Rituximab for Idiopathic Membranous Nephropathy: Who Can Benefit?

Piero Ruggenenti,*† Carlos Chiurchiu,* Mauro Abbate,* Annalisa Perna,* Paolo Cravedi,*† Mario Bontempelli,‡ and Giuseppe Remuzzi*†

**Clinical Research Center for Rare Diseases "Aldo & Cele Daccò," Mario Negri Institute for Pharmacological Research, and Units of †Nephrology and ‡Immunohematology, Azienda Ospedaliera Ospedali Riuniti di Bergamo, Bergamo, Italy*

Rituximab effectively reduces proteinuria in patients with idiopathic membranous nephropathy (IMN), but response to treatment may vary from patient to patient. The association between baseline clinical, laboratory, and histology covariates and proteinuria reduction was evaluated retrospectively by multiple linear regression analysis at 3 mo after rituximab therapy in 14 patients with IMN with proteinuria >3.5 g/24 h while on angiotensin-converting enzyme inhibition for at least 6 mo and no previous remissions. The association strength was expressed by standardized β coefficients (SβC). Glomerular (SβC = 0.48, $P = 0.049$) and tubulointerstitial (TI) scores (SβC = 0.61, $P = 0.003$) predicted the outcome. Among glomerular and TI score components, tubular atrophy (SβC = 0.59, $P = 0.003$) and interstitial fibrosis (SβC = 0.60, $P = 0.001$) were significantly associated with 3-mo proteinuria. Urinary protein excretion decreased from 9.1 ± 4.0 to 4.6 ± 3.5 g/24 h ($P < 0.001$) in eight patients with TI score <1.7 but did not change in six with a score ≥1.7. Nine additional patients with IMN then were allocated prospectively to rituximab treatment on the basis of a TI score <1.7. Three-month proteinuria decreased in all patients from 8.9 ± 5.3 to 4.9 ± 3.9 g/24 h ($P < 0.001$) and serum albumin increased from 2.2 ± 0.6 to 2.8 ± 0.5 mg/dl ($P < 0.01$). Changes in serum albumin and cholesterol were inversely correlated ($P < 0.02$, $r = -0.44$). Rituximab achieved CD_{20} and CD_{19} depletion in all patients. In patients with IMN and nephrotic proteinuria despite angiotensin-converting enzyme inhibition therapy, renal biopsy findings may help in predicting response to rituximab and defining selection criteria for randomized trials that aim to assess the risk/benefit profile of B cell target therapy as compared with aspecific immunosuppressants and/or conservative therapy alone.

Clin J Am Soc Nephrol 1: 738–748, 2006. doi: 10.2215/CJN.01080905

Membranous nephropathy is the leading cause of nephrotic syndrome in adults worldwide (1). The idiopathic form of the disease (idiopathic membranous nephropathy [IMN]) may have a progressive course toward end-stage renal insufficiency, despite favorable outcome in 40 to 70% of cases (1–4). Most of the drugs that currently are in use, including oral glucocorticoids, alkylating agents (chlorambucil and cyclophosphamide), and cyclosporine, can be toxic and not invariably effective in all patients. Although studies have been attempting to identify appropriate protocols (5), very little is known of the factors that underlie different responses among patients. In fact, no variable has been identified as predictor of outcome of a given treatment, and efficacy could not be monitored with respect to markers of specific activity of treatment. Factors that have been shown to influence prognosis, such as the severity of renal dysfunction, nephrotic proteinuria, age, and male gender (4), might play a role. Observational reports and studies of retrospective analyses on heterogeneous populations of patients with IMN focused attention on renal biopsy findings, particularly tubulointerstitial (TI) changes (4,6–9), as prognostic indicators in IMN.

Availability of rituximab, a chimeric mAb to the CD_{20} antigen of B cells, has offered a fully novel therapeutic approach. Rituximab reduced proteinuria and suppressed symptoms over at least 1 yr of follow-up in patients with IMN and persistent nephrotic syndrome that could not be lowered by angiotensin-converting enzyme (ACE) inhibitor therapy for at least 6 mo (10,11). Rituximab also is well tolerated (10,11), and the majority of studies in adults with cancer or autoimmune diseases have not supported concerns about the potential increased risk for opportunistic infections (12). Therefore, rituximab is a promising treatment for IMN, with a better risk/benefit profile as compared with steroids and immunosuppressants. However, rituximab could not lower proteinuria to the same extent in all patients (11). The heterogeneous effects on urinary proteins were not explained by different effect on CD_{19} and CD_{20} lymphocytes that were depleted promptly and persistently from the circulation in all patients (10,11). Therefore, factors that are independent from actual inhibition of immunologic pathways might underlie individual differences in the efficacy of rituximab treatment.

In this study, we sought to investigate the determinants of response to rituximab treatment in patients with biopsy-proven

Received September 21, 2005. Accepted April 12, 2006.

Published online ahead of print. Publication date available at www.cjasn.org.

P.R. and C.C. contributed equally to this work.

Address correspondence to: Dr. Piero Ruggenenti, "Mario Negri" Institute for Pharmacological Research, Via Gavazzeni, 11, 24125 Bergamo, Italy. Phone: +39-035-319-888; Fax: +39-035-319-331; E-mail: manuelap@marionegri.it

ISSN: 1555-9041/104-0738

IMN. The study was organized in two steps: (*1*) Clinical, laboratory, or histology predictors of response to anti-CD_{20} therapy were identified by retrospective analyses in a well-characterized cohort of patients who had IMN and were treated previously with rituximab; and (*2*) identified predictors were used to identify *a priori* patients who had IMN and were expected to benefit from B cell–targeted therapy. These patients then were treated with rituximab, and their outcome was monitored prospectively. The results of our study are relevant to guiding treatment with rituximab and to understanding the mechanisms that underlie resistance to immunosuppressive therapy in patients with IMN.

