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Anti-CD20 monoclonal antibodies immuno-monitoring in primary membranous nephropathy to guide personalized treatment

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UNIVERSITÉ DE NICE SOPHIA-ANTIPOLIS

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**ANTI-CD20 MONOCLONAL ANTIBODIES IMMUNO-MONITORING
IN PRIMARY MEMBRANOUS NEPHROPATHY
TO GUIDE PERSONALIZED TREATMENT**

Thèse de Médecine

Pour l'obtention du grade de Docteur en Médecine (Diplôme d'État)

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Table des matières

LISTE DES ABRÉVIATIONS	19
RÉSUMÉ	20
ABSTRACT	21
BACKGROUND	22
MATERIALS AND METHODS.....	24
RESULTS	28
DISCUSSION.....	39
CONCLUSION	44
REFERENCES	45
SERMENT D'HIPPOCRATE	50

**ANTI-CD20 MONOCLONAL ANTIBODIES IMMUNO-MONITORING
IN PRIMARY MEMBRANOUS NEPHROPATHY
TO GUIDE PERSONALIZED TREATMENT**

LISTE DES ABRÉVIATIONS

ACE	Angiotensin-converting-enzyme
ADA	Anti-drug antibody
ANCA	Anti-neutrophil cytoplasmic antibody
Anti-gp120 antibody	Anti-glycoprotein 120 antibody
Anti-M2 antibody	Anti-mitochondrial of M2 specificity antibody
ARB	Angiotensin II type 1 receptor blocker
CT-scan	Computed-tomography
ELISA	Enzyme linked immunosorbent assay
GI endoscopy	Gastrointestinal endoscopy
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
mAb	Monoclonal antibody
MN	Membranous nephropathy
OBI	Obinutuzumab
OCRE	Ocrelizumab
OFA	Ofatumumab
PCSK9	Proprotein convertase subtilisin–kexin type 9
PLA2R1	Phospholipase A2 receptor 1
PSA	Prostate specific antigen
RTX	Rituximab
SLE	Systemic lupus erythematosus
TNF	Tumor necrosis factor
UPCR	Urine protein/creatinine ratio

RÉSUMÉ

Introduction : Le rituximab (RTX) est un anticorps monoclonal chimérique murin/humain dirigé contre la molécule de surface CD20. Son utilisation dans la glomérulonéphrite extra-membraneuse primitive (GEM) permet l'obtention d'une rémission chez 60-80% des patients. Néanmoins, comme tout anticorps monoclonal, le RTX peut induire une immunogénicité. Celle-ci peut être responsable d'une perte d'efficacité. L'objectif de notre travail était d'étudier l'intérêt de l'immuno-monitorage du RTX dans la GEM et de rechercher une réactivité croisée des anticorps (AC) anti-RTX avec trois nouveaux anti-CD20.

Matériels et méthodes : nous avons mesuré le taux résiduel de RTX à M3 et le taux d'AC anti-RTX par ELISA. Nous avons étudié la cytotoxicité des anti-CD20 sur les lymphocytes B en présence ou non d'AC anti-RTX. Les anti-CD20 testés étaient : le RTX, l'ocrelizumab (OCRE) et l'obinutuzumab (OBI) (anti-CD20 humanisés), l'ofatumumab (OFA) (anti-CD20 totalement humain).

Résultats : le taux résiduel de RTX à M3 corrélait significativement avec le taux d'AC anti-PLA2R1 à M6 ($r = -0.609$, $p = 0.0044$). Il était corrélé avec la protéinurie à M6 ($r = -0.750$, $p = 0.0001$) mais non avec la protéinurie initiale ($r = -0.085$, $p = 0.7136$). Un taux élevé de RTX sérique à M3 et un titre bas d'AC anti-PLA2R à M6 étaient significativement associés à la rémission clinique ($p = 0.0011$ et $p = 0.0095$ respectivement). Il n'existait pas de différence significative pour le résiduel de RTX à M3 en fonction de la présence ou non d'AC anti-RTX. Au sein de notre cohorte de 22 patients, 23% ont développé des AC anti-RTX. Ces patients répondaient partiellement au traitement et nécessitaient d'être re-traités avant M12 ($p = 0.039$). Les AC anti-RTX étaient neutralisants dans 80% des cas. Chez deux patients, les AC anti-RTX inhibaient également la cytotoxicité de l'OBI, l'OCRE et l'OFA. Chez trois patients, il n'existait pas de réactivité croisée avec les nouveaux anti-CD20.

