Supplementary Appendix

RI-CYCLO ClinicalTrials.gov number, NCT03018535

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I. Investigators and committee members

The members of the RI-CYCLO study group are as follows:

Executive Committee – F. Scolari (Principal Investigator), P. Ravani (Co-principal Investigator), G.M. Ghiggeri, C. Ponticelli

Data and Safety Monitoring Board – (Chair) Aldo Roccaro

Study Investigators:

Italy – University of Brescia: F. Scolari, E. Delbarba, N. Dallera, F. Alberici; University of Messina: D. Santoro; University of Bari: L. Gesualdo, P. Protopapa; University of Cagliari: A. Pani, A. Angioi, N. Lepore; Belcolle Hospital, Viterbo: S. Feriozzi; Ospedale Maggiore, Bologna: M. Santostefano; University of Piemonte Orientale: M. Quaglia; Ospedale S Maria della Misericordia, Udine: G. Boscutti G, M. Bosco; Ospedale Policlinico, Milano: P. Passerini; University of Modena: R. Magistroni; Policlinico di Catania: C. Marcantoni; Ospedale Civico Palermo: A. Ferrantelli; Ospedale di Cremona: P. Pecchini

Canada: University of Calgary: P. Ravani

Switzerland: Department of Nephrology and Hypertension, Bern University Hospital, University of Bern, Bern, Switzerland: Mani LY

Anti-PLA2R Laboratory – Institute Giannina Gaslini, Genova, Italy: G.M. Ghiggeri

Data Management and Study Coordination Center – Division of Nephrology, Spedali Civili and University of Brescia: C. Colombi

Statistical Analysis – University of Calgary: P. Ravani

II. Supplementary methods

A. Patient Inclusion and Exclusion Criteria

Inclusion criteria

- Patients aged 18 years and older.
- Biopsy-proven diagnosis of MN performed within 24 months before enrolment.
- Proteinuria >3.5 g/day on three 24-hr urine collections (once a week for 3 weeks following the run-in phase).
- Estimated GFR ≥30 mL/min/1.73 m2 (CKD-EPI equation).
- Postmenopausal females, or females surgically sterile or practising a medically approved method of contraception.
- Blood pressure <130/80 mm Hg.
- Hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor therapy.
- RAS inhibition therapy.

Exclusion criteria

- Serum creatinine >2.0 mg/dL or estimated GFR <30 mL/min/1.73 m2.
- Previous treatment with rituximab, steroids, alkylating agents, calcineurin inhibitors, synthetic ACTH, Micofenolate Mofetil (MMF), azathioprine.
- Presence of active infection.
- Secondary cause of MN (eg, hepatitis B and C, Systemic Lupus Erythematosus (SLE), medications, malignancies; testing for HIV, hepatitis B and C should have occurred <6 months prior to enrolment into the study).
- Type 1 or 2 diabetes mellitus.
- Pregnancy or nursing for safety reasons.
- Renal vein thrombosis documented prior to entry by renal ultrasound (US) or CT scan.

B. Outcomes and follow-up

Primary outcome

The primary outcome measure is complete remission (proteinuria to ≤0.3 g/day) at 1 year. This outcome was selected to inform the first feasibility question related to obtaining the direction of the effect and its size, relative to the standard treatment.

Secondary outcomes

Secondary outcome measures at 6, 12, 18, 24 and 36 months included: the change in proteinuria from baseline, the probability of complete or partial remission (proteinuria at least 50% lower than the baseline and ≤3.5 g/day), and estimated GFR and serum creatinine levels. We summarized data on relapse of proteinuria >3.5 g/day in those who achieved a complete or partial remission. We also assessed the levels of autoantibodies and their relation to therapy and proteinuria at baseline, 6, 12, 18, 24 and 36 months after treatment. We summarized data on serious adverse events, including death, life-threatening events and disability.