Materials and Methods

Study Design and Patients

The study was organized in two steps. Step 1 was a retrospective analysis that aimed to identifying predictors of response to anti-CD_{20} therapy, and step 2 was a prospective analysis on patients who were selected on the basis of histology score. The retrospective analysis included all patients who had IMN and were treated with rituximab in our Nephrology Unit from April 2001 to January 2003. The outcome of eight of these patients was reported previously (10,11). Selection criteria for rituximab treatment were biopsy-proven IMN, creatinine clearance >20 ml/min per 1.73 m^2, persistent urinary protein excretion rate >3.5 g/24 h over at least 6-mo of ACE inhibitor (ACEi) therapy (ramipril 5 to 10 mg/d), and no previous remissions or treatment with steroids or immunosuppressive drugs during the past year (10,11). Treated patients were followed with monthly evaluations of proteinuria. Proteinuria at 3 mo after treatment as compared with baseline was the main outcome of the study. Predictors of response (3-mo proteinuria) then were identified retrospectively by multiple linear regression analysis among a series of baseline clinical, laboratory, and histology parameters. The identified predictors then were used to define the selection criteria for the patients with IMN to be included in the prospective phase of the study. These criteria served to restrict the indication to rituximab treatment to those patients who, on the basis of the results of the retrospective analyses, were expected to benefit form anti-CD_{20} therapy. The outcome of these patients was evaluated prospectively by monthly evaluations of proteinuria. Again, response to treatment was defined as proteinuria reduction at 3 mo after treatment as compared with baseline. It was established *a priori* that, on average, a proteinuria reduction of 40% or more was needed to confirm response to therapy. All patients gave written informed consent according to the Declaration of Helsinki. The study protocol was approved by the Ethical Committee of the Clinical Research Center for Rare Diseases "Aldo & Cele Daccò," Villa Camozzi of the Mario Negri Institute for Pharmacologic Research. This study was investigator initiated and internally funded. No pharmaceutical company was involvement in study design, execution, or analysis.

Baseline Evaluations

All patients who entered the retrospective or the prospective phase of the study had a baseline (pretreatment) physical examination that included body weight and BP evaluation. A series of laboratory parameters also were measured:

- Markers of renal function: 24-h urinary protein excretion (mean of three consecutive urine collections), albuminuria and albumin fractional clearance, serum creatinine, and creatinine clearance
- Routine hematochemistry: Serum lipids (total and HDL cholesterol and triglycerides), serum albumin, and electrolytes
- Blood cell count: White blood cell, red blood cell, and platelet counts and lymphocyte subpopulations, including CD_{20} and CD_{19} B cells, CD_3, CD_4, CD_8, natural killer cells, and CD_4/CD_8 ratios. Lymphocyte subpopulations were assessed by FACS. CD_{19} was used as B cell marker that, in contrast to CD_{20}, is detected by flow cytometry regardless of rituximab binding to its specific receptor (13).

Treatment

Low sodium (2 to 3 g/d Na^+Cl^-) intake and a controlled protein (0.8 g/kg body wt per d) diet were recommended to all patients. All patients received a similar conservative treatment, including loop diuretics to control edema and ACEi (ramipril 5 to 10 mg/d according to BP control and tolerability) combined with β blockers, nondihydropyridine calcium channel blockers, and statins as deemed appropriate to control BP, proteinuria, and hypercholesterolemia. No change in diet and any other concomitant treatment was introduced throughout the whole study period. In addition to conservative treatment, patients received four weekly intravenous infusions of rituximab (375 mg/m^2) as described previously (10,11).

Follow-Up

All the clinical and laboratory parameters that were taken into consideration at baseline then were evaluated every month. No change in concomitant medications, in particular in ACEi therapy, was introduced up to 3 mo after rituximab treatment so as not to affect urinary protein excretion, the main outcome of the study. Patients who at 3 mo had a residual proteinuria of 1 g/24 h or more entered a Remission Clinic protocol (14) that aimed to achieve complete remission of residual proteinuria by combined treatment with maximum tolerated doses of ACEi and angiotensin II receptor antagonist. After month 3 after treatment, all patients, regardless of proteinuria outcome, were maintained on active follow-up on an outpatient basis with monthly or bimonthly visits as deemed appropriate. Patients with a subsequent relapse of proteinuria to nephrotic range (urinary protein excretion 3.5 g/d or more) paralleled by an increase in CD_{20} B cells were asked to repeat a kidney biopsy. Those with biopsy findings that, on the basis of the results of the retrospective phase of the study, were expected to benefit from anti-CD_{20} therapy, were given a second course of rituximab. These patients were followed as described previously and were considered together with novel patients (never treated with rituximab) in the analyses of the prospective phase of the study.

Histology Evaluation

Renal biopsy specimens were processed for light microscopy, immunofluorescence analysis, and electron microscopy using standard techniques. Sections of paraffin-embedded specimens were stained with hematoxylin and eosin, Masson's trichrome, periodic acid-Schiff, and silver stain. Histology changes were evaluated and assigned a score or a numerical value, according to semiquantitative or quantitative criteria (15), by two independent pathologists without knowledge of the identity and clinical features of the patients. The consistency of the scores was assessed by calculating the κ coefficient between the two pathologists for the TI score, as well as for all the other scores that were considered in the histology evaluation (16). The κ value was 0.92 for the TI score and 0.84 for the global score ($P < 0.001$, "very good" for both) and ranged from "good" to "very good" for all the other considered parameters (16). A consensus reading was achieved whenever a score was attributed differently by the two observers.

Glomerular, TI, and Vascular Changes

Glomerular Changes. Percentages of glomeruli with global sclerosis or segmental sclerosis over the total number of glomerular profiles

were assessed. Increase of mesangial matrix was scored as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. The overall severity of glomerular changes was assigned a score as follows: 0, mild increase in mesangial matrix; 1, moderate or severe increase of mesangial matrix; 2, presence of segmental glomerulosclerosis; and 3, association of segmental glomerulosclerosis and at least moderate increase in mesangial matrix.

TI Changes. The severity of tubular atrophy was scored as follows: Tubular atrophy: 0, absent; 1, tubular atrophy in up to 25% of area of cortical tubules; 2, tubular atrophy involving 26 to 50% of cortical area; and 3, tubular atrophy in >50% of cortical area. The severity of interstitial fibrosis was scored as follows: 0, absent; 1, interstitial fibrosis in up to 25% of cortical area; 2, interstitial fibrosis in 26 to 50% of cortical area; and 3, interstitial fibrosis in >50% of cortical area. The severity of interstitial inflammation was scored as follows: 0, absent; 1, up 25% of parenchyma inflamed; 2, 26 to 50% of parenchyma inflamed; and 3, >50% of parenchyma inflamed. The overall severity of TI involvement was quantified as the sum of average score values of tubular atrophy, interstitial fibrosis, and interstitial inflammation.

Vascular Changes. The thickening of the vascular wall, taken as a marker of sclerosing changes and hyalinosis of arteries and arterioles, was quantified with the following vascular score: 0, absent; 1, mild (increase in thickness to a degree that is less than the diameter of the lumen); 2, moderate (increase in thickness equal or slightly greater to the diameter of the lumen); and 3, severe (wall thickness far exceeding the diameter of the lumen).

The glomerular parietal diffuse C3 staining was assigned an intensity score that ranged from 1 (mild) to 3 (strong). Finally, the stage of the disease was graded from 1 to 4 according to the criteria of Ehrenreich and Churg (17).