Discussion et conclusion : le taux résiduel de RTX à M3 pourrait être un paramètre utile au monitoring des patients traités par RTX pour une GEM. Un taux résiduel indétectable à M3 permettrait d'adapter le traitement en encourageant une troisième dose de RTX chez les patients avec des AC anti-PLA2R1 persistants. L'immunogénicité liée au RTX n'est pas rare. La plupart des AC anti-RTX sont neutralisants et altèrent la réponse thérapeutique. L'hypothèse d'un traitement de rattrapage par de nouveaux anti-CD20 paraît séduisante cependant, il existe un risque de réactivité croisée des AC anti-RTX avec les nouveaux anti-CD20. Des études prospectives afin de développer des stratégies thérapeutiques personnalisées basées sur l'immunomonitorage des AC monoclonaux et le dépistage de l'immunogénicité sont nécessaires.

Mots clés : glomérulonéphrite extra-membraneuse, rituximab, anticorps monoclonal anti-CD20, immunogénicité, immuno-monitorage

ABSTRACT

Background: Rituximab (RTX), a murine / human chimeric monoclonal antibody (mAb) directed against the CD20, induced clinical remission in 60 to 80% of patients with primary membranous nephropathy (MN). However, monoclonal antibodies (mAbs) as RTX can elicit an unwanted antibody response, called immunogenicity, resulting in a loss of efficacy of treatment. In this study, we aim to monitor RTX therapy in a cohort of patients treated for primary MN and to assess whether new humanized and fully-human anti-CD20 mAbs could be a therapeutic alternative.

Materials and methods: we measured rituximab levels at month 3 and anti-RTX antibodies by ELISA. We studied anti-CD20 mAbs B cells cytotoxicity in presence or absence of anti-RTX antibodies. Tested anti-CD20 mAbs were: RTX, ocrelizumab (OCRE) and obinutuzumab (OBI) (humanized anti-CD20 mAbs) and ofatumumab (OFA) (fully human anti-CD20 mAb).

Results: residual serum RTX level at month 3 was significantly correlated with anti-PLA2R1 antibodies titer at month 6 ($r = -0.609$, $p = 0.0044$). It was also correlated with proteinuria at month 6 ($r = -0.750$, $p = 0.0001$) whereas there was no correlation with baseline proteinuria ($r = -0.085$, $p = 0.7136$). High serum RTX levels at month 3 and low anti-PLA2R1 antibodies at month 6 were associated with clinical remission at month 6 ($p = 0.0011$ and $p = 0.0095$ respectively). There was no statistical difference between serum RTX concentrations at month 3 between patients with or without anti-RTX antibodies. In our 22 patients cohort, 23% developed anti-RTX antibodies. Patients in whom anti-RTX antibodies developed required to be treated again before month 12 ($p = 0.039$). Anti-RTX antibodies were neutralizing in 80% of cases. For two patients, they also blocked both OBI, OCRE and OFA cytotoxicity whereas no cross-reactivity was observed for three patients.

Discussion and conclusion: measurement of residual serum RTX concentration at 3 months could be a useful adjunct to monitoring of MN patients, while early measurements at day 15 were shown not to be predictive. Undetectable serum RTX at month 3 could impact treatment as encouraging a third RTX infusion in patients with persistent anti-PLA2R1 activity. Neutralizing anti-RTX antibodies are not rare and their presence at month 6 is associated with subsequent MN relapse. They can also neutralize other anti-CD20 mAbs. Further studies are needed to develop personalized therapeutic strategies in primary MN based on drug monitoring and immunogenicity testing.

