec,‡§ Barbara Ruggiero,* Antonietta Chianca,* Timothee Pellé,‡§ Flavio Gaspari,* Flavio Suardi,* Elena Gagliardini,* Silvia Orisio,* Ariela Benigni,* Pierre Ronco,‡§ | and Giuseppe Remuzzi*†

*IRCCS—Istituto di Ricerche Farmacologiche Mario Negri, and †Unit of Nephrology, Azienda Ospedaliera Ospedale Papa Giovanni XXIII, Bergamo, Italy; ‡Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche UMR_S1155, Paris, France; §Sorbonne Universités, Université Pierre and Marie Curie University, Paris 06, Paris, France; and | Assistance Publique-Hôpitaux de Paris, Department of Nephrology and Dialysis, Tenon Hospital, Paris, France

ABSTRACT
Rituximab induces nephrotic syndrome (NS) remission in two-thirds of patients with primary membranous nephropathy (MN), even after other treatments have failed. To assess the relationships among treatment effect, circulating nephritogenic anti-phospholipase A_2 receptor (anti-PLA_2R) autoantibodies and genetic polymorphisms predisposing to antibody production we serially monitored 24-hour proteinuria and antibody titer in patients with primary MN and long-lasting NS consenting to rituximab (375 mg/m²) therapy and genetic analyses. Over a median (range) follow-up of 30.8 (6.0–145.4) months, 84 of 132 rituximab-treated patients achieved complete or partial NS remission (primary end point), and 25 relapsed after remission. Outcomes of patients with or without detectable anti-PLA_2R antibodies at baseline were similar. Among the 81 patients with antibodies, lower anti-PLA_2R antibody titer at baseline (P = 0.001) and full antibody depletion 6 months post-rituximab (hazard ratio [HR], 7.90; 95% confidence interval [95% CI], 2.54 to 24.60; P < 0.001) strongly predicted remission. All 25 complete remissions were preceded by complete anti-PLA_2R antibody depletion. On average, 50% anti-PLA_2R titer reduction preceded equivalent proteinuria reduction by 10 months. Re-emergence of circulating antibodies predicted disease relapse (HR, 6.54; 95% CI, 1.57 to 27.40; P = 0.01), whereas initial complete remission protected from the event (HR, 6.63; 95% CI, 2.37 to 18.53; P < 0.001). Eighteen patients achieved persistent antibody depletion and complete remission and never relapsed. Outcome was independent of PLA_2R and HLA-DQA1 polymorphisms and of previous immunosuppressive treatment. Therefore, assessing circulating anti-PLA_2R autoantibodies and proteinuria may help in monitoring disease activity and guiding personalized rituximab therapy in nephrotic patients with primary MN.


In 2002, rituximab, a monoclonal antibody against the cell surface antigen CD20 of B cells, was reported to safely reduce proteinuria and ameliorate nephrotic syndrome (NS) in eight patients with primary membranous nephropathy (MN) and severe NS unresponsive to prolonged angiotensin-converting-enzyme (ACE) inhibitor therapy.1 Subsequent studies consistently confirmed these preliminary findings,2 even when rituximab was administered as second-line treatment in patients who had previously failed

Received July 2, 2014. Accepted November 28, 2014.
P.R., H.D., and B.R. contributed equally to this work as the first author.
P.Ro. and G.R. contributed equally to this work as the last author.
Published online ahead of print. Publication date available at www.jasn.org.
Correspondence: Dr. Giuseppe Remuzzi, IRCCS—Istituto di Ricerche Farmacologiche Mario Negri, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso, Via Stezzano, 67, 24126 Bergamo, Italy. Email: giuseppe.remuzzi@marionegri.it
Copyright © 2015 by the American Society of Nephrology

to respond to steroids, alkylating agents, or calcineurin inhibitors or who had relapsed after transient remission.\textsuperscript{3,4} Finding that rituximab therapy achieved disease remission and stabilized or even improved renal function in 100 patients at high risk of poor outcomes because of persistent NS pointed to a pathogenic role of antibody-producing lymphocytes in primary MN.\textsuperscript{5} Indeed, experimental and human data converge to indicate that deposition along the glomerular basement membrane of immunoglobulins produced by autoreactive B cells initiates the sequence of events, resulting in secondary injury to the glomerular filtering barrier and proteinuria.\textsuperscript{6} Therefore, agents that specifically interfere with B cell antibody production would ideally be the first step toward selective therapy for primary MN.\textsuperscript{7}

The target of these nephritogenic antibodies, however, remained elusive until 2002 when antibodies from a neutral endopeptidase–deficient mother were found to bind neutral endopeptidase expressed on the podocytes of a newborn with congenital MN.\textsuperscript{8} In 2009, Beck and coworkers\textsuperscript{9} found that in approximately 70% of adult patients with primary MN autoantibodies against an antigen normally expressed on the podocyte cell, the M-type phospholipase A\textsubscript{2} receptor (PLA\textsubscript{2}R) circulates and binds to conformational epitopes present on PLA\textsubscript{2}R, producing in situ deposits characteristic of the disease. The landmark discovery that circulating anti-PLA\textsubscript{2}R autoantibodies are almost exclusively specific to primary MN offered clinicians and scientists a powerful tool to differentiate primary from secondary forms and monitor disease activity and response to therapy.\textsuperscript{10}

Finding that some cases of primary MN tend to cluster in families has been taken to suggest that genetic factors may contribute to the production of anti-PLA\textsubscript{2}R autoantibodies. This hypothesis was recently corroborated by genome-wide association studies revealing significant associations of the 6p21 HLA-DQA1 and 2q24 PLA\textsubscript{2}R1 loci with primary MN in patients of white ancestry\textsuperscript{11} and in ethnically distant populations from Europe\textsuperscript{12} and Asia.\textsuperscript{13} Moreover in a Spanish cohort, these HLA-DQA1 and PLA\textsubscript{2}R1 polymorphisms predicted MN response to immunosuppressive agents and disease progression.\textsuperscript{12} Conceivably, sequence variants within HLA-DQA1 alleles that are unique to primary MN may lead to the presentation of peptides to immunocompetent cells, which facilitates the production of autoantibodies against targets such as variants of PLA\textsubscript{2}R.\textsuperscript{11,14}

To assess the complex interactions among circulating anti-PLA\textsubscript{2}R autoantibodies, HLA-DQA1 and PLA\textsubscript{2}R1 polymorphisms, and disease outcome, we took advantage from a cohort of 132 consecutive patients with primary MN and persistent NS treated with the same rituximab-based regimen who consented to genetic studies and prospective monitoring by standardized serial evaluations of antibody titer, proteinuria, and kidney function. This offered a unique opportunity to assess whether response to B cell–depletion therapy is mediated by inhibited anti-PLA\textsubscript{2}R autoantibody production and whether and to what extent it can be affected by intrinsic patient characteristics, including genetically determined risk factors for disease onset and outcome.