Follow-up

Study visits were completed at baseline, 6, 12, 18, 24 and 36 months, and follow-up continued until complications or relapses occurred. A local study coordinator maintained ongoing contact with the patients to collect potential adverse events and minimize loss to follow-up or dropout. Determination of 24-hr proteinuria and blood count were planned at baseline and after 6, 12, 18, 24 and 36 months. We assessed the levels of anti-PLA2R auto-antibodies at baseline, 6, 12, 18, 24 and 36 months after treatment, using a standardized commercial ELISA method (Euro-immune, Lubeck, Germany); patients were considered to be positive when baseline serum levels were >20 RU/mL; the same cut-off was utilized for defining the immunological response during the post-therapy follow-up (14). The median value of the anti-PLA2R antibodies in the overall population has been used as cut-off to distinguish two subgroups, in order to assess response rates according to baseline anti-PLA2R values. In patients assigned to the cyclical regimen, complete blood count was performed weekly during treatment. We define relapse as

proteinuria >3.5 g/day after complete or partial remission has been obtained at month 6 or 12.

Adverse Events

We recorded any sign, symptom, abnormal laboratory finding or disease that emerged or worsened compared to baseline. Adverse events were defined as any untoward medical occurrence. Serious Adverse Events were defined as any untoward medical occurrence that resulted in death, was life-threatening, required inpatient hospitalization or resulted in persistent or significant disability/incapacity.

C. Study Treatments and Concomitant Medications

Intervention

Patients randomized to the intervention arm received two courses of the chimeric monoclonal anti-CD20 antibody rituximab at a dose of 1 g on days 1 and 15, without concomitant or subsequent drug therapies. Rituximab was diluted in 500 mL of normal saline and administered at 9 mL/hr for the first 30 min; thereafter, the infusion rate was doubled every 30 min up to a maximum of 72 mL/hr. In order to reduce common reactions, patients received a premedication with methylprednisolone (2 mg/kg infused in 30 intravenous diluted in 100 mL of normal saline), oral cetirizine (0.2 mg/kg) and oral paracetamol (15 mg/kg). Registered nurses delivered the premedication and the intervention drug in the nephrology units of participating centers.

Active comparator

Patients randomized to active comparator received cyclical corticosteroid/cyclophosphamide therapy, consisting of three consecutive cycles of 2-month duration each (for a total of 6 months), one based on steroids and one based on cyclophosphamide. The first month of each 2-month cycle (months 1, 3 and 5) began with a 1 g pulse of intravenous methylprednisolone, repeated daily for three consecutive days followed by oral methylprednisolone (0.4 mg/kg/day) or prednisone (0.5 mg/ kg/day) for the remaining days of that month. In the second month of each 2-month cycle

(months 2, 4 and 6), the steroid was stopped and oral cyclophosphamide (2.0 mg/kg/day) was given daily for that month.

Relevant concomitant care

Any medications not listed in the exclusion criteria may be given at the discretion of the investigator. The investigator recorded all concomitant medications taken by the participant in the appropriate section of the case report form.

D. Data and safety monitoring board

The Safety and Monitoring Board at the Spedali Civili di Brescia Hospital was responsible for study monitoring. The Safety and Monitoring Board is an independent team with functional autonomy, specifically created for no profit studies, consisting of qualified personnel expert in clinical research methodology and not directly involved in the clinical study.

E. Sample Size Estimation

The main reason for conducting the present pilot study was gathering preliminary outcome data for the primary outcome measure (disease remission) in order to perform a sample size calculation for a larger trial. To estimate the probability of achieving complete remission in the two treatment groups, we supposed to include 35 participants per arm, following a general rule of thumb (ie, at least 30 statistical unit per parameter) (1). We planned to follow participants for at least 1 year. We expected that each center would have enrolled between 6 to 8 participants over 2 years. This pilot study was designed to provide preliminary effect estimate to inform the design of a larger study, as it would not be powered to address intervention questions.