Outcome Variables

The main outcome variable of the retrospective and of the prospective phases of the study was 24-h proteinuria at month 3 after rituximab treatment. Response to treatment was defined *a priori* as an average 3-mo proteinuria reduction of 40% or more as compared with baseline. Additional outcome variables were 24-h urinary protein excretion, albumin fractional clearance, serum albumin concentration, and serum lipids at months 1, 2, and 3 after rituximab treatment.

Statistical Analyses

Within-patient comparisons were performed by means of repeated measures ANOVA followed by paired *t* test. In the retrospective phase of the study, associations between baseline covariates and response to treatment were evaluated by means of simple and multiple linear regression analysis. In addition to the histology variables listed in Table 1, the following demographic, clinical, and laboratory variables were considered for univariate analysis: Age, gender, systolic and diastolic BP, body weight, serum creatinine, creatinine clearance, urinary protein excretion, albumin fractional clearance, serum albumin, total cholesterol and triglycerides, white blood cell count, total lymphocytes, and $CD_{19/20}$ cells.

A minimal predictive model that included only the strongest risk factors was implemented using stringent statistical criteria. Specifically, the variables were selected on the basis of whether, at univariate analysis, they were associated with the main outcome variable at a significance level of 0.01. At the end of the selection process, the final model included log-transformed proteinuria at baseline and the selected histology covariate. The relative strength of the association of the baseline covariates with the main outcome variable was expressed by means of the standardized β coefficient (SβC), representing the mean change in 24-h proteinuria at 3 mo associated with an increase in the designed baseline factor equal to 1 SD, after controlling for the other baseline variables in the model. A power analysis showed that 14 patients provided the study 80% power (F test, $\alpha = 0.01$, squared multiple correlation coefficient = 0.75) to test the null hypothesis that a number of four covariates included in the multiple linear regression had no linear predictive relationship with 24-h proteinuria at 3 mo.

During the retrospective phase of the study, a cutoff value for the interstitial fibrosis score that segregated responders from nonresponders (no patients with a TI score above the cutoff value achieved a 40% reduction in proteinuria) was identified. Baseline characteristics of the two subgroups of responders and nonresponders were compared by Wilcoxon rank-sums test, χ^2 test, or Fisher exact test as appropriate.

Table 1. Histology changes and stage of the disease at kidney biopsy according to the TI score in IMN patients included in the retrospective and prospective study[a]

	Retrospective			Prospective
	Overall ($n = 14$)	TI Score ≥ 1.7 ($n = 6$)	TI Score < 1.7 ($n = 8$)	TI Score < 1.7 ($n = 9$)
Glomerular	2.5 (0.0 to 3.0)	3.0 (1.0 to 3.0)	1.5 (0.0 to 3.0)	2.0 (0.0 to 3.0)
glomeruli with global sclerosis (%)	1.5 (0.0 to 25.0)	8.5 (0.0 to 25.0)	0.0 (0.0 to 25.0)	0.0 (0.0 to 25.0)
glomeruli with segmental sclerosis (%)	5.0 (0.0 to 30.0)	17.5 (0.0 to 30.0)	0.0 (0.0 to 25.0)	13.0 (0.0 to 27.0)
mesangial matrix (score)	2.0 (1.0 to 3.0)	2.5 (2.0 to 3.0)	1.5 (1.0 to 3.0)[b]	2.0 (1.0 to 3.0)
TI score	1.3 (0.0 to 2.7)	2.3 (1.7 to 2.7)	0.3 (0.0 to 1.3)	1.3 (0.0 to 1.5)
tubular atrophy (score)	1.5 (0.0 to 3.0)	3.0 (2.0 to 3.0)	0.5 (0.0 to 2.0)[c]	1.0 (0.0 to 2.0)[c]
interstitial fibrosis (score)	2.0 (0.0 to 3.0)	3.0 (3.0 to 3.0)	0.5 (0.0 to 2.0)[c]	2.0 (0.0 to 2.0)[c]
interstitial inflammation (score)	0.0 (0.0 to 2.0)	1.0 (0.0 to 2.0)	0.0 (0.0 to 1.0)	1.0 (0.0 to 1.0)
Vascular score	0.0 (0.0 to 3.0)	2.0 (0.0 to 3.0)	0.0 (0.0 to 0.0)	0.0 (0.0 to 2.0)
Stage	2.0 (1.0 to 4.0)	3.0 (1.0 to 4.0)	2.0 (1.0 to 4.0)	2.0 (1.0 to 3.0)

[a]Data are median (interquartile range). IMN, idiopathic membranous nephropathy; TI, tubulointerstitial.
[b]$P < 0.05$ and [c]$P < 0.005$ *versus* TI score ≥1.7; *P* values for comparisons of the TI score are not given because patients were segregated in different groups according to TI score ≥ or <1.7.

Table 2. BP and main clinical and laboratory parameters in responders (TI score < 1.7) and nonresponders (TI score ≥ 1.7) who were included in the retrospective analyses and in patients who had IMN and were included in the prospective study on the basis of a TI score of <1.7[a]