**RESULTS**

**Patient Population**

Among 154 patients with biopsy-proven MN referred to our nephrology unit between March 2001 and September 2013, six were found to have a secondary form of the disease and two were found to have an estimated GFR < 20 ml/min per 1.73 m\textsuperscript{2}. Of the 146 patients entering the 6-month run-in period, three received a kidney transplant and four achieved NS remission by combined ACE inhibitor and angiotensin receptor blocker therapy. When the database was locked, two other patients had not received rituximab, and five out of the 137 treated participants had not yet completed the 6-month follow-up period. Therefore, when the database was closed, a total of 132 participants were available for final analyses (Supplemental Figure 1, Table 1). No one of these patients received steroids or any other immunosuppressive medication during the 6 months preceding rituximab administration and throughout the whole observation period. During the 6-month run-in period, 24-hour urinary protein excretion was persistently in the nephrotic range in all included patients. During this period, proteinuria similarly and significantly increased in the 83 patients who received rituximab as first-line therapy and in the 49 who received rituximab after other immunosuppressive regimens had failed (Supplement Figure 2, left panel). A significant increase was also observed in patients with detectable antibodies at inclusion and in those who after rituximab administration progressed to the combined end point of complete or partial remission or achieved no remission. Proteinuria was stable, but persistently in the nephrotic range, in patients with undetectable antibodies or not available antibody evaluation at inclusion and in those who eventually achieved complete remission considered as a single end point (Supplement Figure 2, middle and right panels).

In no considered subgroup proteinuria decreased throughout the 6-month run-in period.

**Baseline Patient Characteristics**

In 81 out of the 101 patients with baseline anti-PLA\textsubscript{2}R antibody evaluation, the antibody titer exceeded the threshold of 14 RU/ml that identifies subjects with detectable antibodies (Figure 1). In the remaining 20 participants, no antibody was detected or the titer was < 14 RU/ml. Baseline characteristics were similar across the 81, 20, and 31 patients with detectable, undetectable, or not-measured circulating anti-PLA\textsubscript{2}R antibodies, with the exception of less severe proteinuria, hypoalbuminemia and hypercholesterolemia, and shorter time to remission in those with undetectable antibodies than in the other two groups (Table 1). Intermediate values for proteinuria and serum albumin and cholesterol levels were found in subjects without antibody measurements. Among the 81 participants with detectable antibodies, the anti-PLA\textsubscript{2}R titer was marginally correlated with the amount of proteinuria ($P=0.06$, Spearman $Rho=0.214$). Consistently, among the three tertiles of anti-PLA\textsubscript{2}R autoantibody titer, participants in the lowest tertile had lower proteinuria than in the other two subgroups (Table 1).
Baseline characteristics and main outcomes of patients in the study group as a whole (overall) and in patients with undetectable, not available, or detectable anti-PLA2R autoantibodies and according to titer tertile of detectable antibodies considered separately

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall (N=132)</th>
<th>Undetectable (n=20)</th>
<th>Not Available (n=31)</th>
<th>Detectable (n=81)</th>
<th>Lowest Tertile (14–86 RU/ml) (n=27)</th>
<th>Middle Tertile (87–204 RU/ml) (n=27)</th>
<th>Highest Tertile (&gt;204 RU/ml) (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55.7±15.4</td>
<td>55.0±17.0</td>
<td>57.0±18.6</td>
<td>55.4±13.8</td>
<td>54.0±11.9</td>
<td>55.1±15.7</td>
<td>57.2±14.0</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>100 (75.8)</td>
<td>17 (85.0)</td>
<td>21 (67.7)</td>
<td>62 (76.5)</td>
<td>18 (66.7)</td>
<td>24 (88.9)</td>
<td>20 (74.1)</td>
</tr>
<tr>
<td>Previous duration of proteinuria (mo)</td>
<td>25.8 (11.0–70.3)</td>
<td>41.0 (10.0–177.1)</td>
<td>30.6 (12.0–64.2)</td>
<td>25.0 (11.0–64.5)</td>
<td>27.2 (10.2–94.4)</td>
<td>22.1 (10.9–51.9)</td>
<td>21.2 (13.0–50.0)</td>
</tr>
<tr>
<td>Previous immuno-suppression, n (%)</td>
<td>49 (37.1)</td>
<td>7 (35.0)</td>
<td>9 (29.0)</td>
<td>33 (40.7)</td>
<td>10(37.0)</td>
<td>12(44.4)</td>
<td>11(40.7)</td>
</tr>
<tr>
<td>B Cell-driven protocol, n (%)</td>
<td>102 (77.3)</td>
<td>18 (90.0)</td>
<td>13 (42.0)</td>
<td>71 (87.7)</td>
<td>22 (81.5)</td>
<td>23 (85.2)</td>
<td>26 (96.3)</td>
</tr>
<tr>
<td>Clinical parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>76.3±13.1</td>
<td>79.5±10.9</td>
<td>73.8±15.7</td>
<td>76.3±12.7</td>
<td>74.6±14.2</td>
<td>78.5±12.0</td>
<td>75.8±12.0</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>134.3±17.8</td>
<td>132.5±15.4</td>
<td>130.5±19.2</td>
<td>136.0±17.9</td>
<td>134.0±15.3</td>
<td>136.4±18.6</td>
<td>137.9±20.4</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>82.1±10.1</td>
<td>80.9±9.6</td>
<td>81.6±13.2</td>
<td>82.6±9.1</td>
<td>82.3±9.8</td>
<td>82.4±9.1</td>
<td>83.3±8.6</td>
</tr>
<tr>
<td>Laboratory parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.21 (1.00–1.73)</td>
<td>1.13 (0.94–1.99)</td>
<td>1.39 (1.01–1.90)</td>
<td>1.21 (1.00–1.71)</td>
<td>1.09 (0.97–1.60)</td>
<td>1.29 (1.08–1.77)</td>
<td>1.26 (0.97–1.87)</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>2.21±0.59</td>
<td>2.69±0.50</td>
<td>2.28±0.52</td>
<td>2.08±0.56</td>
<td>2.21±0.61</td>
<td>2.06±0.51</td>
<td>1.96±0.57</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>50 (42–63)</td>
<td>44 (40–53)</td>
<td>51 (45–56)</td>
<td>53 (42–66)</td>
<td>59 (44–69)</td>
<td>53 (45–63)</td>
<td>49 (39–69)</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>158 (110–225)</td>
<td>160 (131–209)</td>
<td>180 (131–230)</td>
<td>141 (98–233)</td>
<td>127 (88–180)</td>
<td>161 (118–265)</td>
<td>194 (100–270)</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>9.1 (5.8–12.7)</td>
<td>6.3 (3.4–12.0)</td>
<td>9.2 (6.2–12.0)</td>
<td>9.8 (5.9–13.4)</td>
<td>7.4 (4.3–10.5)</td>
<td>10.8 (6.7–14.8)</td>
<td>11.6 (7.6–13.4)</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined end point</td>
<td>84 (63.6)</td>
<td>12 (60.0)</td>
<td>24 (77.4)</td>
<td>48 (59.3)</td>
<td>22 (81.5)</td>
<td>16 (59.3)</td>
<td>10 (37.0)</td>
</tr>
<tr>
<td>Time to relapse (mo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relapse</td>
<td>7.1 (3.2–12.2)</td>
<td>3.5 (2.4–6.6)</td>
<td>8.6 (2.5–15.5)</td>
<td>7.4 (3.9–12.2)</td>
<td>5.4 (4.1–8.8)</td>
<td>9.1 (3.5–16.4)</td>
<td>11.6 (5.3–24.8)</td>
</tr>
</tbody>
</table>

Variables expressed as mean±SD are compared using one-way ANOVA; variables expressed as median and IQR are compared using Kruskal-Wallis test. Categorical variables are expressed in percentage and compared using Pearson chi-squared test or Fisher exact test, as appropriate. The titer range for each tertile is listed in brackets.