F. Supplementary Statistical Methods

Analyses were mainly descriptive and focused on CI estimation. We adopted standard statistical methods to summarize the sample characteristics overall and by arm assignment, using statistics for quantitative (mean and SD, median and IQR when

appropriate) and qualitative (frequencies) data as appropriate. We did comparative analysis acknowledging the nature and purpose of this pilot study. Given the relatively small study size and its feasibility objective, we treated any comparative analyses as preliminary and interpret them with caution. Following this approach, we considered the following analyses: logistic regression to compare the probability of achieving complete remission at 12 months and other binary outcomes, and methods for continuous, count or survival data for time to event analyses (secondary outcome). In all analyses, we used an intention-to-treat approach, whereby participants were analyzed as randomized regardless of protocol adherence. We replaced missing data by carrying forward the last available measure. If these analyses supported a significant effect of rituximab vs the cyclical regimen we planned to test consistency of findings by considering the missing data in the active comparator group as successes and missing data in the active intervention as failures (worst-case scenario). As an additional analysis, we assessed the probability of complete or partial remission using a per-protocol approach. In this non pre-specified analysis, three patients with severe infusion reaction to rituximab requiring immediate withdrawal of the drug and subsequent treatment with the cyclical regimen were included in the cyclical regimen arm. All patients received less than 50 ml of the infusion, which was insufficient to deplete peripheral CD19+ B-cells.

III. Supplementary results

Patient follow-up

All the patients have been followed until the 12-month mark. After this time point, four patients were lost to follow-up (3 in the cyclical regimen and one in the rituximab arm). For the remaining patients without available follow-up beyond the 12 month, this was due to censoring secondary to events that excluded them as well as insufficient follow-up length due to enrolment during the last years of the study.

In details:

* at 12 months, one patient of the rituximab arm received a non-study intervention (cyclosporine) due to treatment failure; this patient was censored at the 12 months mark

* two patients assigned to the cyclical regimen developed kidney failure requiring renal replacement therapy, respectively at 7 and 24 months; these patients were considered as reaching an event

* one patient in the rituximab arm died between month 24 and 36 of lung cancer; this patient was considered as reaching an event

* eleven patients were enrolled during the last two years and did not have the chance to complete the follow-up at 24 and 36 months.

IV. Supplementary figures and tables referred to in main text

Table S1. Complete remission or composite (complete or partial remission) at 6 to 36 months by per-protocol analysis*

Complete Remission

Study time-points	Rituximab	Cyclical regimen	OR (95% CI)
	No of patients with remission/ total no. (%)		
Per-protocol population			
6 mo	2/32 (6)	3/39 (8)	0.8 (0.13-5.10)
12 mo	4/32 (13)	13/38 (34)	0.28 (0.08-0.95)
18 mo	8/28 (29)	9/37 (24)	1.24 (0.41-3.78)
24 mo	9/23 (39)	13/34 (38)	1.04 (0.35-3.08)
36 mo	6/17 (35)	7/25 (28)	1.40 (0.37-5.27

Complete or Partial Remission

Study time-points	Rituximab	Cyclical regimen	OR (95% CI)
	No of patients with remission/ total no. (%)		
Per-protocol population			
6 mo	17/32 (53)	25/39 (64)	0.63 (0.24-1.65)
12 mo	21/32 (66)	24/38 (63)	1.11 (0.42-2.98)
18 mo	19/28 (68)	29/37 (78)	0.58 (0.19-1.77)
24 mo	20/23 (87)	27/34 (79)	1.72 (0.40-8.86)
36 mo	14/17 (82)	19/25 (76)	1.47 (0.31-7.92)

The per-protocol population included all the patients who received a full course of the trial medications, according to the protocol. Three patients in the rituximab group, who did not tolerate the drug, were switched to the cyclical regimen and considered in this arm in per-protocol analysis. Those patients received less than 50 ml of the infusion and did not achieve CD19+ B-cell depletion.

Table S2. Proteinuria and serum albumin by arm and study time-points*

Proteinuria, g/day			**Serum albumin, g/dL**	
Study time- points	**Rituximab**	**Cyclical regimen**	**Rituximab**	**Cyclical regimen**
Baseline	6.1 (4.0-10.1)	6.2 (5.1-9.3)	2.4 (1.8-2.7)	2.5 (1.9-2.7)
6 months	2.7 (1.0-4.9)	1.6 (0.6-4.8)	3.4 (2.8-3.8)	3.6 (2.8-3.8)
12 months	1.2 (0.4-4.3)	0.8 (0.2-4.4)	3.7 (2.9-4.2)	3.7 (3.2-4.0)
18 months	0.9 (0.3-3.6)	0.6 (0.4-3.0)	3.9 (3.4-4.2)	3.8 (3.3-4.1)
24 months	0.7 (0.2-2.2)	0.7 (0.2-3.0)	4.0 (3.5-4.2)	3.8 (3.4-4.1)
36 months	0.6 (0.2-1.8)	0.6 (0.2-2.2)	3.8 (3.2-4.1)	3.9 (3.3-4.3)