	Months			
	0	1	2	3
Retrospective				
TI > 1.7				
SBP (mmHg)	158.7 ± 36.1	150.8 ± 23.1	147.3 ± 18.4	148.3 ± 15.7
DBP (mmHg)	89.5 ± 12.7	91.2 ± 11.4	89.7 ± 11.3	88.0 ± 10.6
body weight (kg)	75.2 ± 7.6	73.8 ± 8.1	75.0 ± 8.9	75.0 ± 8.2
serum creatinine (mg/dl)	2.1 ± 1.0	2.0 ± 1.0	2.1 ± 0.9	2.1 ± 0.9
creatinine clearance (ml/min per 1.73 m^2)	48.6 ± 17.4	46.9 ± 16.7	47.3 ± 16.6	44.8 ± 14.2
urinary protein excretion (g/24 h)	9.4 ± 4.0	8.2 ± 3.3	8.1 ± 2.5	7.9 ± 3.3
albumin fractional clearance (×1000)	4.0 ± 3.5	3.5 ± 3.5	4.0 ± 2.0	3.2 ± 1.7
serum albumin (mg/dl)	2.6 ± 0.5	2.7 ± 0.5	2.7 ± 0.4	3.0 ± 0.6
serum cholesterol (mg/dl)	290.0 ± 81.6	321.0 ± 68.9	292.3 ± 55.7	322.7 ± 54.8
serum triglycerides (mg/dl)	226.0 ± 94.6	274.4 ± 111.7	283.2 ± 117.8	339.5 ± 152.6[b]
TI < 1.7				
SBP (mmHg)	129.3 ± 8.6	126.9 ± 10.4	126.0 ± 7.7	126.3 ± 8.0
DBP (mmHg)	83.4 ± 7.5	79.3 ± 9.6	82.4 ± 6.0	81.1 ± 6.4
body weight (kg)	76.6 ± 16.61	75.0 ± 15.7	73.9 ± 15.0[b]	74.1 ± 14.8[b]
serum creatinine (mg/dl)	1.3 ± 0.4	1.3 ± 0.3	1.3 ± 0.5	1.3 ± 0.4
creatinine clearance (ml/min per 1.73 m^2)	60.1 ± 35.4	72.4 ± 26.3	69.8 ± 26.3	74.1 ± 20.8
urinary protein excretion (g/24 h)	9.1 ± 4.0	4.2 ± 2.7[c]	4.5 ± 2.8[c]	4.6 ± 3.5[c]
albumin fractional clearance (×1000)	2.7 ± 2.3	1.5 ± 1.5[d]	0.9 ± 1.1[d]	1.0 ± 1.2[d]
serum albumin (mg/dl)	2.6 ± 0.5	2.8 ± 0.3	3.0 ± 0.3[b]	3.0 ± 0.2[b]
serum cholesterol (mg/dl)	237.5 ± 74.0	242.8 ± 80.5	223.4 ± 59.9	215.3 ± 50.9
serum triglycerides (mg/dl)	163.3 ± 74.4	167.0 ± 76.7	156.4 ± 74.6	177.4 ± 82.4
Prospective (TI < 1.7)				
SBP (mmHg)	128.4 ± 11.2	133.1 ± 21.5	130.6 ± 14.9	129.9 ± 13.3
DBP (mmHg)	77.9 ± 11.4	78.1 ± 11.8	75.8 ± 10.6	79.8 ± 11.1
body weight (kg)	78.1 ± 16.6	77.9 ± 16.3	77.8 ± 15.6	77.7 ± 15.2
serum creatinine (mg/dl)	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.2
creatinine clearance (ml/min per 1.73 m^2)	95.6 ± 20.3	102.6 ± 21.3	105.9 ± 26.3	97.8 ± 19.8
urinary protein excretion (g/24 h)	8.9 ± 5.3	6.5 ± 3.5[b]	5.4 ± 3.9[d]	4.9 ± 3.9[c]
albumin fractional clearance (×1000)	2.2 ± 1.4	1.7 ± 0.8	1.2 ± 1.0[b]	1.1 ± 1.0[b]
serum albumin (mg/dl)	2.2 ± 0.6	2.5 ± 0.4[d]	2.6 ± 0.6[d]	2.8 ± 0.5[d]
serum cholesterol (mg/dl)	270.1 ± 73.2	270.9 ± 81.7	246.4 ± 44.2	219.8 ± 46.9
serum triglycerides (mg/dl)	122.0 ± 70.3	140.9 ± 79.8	146.9 ± 98.6	145.1 ± 75.4

[a]DBP, diastolic BP; SBP, systolic BP.
[b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.01$ *versus* month 0.

The two groups were compared at 1, 2, and 3 mo by analysis of covariance (ANCOVA), including the baseline measurements in the model. The primary comparison (3 mo *versus* baseline) was made at the significance level of 0.05. The secondary comparisons at 1 and 2 mo *versus* baseline were made at the significance level of 0.025, using Bonferroni correction.

The outcome of patients who were included in the prospective study was compared with that of responders and nonresponders who were included in the retrospective study. Main and secondary outcome variables in the three groups were compared at 1, 2, and 3 mo by ANCOVA, including the baseline measurements in the model. The primary comparison (proteinuria at 3 mo after treatment) was made at the significance level of 0.05. The secondary comparisons at 1 and 2 mo *versus* baseline were made at the significance level of 0.025, using Bonferroni correction. Comparisons at months 6, 9, and 12 were only explorative in nature and were not adjusted for Bonferroni correction. Continuous variables with skewed distribution were log-transformed before analyses.

All analyses were performed using SAS software (Release 8.2; SAS Institute Inc., Cary, NC). Sample size calculation was carried out using nQuery Advisor Release 3.0. Data are presented as *n* (%), mean ± SD, or median and interquartile range, as appropriate. Primary comparisons that considered proteinuria and other outcome variables at month 3 after treatment were made at the significance level of 0.05. Additional

comparisons that considered proteinuria and other outcome variables at month 1 and 2 after treatment were made at the significance level of 0.025 by using Bonferroni correction. All *P* values were two sided.

Results

Step 1: Retrospective Study

Baseline Characteristics. Fourteen patients (eight men) with biopsy-proven IMN, aged 60.2 ± 18.2 yr, entered and completed the retrospective phase of the study. At baseline, all patients had heavy proteinuria, with hypoalbuminemia and dyslipidemia (Table 2). Their renal function was normal or moderately reduced. The BP was relatively well controlled. All patients were on ACEi therapy. Histology changes and stage of the disease are shown in Table 1. Time from renal biopsy to the beginning of treatment with rituximab therapy was 31 ± 22 and 32 ± 14 mo for patients with a TI score ≥and <1.7, respectively.

Outcome of the Study Group as a Whole. Proteinuria decreased from 9.2 ± 3.9 g/24 h at baseline to 5.9 ± 3.5 g/24 h (−36 ± 30%; $P < 0.001$) at month 1, to 6.0 ± 3.2 g/24 h (−34 ± 28%; $P < 0.01$) at month 2, and to 6.0 ± 3.7 g/24 h (−36 ± 31%; $P < 0.01$) at month 3 after rituximab infusion. Proteinuria reduction was paralleled by an amelioration of edema with a numeric decrease in body weight from 76.0 ± 13.1 to 74.4 ± 12.1 kg ($P = 0.06$) at month 3. At the same time points, serum albumin concentration increased from 2.6 ± 0.5 to 3.0 ± 0.4 ($P < 0.01$). These last two changes resulted in a numeric decrease in albumin fractional clearance from 3.2 ± 2.8 to 1.7 ± 1.7 ($P = 0.09$).

Baseline Predictors of Proteinuria at 3 Months. At simple linear regression analysis that included the demographic and baseline clinical and laboratory parameters listed in Tables 1 and 2, 24-h urinary protein excretion rate ($P = 0.006$) only was selected (Table 3). Among the histology parameters considered (see Materials and Methods), univariate analyses found that glomerular ($P = 0.004$), TI ($P = 0.0007$), interstitial fibrosis ($P = 0.002$), and tubular atrophy ($P = 0.003$) scores were associated with proteinuria at 3 mo (Table 3). In the multiple linear regression analysis, glomerular (SβC = 0.48, $P = 0.049$; Table 4, Model 1) and TI scores (SβC = 0.61, $P = 0.003$; Table 4, Model 2) predicted the outcome. Among glomerular and TI score components, tubular atrophy (SβC = 0.59, $P = 0.003$; Table 4, Model 3) and interstitial fibrosis (SβC = 0.60, $P = 0.001$; Table 4, Model 4) only were significantly associated with 3-mo proteinuria. Furthermore, when three factors—baseline proteinuria, glomerular, and TI scores—were included in the analyses, the TI score was the only significant predictor (SβC = 0.52, $P = 0.025$; Table 4, Model 5). Similar findings were obtained when proteinuria at month 1 or 2 was considered as an outcome variable (data not shown).