*P<0.001 among subgroups (undetectable, not available, and detectable or lowest, middle, and highest tertile).

bP<0.01 for detectable or not available versus undetectable group and for highest or middle versus lowest tertile.

cP<0.05 for detectable or not available versus undetectable group and for highest or middle versus lowest tertile.

dP<0.001 for detectable or not available versus undetectable group and for highest or middle versus lowest tertile.

*P<0.01 among subgroups (undetectable, not available, and detectable or lowest, middle, and highest tertile).
Over a median (range) follow-up of 30.84 (6.00–145.36) months, 84 out of the 132 rituximab-treated patients (63.6%) progressed to the combined end point, including 43 patients (32.6%) who achieved complete remission considered as a single end point. These patients had lower serum creatinine levels and less severe NS at baseline than those never achieving remission (Table 2). Treatment effect on the primary end point and on complete remission considered alone was similar in patients receiving the four-dose or the B cell–driven regimen (Figure 2, right and left panels, respectively). The probability of achieving the combined or single end point did not differ significantly among subjects with or without detectable antibodies and those without antibody measurements (Figure 3, right and left panel, respectively). At multivariable analyses, detectable lower anti-PLA2R titer strongly \((P=0.001)\) predicted the combined end point (Supplement Table 1). Consistently, patients in the higher autoantibody tertile had a lower probability to reach the end point \((P=0.003)\). Therefore, the antibody titer showed a clear trend \((P<0.001)\) to increase from patients achieving complete remission considered as a single end point \((86.7; \text{interquartile range [IQR]}, 36.5–145.7 \text{RU/ml})\) to those achieving partial remission \((129.3; \text{IQR}, 61–256.8 \text{RU/ml})\) or no remission \((212.9; \text{IQR}, 116.5–352.6 \text{RU/ml})\).

Within the cohort of the 81 participants with detectable antibody levels at baseline, the probability of progression to the combined end point was approximately four- and two-folds higher in those in the lowest and middle tertile of antibody titer compared with those in the highest tertile, respectively (Figure 4, left panel). When complete remission was considered as a single end point, the probability was approximately three-folds higher in the lowest and middle tertile compared with the highest one (Figure 4, right panel). Consistently, time to the end point progressively increased from the lowest to the middle and the highest tertile \((P=0.003\) for trend), (Table 1).

### Polynomial Models
Among the 101 subjects with measured anti-PLA2R autoantibody titers at baseline, the probability of progressing to the combined end point was lower in those with undetectable antibodies than in those with the lowest detectable antibody titer (Supplement Figure 3, left panel). Among the 81 patients with detectable antibodies, fitted log-hazard ratios for the probability of complete or partial remission from modified fractional polynomials (FPs) progressively decreased in parallel with progressively increasing anti-PLA2R autoantibody titer. The highest probability was observed among subjects with antibody titer, approximating the detection threshold of the method \((14 \text{RU/ml})\). The probability tended to plateau for antibody levels exceeding 500–600 RU/ml (Supplement Figure 3, left panel). Similar findings were observed when complete remission was considered as a single end point, even if the analysis was less

**Figure 1.** Outcome of the 132 study patients who were available for analyses after rituximab administration. The flow chart shows how many of the 132 patients were or were not screened for anti-PLA2R antibodies and in how many of screened patients, the antibodies were or were not detectable. For each subgroup the flow chart provides detail on patients achieving or not achieving remission after rituximab therapy, progressing or not progressing to a relapse after initial remission, who are completing active follow-up after rituximab administration or are planned for rituximab therapy.

**Primary Outcome**
Over a median (range) follow-up of 30.84 (6.00–145.36) months, 84 out of the 132 rituximab-treated patients (63.6%) progressed to the combined end point, including 43 patients (32.6%) who achieved complete remission considered as a single end point. These patients had lower serum creatinine levels and less severe NS at baseline than those never achieving remission (Table 2). Treatment effect on the primary end point and on complete remission considered alone was similar in patients receiving the four-dose or the B cell–driven regimen (Figure 2, right and left panels, respectively). The probability of achieving the combined or single end point did not differ significantly among subjects with or without detectable antibodies and those without antibody measurements (Figure 3, right and left panel, respectively). At multivariable analyses, detectable lower anti-PLA2R titer strongly \((P=0.001)\) predicted the combined end point (Supplement Table 1). Consistently, patients in the higher autoantibody tertile had a lower probability to reach the end point \((P=0.003)\). Therefore, the antibody titer showed a clear trend \((P<0.001)\) to increase from patients achieving complete remission considered as a single end point \((86.7; \text{interquartile range [IQR]}, 36.5–145.7 \text{RU/ml})\) to those achieving partial remission \((129.3; \text{IQR}, 61–256.8 \text{RU/ml})\) or no remission \((212.9; \text{IQR}, 116.5–352.6 \text{RU/ml})\).

Within the cohort of the 81 participants with detectable antibody levels at baseline, the probability of progression to the combined end point was approximately four- and two-folds higher in those in the lowest and middle tertile of antibody titer compared with those in the highest tertile, respectively (Figure 4, left panel). When complete remission was considered as a single end point, the probability was approximately three-folds higher in the lowest and middle tertile compared with the highest one (Figure 4, right panel). Consistently, time to the end point progressively increased from the lowest to the middle and the highest tertile \((P=0.003\) for trend), (Table 1).

**Polynomial Models**
Among the 101 subjects with measured anti-PLA2R autoantibody titers at baseline, the probability of progressing to the combined end point was lower in those with undetectable antibodies than in those with the lowest detectable antibody titer (Supplement Figure 3, left panel). Among the 81 patients with detectable antibodies, fitted log-hazard ratios for the probability of complete or partial remission from modified fractional polynomials (FPs) progressively decreased in parallel with progressively increasing anti-PLA2R autoantibody titer. The highest probability was observed among subjects with antibody titer, approximating the detection threshold of the method \((14 \text{RU/ml})\). The probability tended to plateau for antibody levels exceeding 500–600 RU/ml (Supplement Figure 3, left panel). Similar findings were observed when complete remission was considered as a single end point, even if the analysis was less
Table 2. Baseline characteristics of patients with combined end point, complete remission alone, or no remission; patients with or without relapse after initial remission; and responders or nonresponders