*Data presented as median (IQR)

Table S3. Serum creatinine by arm and study time-points*

Serum Creatinine, mg/dL				
Study time-points	**Rituximab**	**N°**	**Cyclical regimen**	**N°**
Baseline	1.02 (0.27)	37	0.96 (0.27)	37
6 months	1.00 (0.25)	36	0.98 (0.47)	37
12 months	0.98 (0.29)	36	0.98 (0.48)	36
18 months	0.98 (0.26)	32	1.14 (0.90)	34
24 months	0.94 (0.20)	26	1.12 (0.77)	31
36 months	0.97 (0.20)	20	1.22 (0.77)	22

*Data presented as mean (SD) ° number of patients at each time-point

Table S4. Levels of anti-PLA2R and percentage of immunological remission according to treatment group, in anti-PLA2R positive patients *.

Study time-points	Anti-PLA2R antibodies				Immunological Response		
	All patients *Median (IQR)*	**Rituximab** *median (IQR)*	**Cyclical regimen** *median (IQR)*	**P Value**	**Rituximab** *(%)*	**Cyclical regimen** *(%)*	**P Value**
Baseline	58.6 (42.5-86)	58 (40-81)	63 (52-87)	0.50			
6 months	0 (0-54)	0 (0-44)	13 (0-86)	0.30	63	50	0.71
12 months	0 (0-60)	2 (0-44)	0 (0-73)	0.83	62	56	1.00
18 months	0 (0-2)	0 (0-0)	0 (0-57)	0.21	91	73	0.59
24 months	0 (0-2)	0 (0-0)	0 (0-53)	0.26	90	75	0.59
36 months	0 (0-32)	0 (0-18)	0 (0-45)	0.49	73	64	1.00

*Differences between medians were compared with Mann-Whitney test

Table S5. Clinical response in anti-PLA2R positive patients, by anti-PLA2R levels at baseline

Time points	Anti-PLA2R	All patients	Rituximab	Cyclical regimen	p-value*
Complete remission					
6 months	>58 RU/ml	1/25 (4%)	1/12 (8%)	0/13 (0%)	1
	≤58 RU/ml	3/16 (19%)	2/10 (20%)	1/6 (17%)	1
12 months	>58 RU/ml	4/25 (16%)	2/12 (17%)	2/13 (15%)	1
	≤58 RU/ml	5/16 (31%)	2/10 (20%)	3/6 (50%)	0.61
18 months	>58 RU/ml	3/22 (14%)	2/10 (20%)	1/12 (8%)	0.59
	≤58 RU/ml	7/15 (47%)	4/9 (44%)	3/6 (50%)	1
24 months	>58 RU/ml	6/19 (32%)	4/7 (57%)	2/12 (17%)	0.35
	≤58 RU/ml	6/14 (43%)	3/8 (38%)	3/6 (50%)	1
36 months	>58 RU/ml	5/14 (36%)	2/5 (40%)	3/9 (33%)	1
	≤58 RU/ml	4/14 (29%)	2/8 (25%)	2/6 (33%)	1
Complete or Partial remission					
6 months	>58 RU/ml	14/25 (56%)	6/12 (50%)	8/13 (62%)	0.74
	≤58 RU/ml	10/16 (63%)	5/10 (50%)	5/6 (83%)	0.69
12 months	>58 RU/ml	14/25 (56%)	7/12 (58%)	7/13 (54%)	1
	≤58 RU/ml	14/16 (88%)	8/10 (80%)	6/6 (100%)	0.46
18 months	>58 RU/ml	14/22 (64%)	7/10 (70%)	7/12 (58%)	1
	≤58 RU/ml	12/15 (80%)	6/9 (67%)	6/6 (100%)	0.71
24 months	>58 RU/ml	16/19 (84%)	7/7 (100%)	9/12 (75%)	0.74
	≤58 RU/ml	11/14 (79%)	6/8 (75%)	5/6 (83%)	1
36 months	>58 RU/ml	13/14 (93%)	5/5 (100%)	8/9 (89%)	1
	≤58 RU/ml	11/14 (79%)	6/8 (75%)	5/6 (83%)	1