Baseline Characteristics and Outcomes According to the TI Score. Two subgroups of patients with a TI score <1.7 or ≥1.7 were identified "*a posteriori*" (Figure 1). No patient with a score ≥1.7 achieved a 40% proteinuria reduction after rituximab infusion. As compared with patients with the lower score, those with a score of ≥1.7 more frequently tended to be male

Table 3. Univariate analysis: Association between baseline covariates and 24-h urinary protein excretion at month 3 (retrospective study)[a]

Baseline Covariate	Coefficient	SE	SβC	*P*
Age (yr)	+0.02	0.011	+0.48	0.082
Gender (male/female)	−0.97	0.339	−0.64	0.014
Body weight (kg)	+0.03	0.014	+0.057	0.032
SBP (mmHg)	+0.01	0.008	+0.314	0.275
DBP (mmHg)	+0.04	0.019	+0.530	0.051
Serum creatinine (mg/dl)	+0.37	0.257	+0.381	0.179
Creatinine clearance (ml/min per 1.73 m^2)	+0.001	0.008	+0.023	0.937
Urinary protein excretion (g/24 h)	+1.348	0.406	+0.692	0.006
Albumin fractional clearance (×1000)	+0.160	0.064	+0.585	0.028
Serum albumin (mg/dl)	−0.173	0.491	−0.101	0.731
Serum cholesterol (mg/dl)	+0.005	0.002	+0.497	0.071
Serum triglycerides (mg/dl)	+0.004	0.002	+0.477	0.085
Glomerular score (score)	+0.522	0.148	+0.714	0.004
Glomeruli with global sclerosis (%)	+0.027	0.021	+0.350	0.220
Glomeruli with segmental sclerosis (%)	+0.025	0.017	+0.393	0.165
Mesangial matrix (score)	+0.500	0.249	+0.501	0.068
TI score (score)	+0.636	0.140	+0.794	<0.001
Tubular atrophy (score)	+0.487	0.120	+0.761	0.002
Interstitial fibrosis (score)	+0.450	0.111	+0.761	0.002
Interstitial inflammation (score)	+0.771	0.267	+0.641	0.014
Vascular score (score)	+0.257	0.214	+0.356	0.257
Stage (score)	+0.387	0.194	+0.498	0.070

[a]SβC, standardized β coefficient.

Table 4. Multiple linear regression analysis: Association between selected covariates and 24-h urinary protein excretion at month 3 (retrospective study)

Selected Covariate	Coefficient	SE	SβC	*P*
Model 1				
urinary protein excretion (g/24 h)	+0.839	0.421	+0.440	0.072
glomerular score (score)	+0.350	0.158	+0.478	0.049
Model 2				
urinary protein excretion (g/24 h)	+0.821	0.313	+0.421	0.024
TI score (score)	+0.486	0.128	+0.607	0.003
Model 3				
urinary protein excretion (g/24 h)	+0.929	0.299	+0.477	0.010
tubular atrophy (score)	+0.375	0.098	+0.586	0.003
Model 4				
urinary protein excretion (g/24 h)	+0.975	0.273	+0.501	0.004
interstitial fibrosis (score)	+0.356	0.083	+0.603	0.001
Model 5				
urinary protein excretion (g/24 h)	+0.720	0.343	+0.369	0.062
glomerular score (score)	+0.122	0.154	+0.167	0.449
TI score (score)	+0.416	0.158	+0.519	0.025

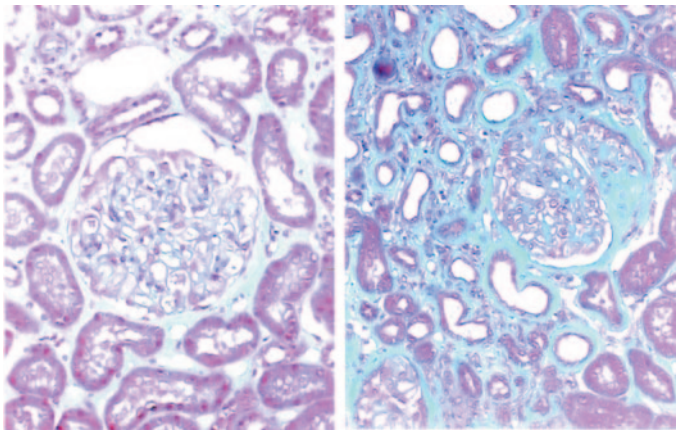

Figure 1. Histology changes at kidney biopsy representative of idiopathic membranous nephropathy with a score of interstitial fibrosis <1.7 (left) or ≥1.7 (right).

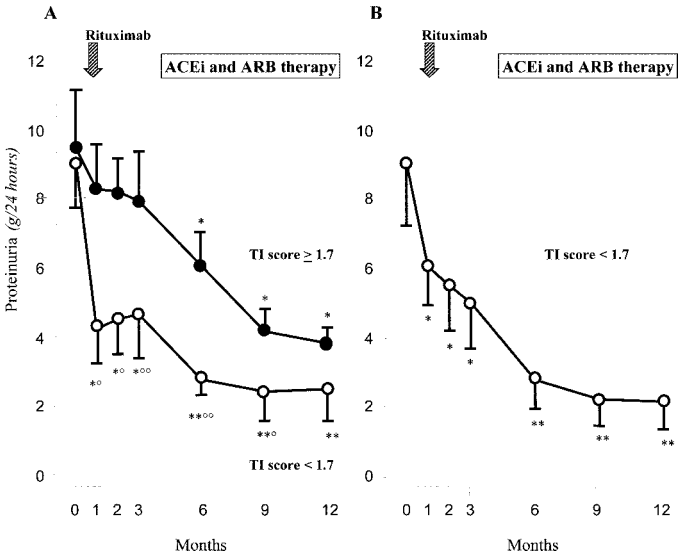

Figure 2. Twenty-four-hour proteinuria from baseline (month 0) to 12 mo after rituximab administration in two cohorts of patients with idiopathic membranous nephropathy (IMN) and a baseline tubulointerstitial (TI) score of <1.7 (responders) or ≥1.7 (nonresponders) who were included in the retrospective study (left) and in the cohort of patients who had IMN and were included in the prospective study on the basis of a baseline TI score of <1.7 (right). ***$P \leq 0.001$, **$P < 0.01$, and *$P < 0.01$ *versus* month 0; °°$P < 0.01$ and °$P < 0.05$ *versus* nonresponders (by ANCOVA with Bonferroni adjustment). Starting from month 3 after rituximab administration, patients with residual proteinuria >1 g/24 h (five of the six patients with TI score of ≥1.7 as compared with two of the eight with a TI score of <1.7 in the retrospective analysis and six of the nine with a TI score of <1.7 in the prospective analysis) received combined therapy with angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB).