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Combined End Point (n=84)</th>
<th>Complete Remission (n=43)</th>
<th>No Remission (n=48)</th>
<th>Relapse (n=25)</th>
<th>No Relapse (n=59)</th>
<th>Responders (n=18)</th>
<th>Nonresponders (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55.0±16.3</td>
<td>55.7±16.8</td>
<td>57.0±13.9</td>
<td>51.3±15.2</td>
<td>56.6±16.6</td>
<td>59.6±16.9</td>
<td>54.0±12.9</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>54 (64.3)</td>
<td>24 (55.8)</td>
<td>46 (95.8)</td>
<td>17 (68.0)</td>
<td>37 (62.7)</td>
<td>11 (61.1)</td>
<td>15 (93.8)</td>
</tr>
<tr>
<td>Previous duration of proteinuria (mo)</td>
<td>25.2 (10.8–68.6)</td>
<td>21.1 (9.3–64.2)</td>
<td>34.3 (11.5–75.8)</td>
<td>25.2 (11.9–64.5)</td>
<td>25.3 (10.7–68.6)</td>
<td>15.7 (8.8–27.5)</td>
<td>18.8 (11.2–50.4)</td>
</tr>
<tr>
<td>Previous immunosuppression, n (%)</td>
<td>27 (32.1)</td>
<td>14 (32.6)</td>
<td>22 (45.8)</td>
<td>8 (32.0)</td>
<td>19 (32.2)</td>
<td>4 (22.2)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>B cell–driven protocol, n (%)</td>
<td>60 (71.4)</td>
<td>30 (69.8)</td>
<td>42 (87.5)</td>
<td>15 (60.0)</td>
<td>45 (76.3)</td>
<td>13 (72.2)</td>
<td>15 (93.8)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>75.3±13.6</td>
<td>73.5±15.6</td>
<td>78.2±12.2</td>
<td>75.3±10.4</td>
<td>75.6±14.8</td>
<td>75.6±14.8</td>
<td>80.9±13.0</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>133.4±17.9</td>
<td>131.2±14.6</td>
<td>135.8±17.9</td>
<td>128.4±16.6</td>
<td>135.7±18.1</td>
<td>138.1±15.0</td>
<td>132.1±15.1</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81.6±9.6</td>
<td>79.3±8.3</td>
<td>83.0±11.0</td>
<td>81.3±11.7</td>
<td>81.8±8.6</td>
<td>80.5±7.7</td>
<td>83.1±11.1</td>
</tr>
<tr>
<td>Laboratory parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.10 (0.97–1.57)</td>
<td>1.09 (0.90–1.21)</td>
<td>1.65 (1.11–2.26)</td>
<td>1.03 (0.95–1.41)</td>
<td>1.14 (0.97–1.58)</td>
<td>1.12 (0.91–1.29)</td>
<td>1.40 (1.13–1.94)</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>2.31±0.61</td>
<td>2.41±0.55</td>
<td>2.06±0.50</td>
<td>2.30±0.62</td>
<td>2.31±0.62</td>
<td>2.19±0.57</td>
<td>2.03±0.49</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>241 (208–311)</td>
<td>233 (208–294)</td>
<td>288 (235–354)</td>
<td>280 (212–318)</td>
<td>235 (208–303)</td>
<td>270 (216–311)</td>
<td>272 (215–320)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>51 (42–65)</td>
<td>55 (50–72)</td>
<td>47 (39–60)</td>
<td>59 (49–73)</td>
<td>50 (42–63)</td>
<td>58 (50–64)</td>
<td>47 (41–60)</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>134 (100–182)</td>
<td>115 (87–166.5)</td>
<td>246.5 (189–302.5)</td>
<td>147 (113–198)</td>
<td>131 (95–177)</td>
<td>105 (80–177)</td>
<td>270 (110–347)</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>7.6 (4.9–10.8)</td>
<td>6.3 (3.8–9.3)</td>
<td>12.5 (9.0–14.8)</td>
<td>7.5 (4.9–10.6)</td>
<td>7.8 (5.5–10.8)</td>
<td>7.6 (5.6–10.5)</td>
<td>12.9 (10.9–14.8)</td>
</tr>
<tr>
<td>Anti-PLA2R antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undetectable, n (%)</td>
<td>12 (14.3)</td>
<td>7 (16.3)</td>
<td>8 (16.7)</td>
<td>3 (12.0)</td>
<td>9 (15.3)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Detectable, n (%)</td>
<td>48 (51.1)</td>
<td>28 (65.1)</td>
<td>33 (68.7)</td>
<td>14 (56.0)</td>
<td>34 (57.6)</td>
<td>18 (100)</td>
<td>16 (100)</td>
</tr>
<tr>
<td>Not available, n (%)</td>
<td>24 (28.6)</td>
<td>8 (18.6)</td>
<td>7 (14.6)</td>
<td>8 (32.0)</td>
<td>16 (27.1)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Titer (RU/ml)</td>
<td>101.2 (46.6–181.9)</td>
<td>86.7 (36.5–145.7)</td>
<td>212.9 (116.5–352.6)</td>
<td>65.2 (45.8–152.4)</td>
<td>103.0 (47.4–186.5)</td>
<td>101.2 (45.3–139.1)</td>
<td>351.3 (192.7–429.0)</td>
</tr>
<tr>
<td>Re-emergence after</td>
<td>10/44 (22.7)</td>
<td>8/26 (30.8)</td>
<td>NA</td>
<td>8/13 (61.5)</td>
<td>2/31 (6.5)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>depletion, n (%)</td>
<td>13/44 (29.6)</td>
<td>9/26 (34.6)</td>
<td>NA</td>
<td>10/13 (76.9)</td>
<td>3/31 (9.7)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Variables expressed as mean±SD are compared using one-way ANOVA; variables expressed as median and IQR are compared using Kruskal-Wallis test. Categorical variables are expressed in percentage and compared using Pearson chi-squared test or Fisher exact test, as appropriate. NA, not applicable; Nonresponders, patients with persistent nephrotic syndrome without autoantibody depletion; Responders, patients with persistent remission and anti-PLA2R autoantibody depletion.

aP<0.001 for combined end point or complete remission versus no remission.
bP<0.05 for combined end point or complete remission versus no remission.
cP<0.01 for combined end point or complete remission versus no remission.
dP<0.001 for no relapse versus relapse or nonresponders versus responders.
eP<0.05 for no relapse versus relapse or nonresponders versus responders.
fP<0.01 for no relapse versus relapse or nonresponders versus responders.
powerful because of the smaller number of events (Supplement Figure 3, right panel).

Longitudinal Analyses of Continuous Variables
After rituximab therapy, proteinuria considered as a continuous variable progressively decreased in parallel with a progressive increase in serum albumin levels (Figure 5). The titer of circulating anti-PLA2R antibodies promptly decreased after rituximab administration. It achieved the nadir at 6 months post-treatment and stabilized thereafter. Percent reduction of anti-PLA2R titer approximated 70% by 1 month after rituximab administration and preceded similar changes in proteinuria by approximately 2 years. Changes versus baseline for each considered variable at each considered time point were highly significant ($P<0.001$). CD20 cell counts promptly depleted by 1 month post-treatment and progressively recovered approximating baseline values over 18–24 months of follow-up (Figure 5). Changes in anti-PLA2R antibody titer and, as expected, proteinuria were significantly larger in patients progressing to the combined end point or to complete remission alone than in those without remission (Figure 6, highest and lowest panels, respectively). In both groups, however, reduction in antibody titer was prompter and larger than reduction in proteinuria, with the largest antibody reduction being observed in those achieving complete remission considered as a single end point (Figure 6, lowest panel). Conversely, changes in CD20 cell counts were similar across all considered groups, including patients without remission (Figure 6, middle panel).

On the basis of the equations describing fitted values of mean percent changes at different visits versus baseline estimated by FP simple regression, anti-PLA2R autoantibody levels and proteinuria were estimated to decrease by 50% in 0.65 and 10.5 months, respectively, whereas serum albumin concentration was estimated to increase by 50% in 11.4 months (Supplement Figure 4).