Data presented as number of patients showing the event on number of the patients assessed; in brackets reported percentage)

* Comparison between rituximab and cyclical regimen groups

Table S6. Proteinuria and anti-PLA2R titer throughout follow-up, in patients who experienced a relapse

Rituximab												
Pts	**Baseline**		**6 months**		**12 months**		**18 months**		**24 months**		**36 months**	
	Prot*	Anti-PLA2R°	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R
1	4.6	40	2.1	18	0.7	14	5.1	76	5.4	0	5.3	0
2	14.7	203	1.0	0	1.5	NA	5.0	NA	1.3	0	1.7	32
3	9.6	0	0.8	0	0.5	0	4.2	0	2.6	0	NA	0
Cyclical regimen												
Pts	**Baseline**		**6 months**		**12 months**		**18 months**		**24 months**		**36 months**	
	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R	Prot	Anti-PLA2R
1	5.7	900	1.6	6	9.8	81	3.7	66	2.5	NA	NA	NA
2	10	NA	2.5	NA	4.7	NA	3.5	NA	3.2	NA	NA	NA
3	8.6	71	1.9	0	5.4	40	1.8	0	0.3	0	NA	NA
4	14.6	30	2.3	0	1.2	0	0.5	0	0.1	0	7.7	NA
5	3.5	56	1.0	NA	0.2	0	0.3	NA	5.2	NA	1.2	NA
6	8.2	105	0.5	NA	4.0	NA	NA	NA	NA	NA	NA	NA