(five of six *versus* three of eight; $P = 0.14$) and to have higher BP and serum creatinine levels at baseline (Table 2). None of the numeric differences between the two groups, however, achieved statistical significance. In particular, baseline 24-h urinary protein excretion was very well comparable in the two groups (Table 2). All patients were on full-dose ACEi therapy from at least 6 mo.

As expected, patients with a score of ≥1.7 had more severe histology changes as compared with those with a score of <1.7 (Table 1). In particular, patients with a high TI score had more severe glomerular and vascular changes as compared with those with a low TI score (Table 1). Global or segmental sclerosis tended to be more frequent—but not to a significant degree—among those with a high TI score (Table 1).

The outcome of the two groups was remarkably different. Proteinuria significantly decreased by 55, 52, and 54% at

months 1, 2, and 3, respectively, after rituximab infusion as compared with baseline in patients with a TI score of <1.7 (Table 2, Figure 2) but did not significantly change in those with a TI score of ≥1.7 (Table 2, Figure 2). Five (63%) patients with a TI score of <1.7 had a proteinuria <3 g/24 h at 3 mo after rituximab therapy. By ANCOVA accounting for baseline proteinuria, at each time point on follow-up, proteinuria was significantly lower in patients with a score of <1.7 than in those with a score of ≥1.7 (Table 2, Figure 2). The same trend was observed for albuminuria and albumin fractional clearance (Table 2). By ANCOVA accounting for baseline albumin fractional clearance, at 3 mo after treatment, albumin fractional clearance was significantly lower ($P < 0.05$) in patients with a score of <1.7 than in those with a score of ≥1.7. In patients with a TI score of <1.7, proteinuria reduction was paralleled by a significant decrease in body weight and by a concomitant increase in serum albumin concentration (Table 2). BP, renal function, and routine laboratory parameters did not change significantly (Table 2). No significant change was observed in patients with a TI score of ≥1.7, who, however, showed a trend of creatinine clearance to progressively decline and of serum lipids to increase over time. By ANCOVA accounting for baseline serum cholesterol, at month 3 after treatment, serum cholesterol levels were significantly lower ($P = 0.0001$) in patients with a TI score of <1.7 than in those with a TI score of ≥1.7. These different outcomes were not explained by different changes in CD_{20} and CD_{19} lymphocytes that decreased to undetectable numbers by month 1 after rituximab infusion and remained below normal ranges up to 1 yr in both treatment groups (Table 5). In both groups, there were NS changes in white blood cell, platelet, and lymphocyte counts, as well as in other considered lymphocyte subpopulations and in serum immunoglobulins (data not shown).

Step 2: Prospective Study

Baseline Characteristics and Outcomes. Nine consecutive patients (four men), aged 51.2 ± 13.2 yr, with a TI score of <1.7 entered the prospective study from August 2004 to January 2005. Two patients had received a second course of rituximab after their proteinuria had increased to pretreatment values 29 and 39 mo after the first course of rituximab, respectively. In these two patients, worsening of proteinuria was paralleled by a progressive increase in CD_{20} counts in the peripheral blood. A second kidney biopsy revealed a TI score of 1.3 in both cases and excluded causes of proteinuria different from IMN. Their baseline characteristics were very well comparable with those of patients of the retrospective study with a score of <1.7, except for a trend to lower serum creatinine levels and higher albuminuria.

Proteinuria decreased by 23 ± 27, 39 ± 24, and 48 ± 20% at months 1, 2, and 3, respectively, after rituximab infusion (Table 2, Figure 2). Proteinuria decreased in all patients. In six cases, proteinuria decreased by >40%, and in four of those cases, it decreased to <3 g/24 h. By ANCOVA accounting for baseline proteinuria, at each time point on follow-up, proteinuria was comparable to that of patients with a score of <1.7 included in the retrospective study and was significantly lower than that of those with a score of ≥1.7 (Table 2, Figure 2). Proteinuria reduction was paralleled by a significant decrease in albuminuria and a significant increase in serum albumin concentration. These last two changes resulted in a significant decrease in albumin fractional clearance (Table 2). By ANCOVA account-

Table 5. Absolute number and percentage of circulating CD_{19}- and CD_{20}-positive B cells at different time points from rituximab infusion[a]

	Months					
	0	1	2	3	6	12
Retrospective						
TI ≥ 1.7						
CD_{19} (*n*)	280.0 ± 199.7	10.3 ± 4.4	11.8 ± 5.1	14.3 ± 8.4	36.4 ± 42.5	148.4 ± 138.3
CD_{19} (%)	13.4 ± 3.3	0.7 ± 0.8	0.8 ± 0.5	1.0 ± 0.8	3.0 ± 2.1	7.2 ± 5.4
CD_{20} (*n*)	225.4 ± 154.6	0.8 ± 1.1	2.7 ± 2.5	10.3 ± 12.0	13.2 ± 14.7	54.4 ± 62.3
CD_{20} (%)	11.1 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.9	1.3 ± 0.7	2.4 ± 1.9
TI < 1.7						
CD_{19} (*n*)	225.6 ± 83.7	6.0 ± 3.7	5.3 ± 4.2	20.1 ± 20.0	53.0 ± 28.6	137.1 ± 88.3
CD_{19} (%)	13.0 ± 0.7	0.2 ± 0.4	0.3 ± 0.6	0.7 ± 1.0	3.0 ± 1.9	7.0 ± 3.5
CD_{20} (*n*)	161.0 ± 89.9	1.2 ± 1.8	26.5 ± 27.4	34.0 ± 18.3	63.5 ± 51.9	101.6 ± 89.3
CD_{20} (%)	7.9 ± 3.2	0.0 ± 0.0	1.4 ± 1.2	2.7 ± 2.3	3.1 ± 1.9	5.1 ± 4.2
Prospective (TI < 1.7)						
CD_{19} (*n*)	358.5 ± 120.9	6.8 ± 9.4	30.2 ± 36.0	25.2 ± 37.5	10.6 ± 7.6	42.5 ± 37.5
CD_{19} (%)	15.5 ± 6.4	0.5 ± 0.8	0.6 ± 0.5	1.2 ± 1.2	0.6 ± 0.5	3.5 ± 3.5
CD_{20} (*n*)	165.5 ± 20.5	3.5 ± 2.1	20.6 ± 25.9	13.5 ± 18.4	6.3 ± 7.1	32.0 ± 32.5
CD_{20} (%)	7.8 ± 4.7	0.7 ± 0.4	0.5 ± 0.5	0.4 ± 0.5	0.9 ± 0.7	2.7 ± 3.0