Predictive Value of Anti-PLA2R Antibody Depletion
Over 6 months after rituximab administration, anti-PLA2R antibodies were fully depleted from the circulation (antibody titer reduction to $<14$ RU/ml) in 46 out of the 64 patients (71.9%) with detectable antibodies at baseline and in at least one additional evaluation within 6-month follow-up. Of these 46 patients, 41 (89.1%) subsequently achieved complete or partial remission compared with only two of the 18 patients (11.1%) without antibody depletion ($P<0.001$). Complete antibody depletion was observed in 25 out of the 25 patients achieving complete remission compared with 16 out of the 21 patients achieving partial remission (76.2%), considered separately ($P=0.01$). Six-month depletion of anti-PLA2R autoantibodies independently and strongly predicted an increased probability of achieving the combined end point or complete remission considered alone (Figure 7, right panel).

Figure 2. Kaplan–Meier curves for the proportion of participants with primary MN who progressed to the combined end point of complete or partial remission (left panel) or complete remission considered as a single end point (right panel) according to treatment with the four-dose or the B cell–driven regimen. In the unadjusted analysis of complete remission, we included a time-varying covariate to account for the unmet proportional hazard assumption. Progression to both end points did not differ appreciably between groups. Adjustments were by sex and log-transformed serum creatinine, and proteinuria. 95% CI, 95% confidence interval; HR, hazard ratio.

Figure 3. Kaplan–Meier curves for the proportion of participants with primary MN who progressed to the combined end point of complete or partial remission (left panel) or complete remission considered as a single end point (right panel) in three subgroups with or without detectable anti-PLA2R autoantibodies or without anti-PLA2R antibody evaluations at baseline. Progression to both end points did not differ appreciably among the groups. The upper and lower HRs refer to not available and the detectable group versus the undetectable group (reference), respectively. 95% CI, 95% confidence interval; HR, hazard ratio.

Figure 4. FP simple regression for the fitted values of anti-PLA2R titer versus baseline with 95% CI.

Figure 5. Longitudinal analyses of proteinuria and serum albumin levels and anti-PLA2R antibody titer.
left and right panels, respectively). On average, anti-PLA2R depletion preceded the primary end point by a median (IQR) of 2.66 (1.54 to 8.03) months.

### Receiver Operating Characteristic Analyses

Six-month percent reduction of anti-PLA2R autoantibody predicted progression to the combined end point (area under the curve: 0.81, P < 0.01) and complete remission considered alone (area under the curve: 0.68, P = 0.04) on subsequent follow-up (Supplement Figure 5, left and right panels, respectively). Similar analyses failed to detect any predictive value for concomitant changes in CD20 cell counts (data not shown).

## Predictors of Disease Relapse

Twenty-five of the 84 patients (29.8%) who had achieved the combined end point had a NS relapse over a median (IQR) of 37.7 (24.8–49.6) months after rituximab administration. The risk of relapse was similar between patients who had received the standard four-dose or B cell–driven regimen (Figure 8). A second course of rituximab (375 mg/m², one single dose) was administered to 22 of these patients and was planned in the remaining three patients. At database closure, 14 of the treated patients had already achieved remission, and eight were on active follow-up (Figure 1).

Baseline characteristics of relapsers and nonrelapsers were similar (Table 2). Rituximab-induced complete remission strongly predicted a significantly reduced risk of relapse compared with partial remission (P = 0.003, Figure 9).

Among the 84 patients achieving complete or partial remission after rituximab administration, 44 had serial anti-PLA2R autoantibody evaluations after the event. Thirteen of the 44 patients (29.6%) subsequently relapsed. Anti-PLA2R autoantibodies increased/re-emerged into circulation after initial reduction/depletion in ten of the 13 patients who relapsed, but only in three of the 31 who did not relapse (P < 0.001, Table 2). In one out of the three patients with undetectable antibodies at inclusion who eventually relapsed after initial remission, anti-PLA2R autoantibodies emerged into circulation throughout the relapse episode. At Cox regression analysis, antibody increase and/or re-emergence (Figure 10, left panel) or re-emergence only after initial depletion (Figure 10, right panel) strongly and independently predicted disease relapse. Antibody increase or re-emergence preceded disease relapse by a median (IQR) of 2.69 (0.89–6.53) months.

Forty-three out of the 64 patients (76.2%) with detectable anti-PLA2R autoantibodies at baseline and at least one additional evaluation of the antibody titer within 6-month follow-up progressed to the end point. Thirty of them (69.8%) never relapsed throughout the whole observation period after initial remission. In 18 of these patients (60%), complete and persistent remission of the NS was consistently preceded by complete and persistent antibody depletion, whereas 16 patients (25%) never achieved either antibody depletion or disease remission (responders and nonresponders, respectively). Compared with responders, nonresponders had more severe proteinuria and hypertriglyceridemia, higher serum creatinine levels, and more than three-fold higher anti-PLA2R antibody titers (Table 2).

Progression to the combined end point (and NS relapse) was independent of previous exposure to steroid or other immunosuppressive medications (Table 2).
Predictive Value of PLA2R1 and HLA-DQA1 Risk Alleles

Among the 102 participants who consented to genetic analyses, the PLA2R1 and HLA-DQA1 genotypes AA, AG, and GG were observed in 62, 39, and one patient and in nine, 42, and 51 patients, respectively. Homozygous carriers of the A PLA2R1 risk allele had two-fold higher anti-PLA2R antibody titers than AG and GG carriers. When the progression to the combined end point of participants with the AA genotype of the PLA2R1 polymorphism was compared with the progression of those with the AG and GG genotype considered as a whole (dominant model), no significant difference was observed between the two groups (data not shown). Similar findings were observed when the analysis was restricted to the 72 subjects with genetic analyses and detectable anti-PLA2R autoantibodies at baseline (Supplement Figure 6, left panel). When HLA-DQA1 polymorphism was considered, a nonsignificant trend to more remissions was found among GG homozygotes compared with AA or AG carriers considered as a whole (Supplement Figure 6, right panel).

**DISCUSSION**

In this longitudinal, observational study we found that over a >12-year observation period, rituximab therapy achieved a combined end point of complete or partial remission of the disease in 84 out of 132 subjects with primary MN and long-lasting persistent NS. Treatment effect did not appreciably differ between participants with or without detectable anti-PLA2R antibodies into circulation. Among subjects with detectable antibodies, however, a lower antibody titer strongly predicted a higher rate of remissions and a shorter time to remission. Furthermore, 6-month reduction or depletion of anti-PLA2R autoantibodies strongly predicted progression to the combined end point, and a 50% decrease in antibody titer preceded an equivalent decrease in proteinuria or an increase in serum albumin concentration by approximately 10 and 11 months, respectively. Similar findings were observed at sensitivity analyses considering complete remission as a single end point or complete and partial remission combined to serum albumin increase to a normal range as a more restrictive end point in the subset of subjects with hypoalbuminemia at inclusion. Finally, progression to the end point was similar in the three subgroups with detectable or undetectable antibodies or not available antibody data at inclusion, even when six ELISA negative, but Western blot positive, patients were considered in the detectable antibody group. Even in this context, among patients with detectable antibodies, a lower antibody titer at inclusion significantly predicted a higher probability of disease remission.