* Proteinuria is given in g/day °Anti-PLAR levels are expressed in RU/mL

Figure S1. Design of the study

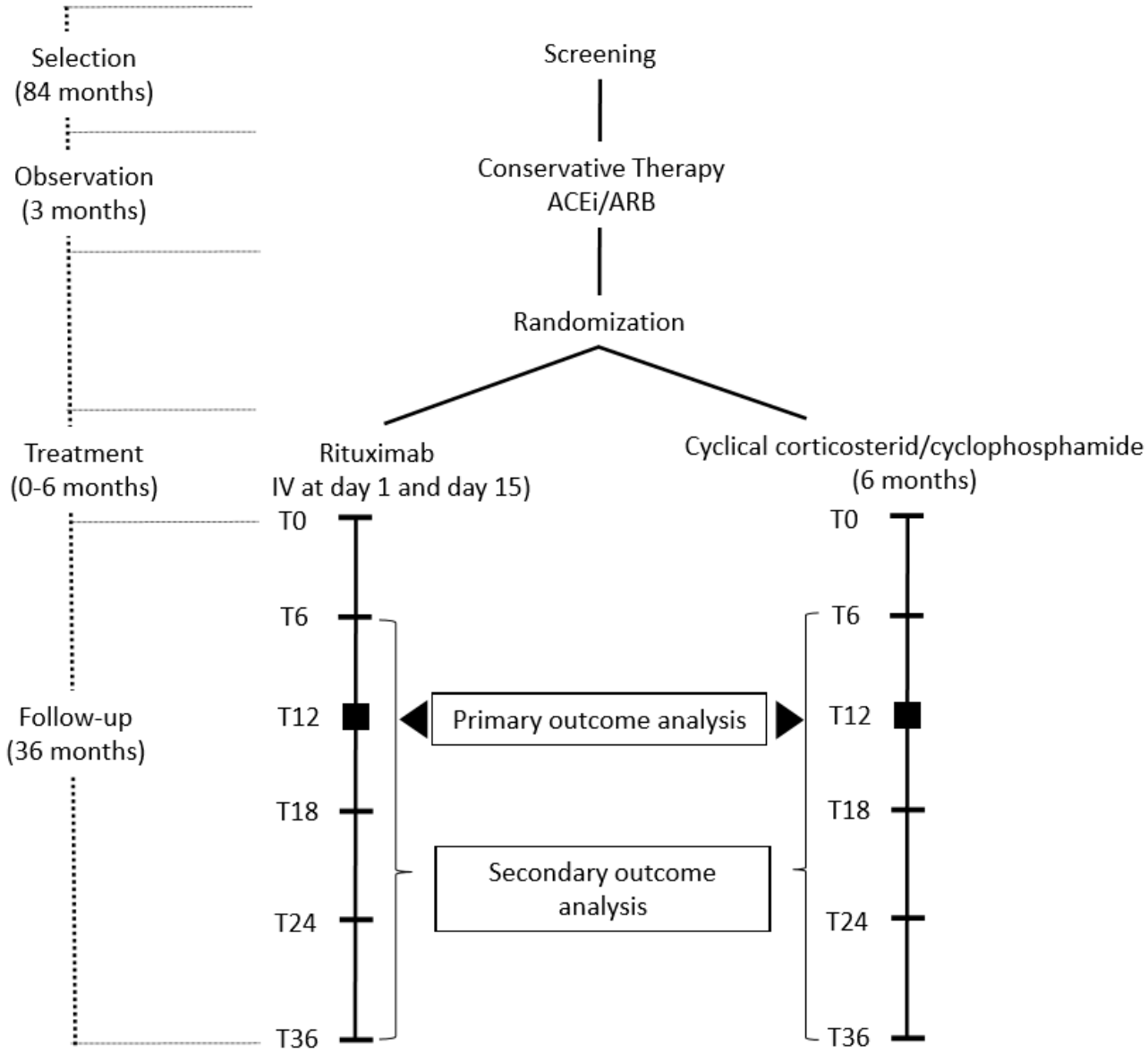
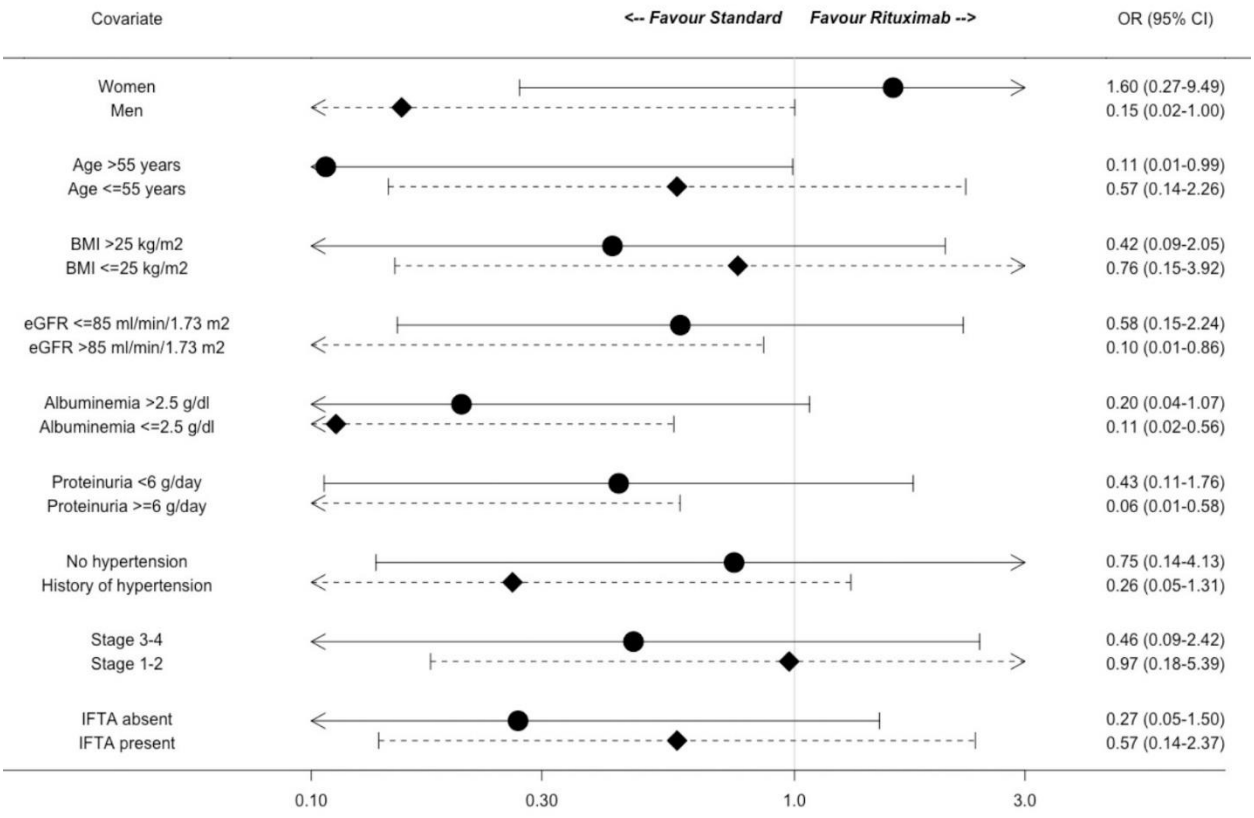