[a]Data are mean ± SD. Normal ranges at our laboratory: CD_{19} (*n*: 35 to 506 cell/mm^3; %: 3 to 22), CD_{20} (*n*: 61 to 574 cell/mm^3; %: 4 to 25).

ing for baseline albumin fractional clearance, at month 3 after treatment, albumin fractional clearance was significantly lower ($P < 0.05$) than in patients who had a TI score of ≥1.7 and were included in the retrospective study.

The progressive increase in serum albumin was paralleled by a progressive decrease in serum cholesterol levels (Figure 3), and changes in serum albumin and cholesterol at different time points after rituximab treatment (*versus* baseline) were significantly correlated ($P < 0.02$, $r = -0.44$). By ANCOVA accounting for baseline serum cholesterol, at month 3 after treatment, serum cholesterol levels were significantly lower ($P = 0.001$) than in patients who had a TI score of ≥1.7 and were included in the retrospective study. CD_{20} and CD_{19} lymphocytes uniformly decreased to undetectable numbers by month 1 after infusion and remained below normal ranges up to 1 yr in all patients (Table 5).

***Post Hoc* Analysis at 1 Yr after Rituximab Therapy.** At 3 mo after rituximab therapy, patients who had a residual proteinuria ≥1 g/24 h entered a Remission Clinic protocol (14) that aimed to achieve remission of residual proteinuria by combined treatment with ACEi and angiotensin II receptor blockers (ARB). In the retrospective group as a whole, proteinuria at 12 mo decreased by 64% from baseline ($P < 0.0001$; Table 6). At 12 mo after rituximab therapy, proteinuria decreased to <3 g/24 h in four patients and to <1 g/24 h in two additional patients with a TI score of <1.7. All these patients were confined in the group with a TI score of <1.7. At 1 yr, five of the six patients with a TI score of ≥1.7 were on ACEi/ARB combined therapy as compared with only two of the eight patients with a TI score of <1.7 ($P = 0.10$). The remaining patients, including those with proteinuria <1 g/24 h, were on ACEi therapy alone. At the same time point, all patients with TI score of ≥1.7 were on statins as compared with three patients with a TI score of <1.7.

Proteinuria significantly decreased also in the nine patients of the prospective group. In two patients, proteinuria decreased to <3 g/24 h and in four additional patients to <1 g/24 h. Proteinuria reduction was paralleled by a raise in serum albumin concentration that increased by 76% from baseline ($P = 0.0017$) and a lowering in serum cholesterol (−28% *versus* baseline; $P = 0.09$). Serum creatinine remained stable up to 1 yr of follow-up. No significant difference in any of the measured variables was observed between the two groups of patients who had a TI score of <1.7 and were included in the retrospective and prospective studies, respectively.

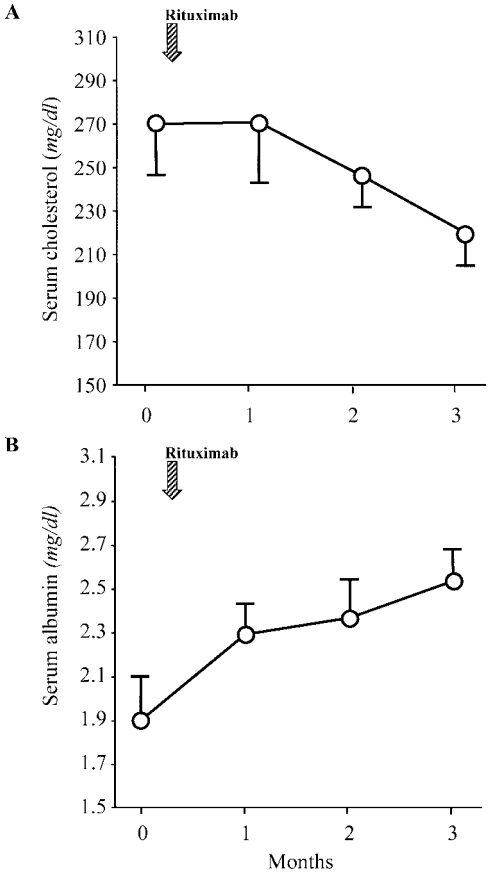

Figure 3. Total cholesterol (top) and serum albumin (bottom) levels at baseline (month 0) and at 1, 2, and 3 mo after rituximab administration in the cohort of patients who had IMN and were included in the prospective study on the basis of a baseline TI score of <1.7. $^{**}P < 0.01$ *versus* month 0.

Discussion

Here we found that in patients with IMN and persisting nephrotic-range proteinuria despite ACEi, 3-mo proteinuria in response to rituximab therapy was strongly dependent on renal biopsy findings before rituximab therapy. In step 1 (retrospective study), we identified a score of TI changes that segregated responders from nonresponders. In step 2 (prospective study), we established the predictive value of the histology score, thereby validating the findings of the step 1 analysis. Actually, 3-mo proteinuria decreased by 48% in the nine patients who were treated prospectively with rituximab on the basis of a TI score of <1.7. Importantly, proteinuria reduction was accompanied by a consistent amelioration of components of the nephrotic syndrome, such as hypoalbuminemia and hypercholesterolemia. The further reduction in proteinuria that we observed at 1 yr in all study groups might reflect a time-dependent effect of rituximab that, over a longer follow-up, may become apparent also in those with more severe histology changes. However, we cannot exclude a beneficial effect of combined ACEi and ARB therapy in patients who at 3 mo still had a residual proteinuria of ≥1 g/24 h (14). Regardless of the above, those with less severe histology changes had a more consistent proteinuria reduction also in the long term. At 12 mo after rituximab administration, all patients who achieved a reduction of proteinuria to subnephrotic ranges, or subclinical ranges, segregated in the two groups with a TI score <1.7.