Among the 84 subjects achieving remission, 25 had a relapse of NS on average approximately 3 years after rituximab administration. At study end, 14 of these patients had already achieved remission after one single 375-mg/m² dose of rituximab. The risk of relapse was remarkably higher among participants who had previously achieved only a partial remission of NS compared with those who had achieved complete remission. Independent of previous outcomes, antibody titer increase, after initial reduction or complete depletion, strongly predicted disease relapse and preceded the onset of nephrotic range proteinuria by approximately 3 months on average. Subjects who were homozygous for the PLA2R1 risk allele A had significantly higher titers of anti-PLA2R autoantibodies. However, multivariable analyses adjusted for baseline anti-PLA2R autoantibody levels and other baseline covariates, failed to detect any significant association between PLA2R1 or HLA-DQA1 polymorphisms and the probability of achieving disease remission after
The probability of achieving remission and the risk of subsequent relapses were similar between patients who had received rituximab as first-line therapy and those who had previously failed treatment with steroids or other immunosuppressive medications and between patients treated with the standard four doses of rituximab or with a B cell–driven regimen. Circulating CD20+ B cells were similarly depleted and similarly re-emerged into circulation after initial depletion in all patients independent of previous immunosuppression, treatment protocol, and outcome.

Altogether, the aforementioned findings converge to indicate that in subjects with primary MN and long-lasting NS, serial evaluation of circulating anti-PLA2R antibodies may help predict response to rituximab and risk of relapse, in particular after initial partial remission. Therefore, in addition to clinical biomarkers, such as proteinuria and kidney function, longitudinal anti-PLA2R antibody evaluation may be of help in clinic and in research to monitor disease outcome and guide rituximab therapy in subjects with primary MN. Conversely, monitoring for circulating CD20+ cells is instrumental to guide B cell–driven rituximab therapy, but it does not add to the predictive value of autoantibodies evaluation. Indeed, CD20+ B cells fail to predict disease outcome most likely because they reflect the whole population of circulating B cells rather than the specific autoreactive clones that can be definitively eradicated by rituximab, at least in patients achieving persisting remission.

Previous studies described small and quite heterogeneous populations of patients with different amounts of proteinuria, even no proteinuria in some cases, with or without concomitant immunosuppression at inclusion and with spontaneous remissions or treatment-induced remissions on exposure to heterogeneous immunosuppressive regimens in the context of an uncontrolled clinical setting. Of interest, one of the aforementioned studies found increased antibody titer during disease relapse in six of seven patients with previous antibody depletion associated with remission of NS. None of the aforementioned studies provided information about predictors of disease relapse or systematically evaluated the interactions among clinical outcomes, circulating anti-PLA2R autoantibodies, and underlying genotypes. Finding that in 81 patients antibody depletion strongly predicted disease remission confirmed and extended previous evidence that in 17 rituximab-treated patients with primary MN, antibody titer reduction was associated with remission of NS. In this study, however, patients were followed for 2 years, which did not allow for assessment of the risk of relapse after initial remission. On the other hand, finding that circulating anti-PLA2R autoantibodies decreased in six patients achieving remission of NS by steroid and cyclophosphamide therapy and eventually increased close to pretreatment levels during disease relapse can be taken to suggest that measuring the antibody titer may help monitor disease activity independent of patient treatment. Here we found that autoantibody re-emergence into circulation after rituximab-induced depletion or titer increase after initial reduction preceded disease relapses. This is consistent with evidence that in active Heymann nephritis, proteinuria reduction is a slow and progressive phenomenon.
that may be observed over extended periods of time after glomerular antibody deposition and once in situ immune deposit formation have been exhausted.\textsuperscript{21} Therefore, the temporal association we found in our patients between early rituximab-induced antibody depletion or reduction and subsequent progressive reduction in proteinuria over several months was consistent with the possibility of a causal relationship between early exhaustion of the pathogenic events sustained by glomerular deposition of nephritogenic anti-PLA\textsubscript{2}R autoantibodies and subsequent remission of NS. Of interest, patients with complete remission of NS more frequently achieved depletion of circulating anti-PLA\textsubscript{2}R autoantibodies than those achieving only partial remission and appeared to be relatively protected from subsequent re-emergence of circulating autoantibodies and disease relapse. A remarkable and rather unexpected finding of this study was that 18 patients achieved persistent antibody depletion and never relapsed after initial complete remission. Conceivably, in this subgroup, rituximab therapy might have definitely eradicated autoreactive B-cell clones and cured the disease. Actually, rituximab can be definitely curative in subjects with aggressive lymphomas, including in those who did not achieve complete response after chemotherapy or later developed recurrent disease.\textsuperscript{22} Our data can be taken to suggest that this evidence can be extended at least to a subgroup of patients with primary MN, including those, as in our study, who had previously failed to respond to steroids, alkylating agents, or calcineurin inhibitors or who had relapsed after transient remission. In other patients with transient remissions and antibody depletion, rituximab appeared to mitigate the disease without definitely stifling the pathogenic mechanisms that underlie proteinuria. In these subjects, a second dose of rituximab allowed achievement of further remission of NS. This provides the background for studies designed to assess whether repeated courses of rituximab may help in achieving sustained disease remission and at the same time may protect patients from prolonged and repeated exposure to steroids and other immunosuppressants and from treatment-related side effects.

On the other hand, evidence that rituximab is not uniformly effective, as previously observed in patients with steroid-dependent or frequently relapsing NS,\textsuperscript{23} may imply different etiologies in some patients, despite similar clinical presentation and underlying pathology of the disease. In particular, why in a small subgroup of patients rituximab therapy failed to achieve remission and to reduce circulating anti-PLA\textsubscript{2}R autoantibodies despite full B cell depletion remains elusive. In rheumatoid arthritis and systemic lupus erythematosus,\textsuperscript{24} rituximab administration reduces circulating autoantibodies much more than total immunoglobulins or antimicrobial antibodies. These data have been taken to suggest that in the aforementioned autoimmune diseases autoantibodies are predominantly produced by short-lived B cells that express the CD20 antigen, whereas other immunoglobulins emerge from differentiated plasmablasts or plasmacells that do not express the antigen.\textsuperscript{25} Consistently, in

Figure 9. Kaplan–Meier curves for the proportion of participants with primary MN with a relapse of the NS after initial remission considered according to previous complete or partial remission. Previous complete remission is associated with a significant protective effect against subsequent disease relapse even after adjusting for age, sex, and log-transformed proteinuria. 95% CI, 95% confidence interval; HR, hazard ratio.

Figure 10. Kaplan–Meier curves for the proportion of participants with primary MN with a relapse of the NS after initial complete or partial remission considered according to previous anti-PLA\textsubscript{2}R autoantibody titer increase/re-emergence after initial rituximab-induced reduction/depletion (left panel) or re-emergence only into circulation after initial depletion (right panel). In both cases, titer increase or antibody re-emergence are associated with a significant excess risk of subsequent disease relapse, even after adjusting for other risk factors (age). 95% CI, 95% confidence interval; HR, hazard ratio.
most patients with primary MN, depletion in circulating anti-PLA2R autoantibodies achieved by rituximab therapy is not paralleled by a concomitant reduction in circulating immunoglobulins, which actually tend to increase in parallel with reduction in proteinuria and amelioration of the NS. Therefore, even in membranous patients, autoantibodies might be predominantly produced by short-lived B cells that express the CD20 antigen and are efficiently depleted by rituximab, whereas other immunoglobulins would be predominantly produced by mature plasma cells that do not express the antigen and are not affected by rituximab. A plausible speculation is that in the minority of patients without appreciable changes in anti-PLA2R antibody titer, nephritogenic autoantibodies could be preferentially produced by differentiated plasmablasts or plasmacells that do not express the CD20 antigen and are not affected by rituximab. Should this be the case, plasmablast- or plasmacell-targeting treatments might be a valuable option for patients with primary MN who do not benefit from rituximab therapy.