Figure S2. Pre-specified subgroup analysis of the composite outcome (complete or partial remission) at 12-month follow-up



Statistical tests for interactions all non-significant

Figure S3. Serum creatinine and eGFR over time. Data is presented as mean ± standard deviation over time by assigned treatment.

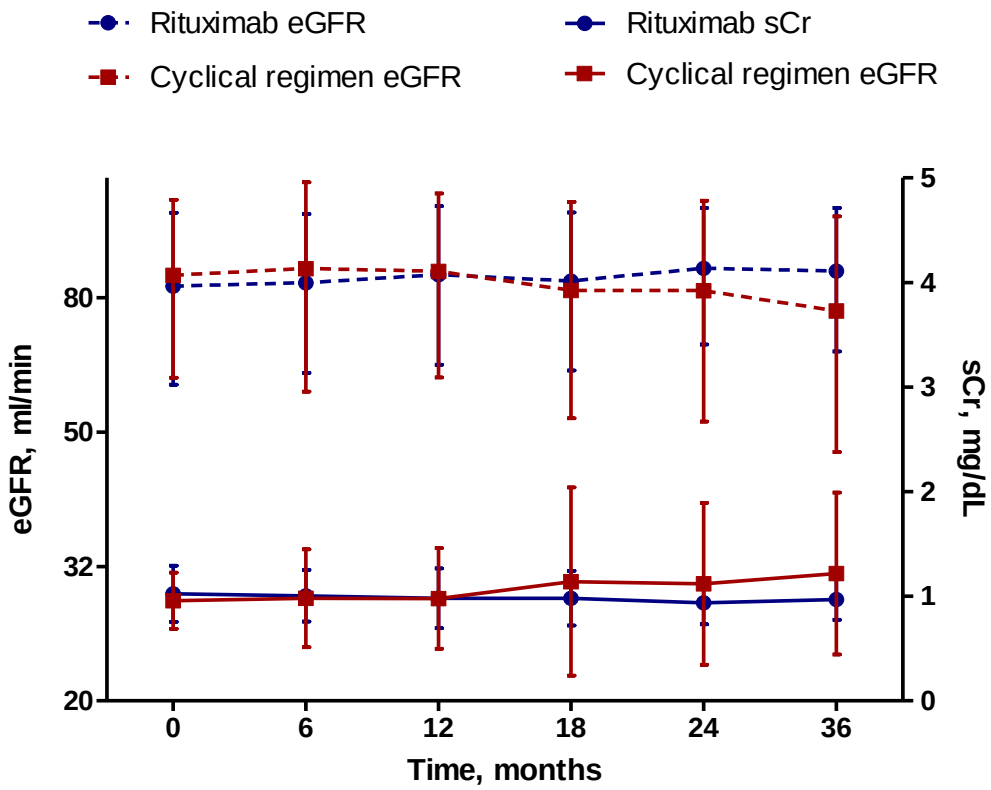
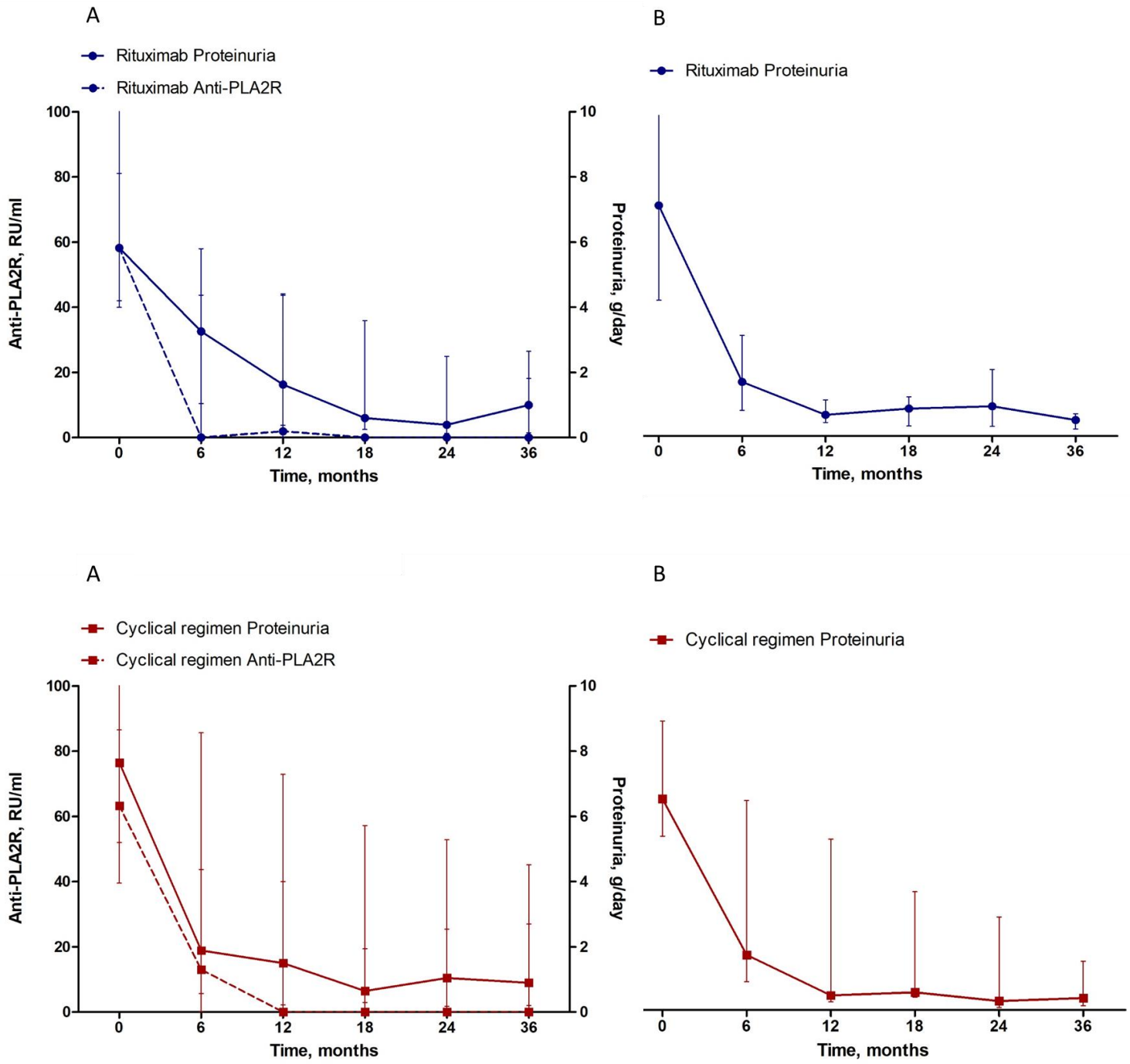


Figure S4. Anti-PLA2R levels and proteinuria by treatment group and time in anti-PLA2R positive patients (A). Proteinuria by group and time in anti-PLA2R negative patients (B)



Supplementary References

1. Lancaster GA, Dodd S, Williamson PR. Design and analysis of pilot studies: recommendations for good practice. J Eval Clin Pract 2004;10:307–12