In membranous nephropathy, proteinuria ensues as a consequence of activation of antibody-mediated pathways that lead to injury to the glomerular barrier (18). Although CD_{20} cells, precursors to the antibody-producing plasma cell, were uniformly depleted from blood in all patients, reduction in protein loss after rituximab therapy differed among patients. In the

Table 6. Main parameters at 0 and 12 months from rituximab infusion

	Retrospective				Prospective	
	TI ≥ 1.7		TI < 1.7		TI < 1.7	
	0 Months	12 Months	0 Months	12 Months	0 Months	12 Months
Systolic blood pressure (mmHg)	158.7 ± 36.1	137.2 ± 19.7	129.3 ± 8.6	133.0 ± 7.1	128.4 ± 11.2	128.1 ± 14.3
Diastolic blood pressure (mmHg)	89.5 ± 12.7	79.2 ± 15.4[a]	83.4 ± 7.5	82.4 ± 9.1	77.9 ± 11.4	75.7 ± 11.9[a]
Body weight (kg)	75.2 ± 7.6	74.1 ± 8.9	76.6 ± 16.6	76.1 ± 16.7	78.1 ± 16.6	82.0 ± 15.1
Serum creatinine (mg/dl)	2.1 ± 1.0	2.0 ± 1.0	1.3 ± 0.4	1.2 ± 0.4	1.0 ± 0.3	1.0 ± 0.3
Urinary protein excretion (g/24h)	9.4 ± 4.0	3.7 ± 1.1[b]	9.1 ± 4.0	2.5 ± 2.6[b]	8.9 ± 5.3	2.0 ± 2.3[b]
Serum albumin (mg/dl)	2.6 ± 0.5	3.2 ± 0.4	2.6 ± 0.5	3.4 ± 0.5[a]	2.2 ± 0.6	3.4 ± 0.4[b]
Serum cholesterol (mg/dl)	290.0 ± 81.6	223.2 ± 48.2[a]	237.5 ± 74.0	173.1 ± 34.1[a]	270.1 ± 73.2	181.0 ± 38.3
Serum triglycerides (mg/dl)	226.0 ± 94.6	226.7 ± 100.3	163.3 ± 74.4	131.5 ± 62.7[a]	122.0 ± 70.3	92.4 ± 45.6

Data are mean ± standard deviation.
[a]$P < 0.05$, [b]$P < 0.01$ vs month 0.

presence of chronic structural damage, factors other than failure to deplete CD_{20} cells and thus presumably to inhibit the immunologic pathway accounted for failure of treatment.

One may consider that the kidney of a patient who has IMN and is on rituximab mimics the situation of the kidney taken from a rat with active Heymann nephritis and transplanted into a normal syngeneic recipient, a surgical way to abrogate the immune pathway that is elicited by immunization with renal target antigen(s) (18). Those experiments revealed a dramatic drop in proteinuria of donor kidney origin within 4 wk after transplantation without yet completely subsiding. Residual proteinuria could be attributed to persistent glomerular damage that did not recover despite abrogation of the underlying immunologic insult (18). Functional and morphometric data actually revealed that the magnitude of urinary protein traffic in IMN is strongly related to changes in the epithelial layer of the glomerular capillary wall but not to the physical presence of subepithelial immune deposits (19).

So far, no variable, including renal biopsy findings, has been demonstrated to predict the response to the more conventional therapies, such as steroids plus alkylating agents or cyclosporine. The main finding of our study was that the degree of TI changes segregated responders from nonresponders. TI changes, in particular interstitial fibrosis, are predictors of worse prognosis (4,6–9). Interstitial injury depends at least in part on sustained exposure of renal epithelial cells to ultrafiltered proteins, complement components, and chemokines, shown by abundant experimental evidence to promote synthesis of proinflammatory and fibrogenic molecules (20). Chemokines and fibrogenic cytokines that contribute to such response (monocyte chemoattractant protein-1, RANTES, osteopontin, and TGF-β1) have been detected in biopsy studies of progressive IMN (21) or experimental nephritis (22,23). High degrees of TI damage in patients who are less responsive to rituximab may reflect sustained activation of such mechanisms. Additional putative factors can be chronic hypoperfusion or the long-term toxicity of nephritogenic antibodies on proximal tubular cells that might promote dysfunction after the immune attack has ceased (24). The finding that vascular changes were strictly associated with high TI score would be in support of a contributory role of chronic ischemic damage.

At the glomerular level, segmental sclerosis is associated with a high rate of disease progression in IMN (4). Here, the severity of glomerular involvement predicted response to treatment in simple and multiple regression analyses. It may become important to understand whether individual variations in susceptibility to glomerular injury—that can be genetically determined (25)—occur in IMN, as recently suggested for HIV-1–associated nephropathy (26), and how they modify therapeutic responses. Differences in glomerular structure might explain the diverse responses to rituximab therapy also within patients with low TI changes.

The scoring system was particularly reliable, as documented by the very good κ coefficient values between the two pathologists not only for the TI score but also for all the other considered parameters (16). However, some findings must be taken with caution because the relatively small number of cases reduced the power of the analyses to detect relatively weaker predictors of outcome, such as male gender or higher serum creatinine levels at study entry. Notwithstanding, here we showed that patients who had IMN and were treated on the basis of the TI score invariably responded to rituximab therapy. These study findings might provide useful information to identify people who may benefit the most from rituximab treatment and to define the selection criteria of randomized trials that aim to assess the risk/benefit profile of B cell target therapy as compared with less specific immunosuppressive treatments and/or conservative therapy alone.

Conclusion

Renal biopsy findings possibly should be used to guide rituximab treatment for IMN. Rituximab reduces proteinuria in patients with no or mild TI changes. Up to 50% of these patients achieve remission of the nephrotic syndrome or even a full remission of proteinuria within 3 mo after treatment. In those with residual proteinuria, a further reduction in urinary proteins can be achieved by add-on combined treatment with ACEi and ARB. This sequential approach may be particularly effective in those with severe interstitial fibrosis and tubular atrophy, as it effectively reduced proteinuria in the advanced phase of experimental (27) and human (14) disease.

This was a pilot, explorative study, and the preliminary results of our analysis may represent the rationale for a large, prospective trial that aims to assess the long-term risk/benefit profile of B cell targeted therapy as compared with aspecific immunosuppressants and/or conservative treatment alone in patients with homogeneous histology changes.

Acknowledgments

We are grateful to Dr. Varuska Brusegan and Dr. Anna Fassi and the research nurses Franca Gamba, Grazia Natali, Elena Carozzi, and Anna Ferrari for patient management and monitoring; to Dr. Flavio Gaspari and Dr. Claudia Filippi for the laboratory measurements; to Dr. Alessandro Bonomi for patient assistance during rituximab infusion; to Dr. Gaetano Bonini for performing the kidney biopsies; and to Franco Marchetti for preparation of the histology material. Manuela Passera helped to prepare the manuscript.

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