Overall, the probability of achieving remission, including complete remission as a single end point, was similar between participants with or without anti-PLA2R autoantibodies at baseline. However, the probability in those without antibodies was lower than in those with the lowest titers of detectable antibodies. We speculate that among subjects with detectable anti-PLA2R autoantibodies, the antibody titer may reflect disease activity, and better outcome of subjects with lower antibody titers might be explained by less active disease. Conversely, the group with undetectable antibodies might include two subgroups. One might include patients with PLA2R-related disease but an extremely low titer of antibodies that can be detected only by particularly sensitive tests, such as Western blot analysis, or patients with detectable antibodies only at kidney biopsy immunostaining (possibly because in these cases circulating autoantibodies are rapidly cleared by the kidney tissue). The other subgroup might include a minority of subjects who, independent of disease activity, are affected by different forms of primary MN sustained by still unknown antibodies different from those targeting the PLA2R antigen. Independent of these considerations, finding that, during the 6-month run-in period, patients without detectable antibodies had a stable nephrotic-range proteinuria, reasonably excluded that failure to detect circulating antibodies could be explained by initial immunologic remission in this subgroup.

Consistent with data from previous studies in Caucasian and Asian populations, we found a significant association of PLA2R1 risk allele A with higher anti-PLA2R antibody titers at baseline; however, we failed to capture any significant association between PLA2R1 and HLA-DQA1 risk alleles and outcome. At variance with previous evidence that the underlying genotype may affect response to steroid and alkylating agents, our present findings suggest that the effect of rituximab therapy is not appreciably affected by either PLA2R1 and HLA-DQA1 polymorphism. Independent of this, our present data clearly show that in this context screening for PLA2R1 and HLA-DQA1 risk alleles do not appear to add substantially to the predictive value of anti-PLA2R autoantibody levels at the time of rituximab treatment and antibody titer changes on follow-up.

An ancillary finding of our study was that the kinetics of circulating CD20+ cells or anti-PLA2R autoantibody depletion and proteinuria reduction after rituximab therapy were very much the same between patients receiving the standard four 375-mg/m² dose protocol or the B cell–driven regimen. These data may be of clinical relevance because avoiding unnecessary re-exposure to rituximab, in addition to being extremely cost-saving, might also limit the production of antichimeric antibodies that may increase the risk for adverse reactions and prevent retreatment of disease recurrences.

### Limitations and Strengths

This was a post hoc analysis of outcome data in patients originally identified and monitored for other purposes. The relatively small number of patients with either detectable anti-PLA2R autoantibodies and PLA2R1 and HLA-DQA1 polymorphism evaluation might have reduced the power of multivariable models to detect the predictive value of considered genotypes. A major strength was that all participants were exposed to the same B cell–depletion regimen by rituximab in the context of a predefined and standardized treatment and monitoring protocol. Proteinuria was measured at each time point in three consecutive urinary collections. This limited random data fluctuations and increased the power of the outcome analyses. Finally, the combined evaluation of anti-PLA2R autoantibody titer (at the time of rituximab administration and subsequent follow-up) and PLA2R1 and HLA-DQA1 polymorphisms allowed an integrated evaluation of the complex interactions of clinical parameters, immunity, and triggering gene variants with disease outcome and response to therapy. Screening for circulating anti-PLA2R autoantibodies by ELISA allowed for the generation of continuous data on antibody titer on the basis of a test that is by far the most frequently used in everyday clinical practice and research. Therefore, compared with Western blot assay, this approach allowed more informative analyses and, at the same time, generated remarkably more generalizable results. In a large cohort of patients with primary MN and long-lasting persistent NS we found that baseline anti-PLA2R autoantibody levels and changes in antibody titer on follow-up independently predicted remission of the disease after rituximab therapy and risk of relapse after initial remission. Screening for PLA2R1 and HLA-DQA1 risk alleles did not appear to add substantially to the predictive value of antibody monitoring, whereas serial counts of circulating CD20+ cells are instrumental to guide rituximab therapy independent of disease activity. In other autoimmune diseases such as thrombotic thrombocytopenic purpura, combined monitoring of pathogenic autoantibodies against the disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), and of markers of microangiopathic hemolysis may help to monitor disease activity and specific intervention (29). Along the same line, combined assessment of
circulating anti-PLA2R autoantibodies and proteinuria might be instrumental to monitor disease activity and guide personalized therapy in patients with primary MN. This can now be addressed in prospective studies designed to test whether titration of rituximab therapy to autoantibody titer may help increase the probability of achieving remission of NS and prevent relapses in patients with initial proteinuria reduction.

**CONCISE METHODS**

**Study Population**
Since April 2001, all consecutive patients referred to our nephrology unit were elected for rituximab treatment if presenting with the following criteria: (1) biopsy-proven MN; (2) creatinine clearance ≥ 20 ml/min per 1.73 m²; (3) 24-hour proteinuria persistently exceeding 3.5 g despite at least 6-month therapy with full-dose ACE inhibitors and optimized conservative therapy, but without steroid or any immunosuppressive medication; and (4) no circulating hepatitis B surface antigens. Patients with secondary forms of MN were not considered (Supplemental Figure 1).

The treatment protocol was approved by the Ethical Committee of the Clinical Research Center of the Mario Negri Institute and the Azienda Ospedaliera, Ospedali Riuniti, Bergamo, Italy. Patients gave written informed consent to rituximab treatment and genetic analyses according to the Declaration of Helsinki guidelines. Rituximab was supplied by the Pharmacy of the Azienda Ospedaliera. No pharmaceutical company was involved.

**Treatment and Monitoring**
Before rituximab administration, proteinuria was measured in three consecutive 24-hour urine collections, and the average value was recorded. Creatinine excretion was measured in the last collection. A blood sample was collected for hematochemistry and blood cell counts. Up to October 2005, patients received four 375 mg/m² weekly doses of rituximab; thereafter, they received a second infusion only when more than five circulating B cells per cubic millimeter were detected 1 week after completion of the first rituximab administration.¹⁵ All of the clinical and laboratory parameters evaluated at baseline were then evaluated at month 1, 2, 3, 6, 9, and 12 after rituximab administration and at least every 6 months thereafter. Anti-PLA2R autoantibodies were evaluated in all available biologic samples at baseline (before rituximab administration) and on subsequent follow-up by ELISA according to manufacturer instructions (EUROIMMUN).

**Anti-PLA2R Autoantibody Evaluation**
After sampling, all sera were immediately aliquoted, frozen, and stored at −20°C. They were thawed only at the time of ELISA measurements. Previously unfrozen samples were never used for the tests. After thawing, all serum samples were tested for the presence of anti-PLA2R total IgG antibodies using the recently developed and validated quantitative ELISA test commercialized by EUROIMMUN (Lübeck, Germany). In brief, sera diluted to 1:100 were incubated with PLA2R already-coated microplates and detected by incubation with anti-human IgG horse-redish peroxidase conjugate. The final concentrations for each sample were calculated from the calibration curve extinction values plotted against the concentration for each calibrator. ELISA cutoff values were established according to manufacturers’ protocol, and the results were considered as negative for < 14 RU/ml and positive for ≥ 14 RU/ml. The coefficients of variation (CV) were assessed by using three selected serum samples covering the measuring range. The intra-assay and inter-assay CVs were on the basis of 20 measurements for each serum in one set or on three-fold replica in ten sets, respectively. In our laboratory, the calculated intra- and interassay CVs are <4% and <9%, respectively. Up to five freeze/thaw cycles were found not to affect anti-PLA2R binding by ELISA.

**HLA-DQA1 and PLA2R1 Single Nucleotide Polymorphisms Genotyping**
Since May 2011, blood samples were collected for genetic analyses. Genomic DNA was isolated from peripheral blood using a standard method. Single nucleotide polymorphisms (SNPs) rs2187668 (located within the first intron of the HLA-DQA1 gene) and rs4664308 (located within the first intron of the PLA2R1 gene) were genotyped using TaqMan SNP Genotyping Assays (C_58662585_10 and C_27902747_10, respectively) according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Amplification reactions were performed on an ABI 7000 Real-Time PCR System (Applied Biosystems). Internal controls for each genotype were included in all runs. Genotype frequencies for both SNPs were within Hardy–Weinberg equilibrium in controls.

**Outcomes**
Primary outcome was a combined end point of complete or partial remission defined as 24-hour urinary protein excretion < 0.3 g or < 3.0 g (with at least 50% reduction versus baseline), respectively.⁵

Secondary outcome included complete remission considered as a single end point; relapse of the NS defined as 24-hour proteinuria increase to ≥ 3.5 g in subjects with previous complete or partial remission; depletion of circulating anti-PLA2R autoantibodies after rituximab administration and re-emergence into the circulation after their initial depletion or increase after initial reduction; and changes over time in 24-hour proteinuria and in serum albumin and anti-PLA2R antibody levels.

Main outcome of sensitivity analyses was a combined end point of complete or partial remission of proteinuria plus serum albumin increase to ≥ 3.2 mg/dl (lower limit of normal range) in subjects with serum albumin levels < 2.5 mg/dl at inclusion.

**Safety**
Serious and nonserious adverse events and unexpected changes in clinical or laboratory parameters observed throughout the whole follow-up period were reported in patient case report forms and monitored up to complete resolution. Any possible causal relationship with rituximab or previous immunosuppressive therapy was evaluated.

**Statistical Analyses**
All patients with at least 6 months of follow-up were considered for the analyses. The Kaplan–Meier method was used to plot the probability of progressing to the primary composite end point, complete remission, or relapse after initial remission. Survival time was determined using the log-rank test and Cox proportional hazards method.
from the beginning of the first treatment until the event of interest. Patients not achieving remission were considered as censored at the time of the last visit with a nonmissing value of proteinuria. Predictors of progression to the composite end point, complete remission, or relapse were modeled by univariable and multivariable Cox regression analyses. The multivariable Cox model for the combined end point included sex and proteinuria and serum creatinine and anti-PLA2R antibody levels at baseline. Proportional-hazards assumption was tested by Schoenfeld residuals. In case of significant results, a time-varying covariate was introduced in the model. The anti-PLA2R autoantibody titer had a semicontinuous distribution because all values not achieving the 14 RU/ml detection threshold of the method were spiked at zero. In the study group as a whole, the titer was considered as a binary variable to identify subjects with detectable or undetectable antibodies. Among patients with detectable autoantibodies, the titer was considered as a continuous variable or as a categorical variable identifying three tertiles of patients with baseline antibody titer 14–86 (lowest), 87–204 (middle), or >204 (highest) RU/ml.

Modified FPs were used to model the antibody titer in a multivariate Cox analysis to estimate the probability of the primary end point, including patients with undetectable antibodies who had their antibody titer spiked at zero.30 The best-fitting model was the one with minimum deviance between a univariate modified FP with spike at zero for antibody titer. The best transformation was included in the multiple modified FP where the confounders considered in the model (sex, log proteinuria, and log creatinine centered on the mean) were selected independently by antibody titer.30–32 According to the deviance criterion for the FP comparison, the fit, including log-transformed antibody titer, was only slightly worse than the minimum deviance model (468.30 versus 467.81). Therefore, the modified multivariate model of choice included log-transformed anti-PLA2R autoantibody titer adjusted by sex and log-transformed creatinine and proteinuria. Fitted log-hazard ratio for the risk of combined end point or complete remission in FP-spike models was plotted against antibody titer.

Baseline parameters were shown as numbers and percentages, means and SDs, or medians and IQRs, as appropriate. Groups were compared by one-way ANOVA, Kruskal–Wallis test, Pearson chi-squared test, Fisher exact test, or nonparametric test for trend, as appropriate. Mean percent changes in proteinuria and serum albumin and anti-PLA2R antibody levels at different visits versus baseline were plotted and compared using paired t tests. Proteinuria at inclusion and baseline evaluation were plotted by box plots and compared by Wilcoxon matched-pair signed-rank test. Fitted values of mean percent changes estimated by FP simple regression were plotted, and relative equations were represented. The associations between 6-month percent reduction in anti-PLA2R autoantibody titer and progression to the combined end point or complete remission considered as a single end point were evaluated by receiver operating characteristic curve and logistic regression analysis. Normality for continuous variables was assessed by means of the Q-Q plot. Correlation analyses between continuous not normally distributed variables were carried out by Spearman correlation coefficient (Rho). All P values were two sided. Data were analyzed using SAS version 9.1 and Stata version 13 software.

**ACKNOWLEDGMENTS**

We are indebted to the staff of the Nephrology Unit of the Bergamo Hospital and the Clinical Research Center “Aldo e Cele Daccò” of the Mario Negri Institute, Bergamo, Italy, for assistance in the selection and care of the subjects of this study. We are grateful to Dr. Paolo Cravedi, Dr. Maddalena Marasà, Dr. Alessia Gennarini, and Dr. Giorgio Gentile, who were in charge of patient care and follow-up and Silvia Prandini and Veruska Lecchi for their nursing support. We also wish to thank Dr. Mariano Marchesi, who was alert for acute adverse reactions during each rituximab infusion; Annalisa Perna, who supervised data analysis; Giulia Gherardi, who helped in data entry; and Sergio Carminati and Bogdan Ene-lordache, who assisted in data handling. Manuela Passera assisted in preparing the manuscript.

Supported by the European Research Council (grant no. ERC-2012-ADG_20120314, grant agreement no. 322947), the Agence Nationale pour la Recherche Programme Blanc SVSE1 (2012) (decision no. ANR-12-BSE1-0002-01), and a Fondation pour la Recherche Médicale Equipe FRM 2012 grant.

No pharmaceutical company or any other sponsor was involved in this fully academic study.

No medical writer was involved in the creation of the manuscript.

**DISCLOSURES**

None

**REFERENCES**