Keywords: membranous nephropathy, rituximab, anti-CD20 monoclonal antibody, immunogenicity, immune-monitoring

BACKGROUND

Rituximab (RTX) is a chimeric antibody with human IgG1 constant regions and a murine monoclonal anti-human CD20 variable region that binds to the CD20 surface marker expressed on B cells (1). This includes precursor B cells (pre-B cells) and mature and memory B cells. Following antibody binding, RTX can trigger B-cell death by direct signaling (1,2), complement mediated cytotoxicity (1,3) and antibody-dependent cellular cytotoxicity (1,4). Although the loss of B cells from the circulation is transient, the duration of depletion can be highly variable between individuals. RTX was first developed for treatment of non-Hodgkin lymphoma (5). In the last 20 years, it has emerged as a potent immunosuppressor for many immune-mediated diseases, beginning initially with rheumatoid arthritis (6), and now extending into several other fields, including clinical nephrology (7). Based on recent large clinical trials, RTX is approved for induction and maintenance therapy for ANCA-associated vasculitis (8,9) and continues to be studied in off-label usage for many glomerular diseases including membranous nephropathy (MN) (10–14), lupus nephritis (15) and mixed cryoglobulinemia (16). It has been used as a treatment in nephrotic syndrome in children and adults, including both minimal change disease and focal segmental glomerulosclerosis (17,18). Given its efficacy, tolerability and safety profile in comparison to more conventional treatment regimens (19), RTX is rapidly emerging as a critical treatment modality in glomerular diseases. In fact, RTX induced clinical remission in 60-80% of patients with primary MN in several non-randomized studies (10,11,13).

The improved patient-outcomes and cost-effectiveness have led to the development of other anti-B cells agents (20). New monoclonal antibodies (mAbs) targeting CD20 are currently studied in non-Hodgkin lymphomas and autoimmune diseases (rheumatoid arthritis, pemphigus, multiple sclerosis...)(5,21).

However, mAbs such as RTX can elicit an unwanted antibody response in a substantial number of patients, resulting in a lack of therapeutic response. The first generation of anti-CD20 antibodies was of murine origin and the immunogenicity of those monoclonal antibodies has been studied extensively. Some 90% of patients treated with murine antibodies produced human anti-mouse antibodies. Immunogenicity of therapeutic antibodies has been markedly reduced by replacing murine constant regions with human ones, resulting in chimeric antibodies such as in RTX and infliximab, an anti-TNF α . Immunogenicity is another reason for producing humanized or human mAb. For example, IgG antibodies to infliximab developed in 60% of patients with Crohn's disease blunting treatment response (22). In

pemphigus vulgaris and ANCA-associated vasculitis, patients with anti-RTX antibodies presented disease relapses (23,24). In systemic lupus erythematosus (SLE), antibodies to RTX correlated with poor B-cell depletion and negative outcomes (25). In six multinational trials evaluating bococizumab, a humanized monoclonal antibody targeting PCSK9, antidrug antibodies (ADAs) developed in a large proportion of the patients and significantly attenuated the lowering of LDL cholesterol levels (26). These findings have not been well described yet in primary MN treated with RTX.

Among these new generation of anti-CD20 mAb we can mention two humanized IgG1: obinutuzumab (OBI) and ocrelizumab (OCRE) (Roche[®]); and a fully-human IgG1: ofatumumab (OFA) (GSK[®]).

We hypothesized that decreased effectiveness, or lack of response to RTX among patients with active nephrotic syndrome due to MN is partially explained by the presence or development of anti-RTX antibodies. We aim to monitor RTX therapy in a cohort of patients treated for primary MN and to assess whether new humanized (OBI and OCRE) and fully-human (OFA) anti-CD20 mAbs could be a therapeutic alternative.

MATERIALS AND METHODS

Patient population

Patients were enrolled after signing informed consent from a previous prospective study. They were recruited in the Department of Nephrology at Pasteur University Hospital in Nice between January 2011 to September 2017. The inclusion criteria were: (a) biopsy-proven diagnosis; (b), primary MN defined by the absence of anti-nuclear antibodies, negative hepatitis B and C serologies, and negative cancer workup (whole-body CT-scan, GI endoscopy, PSA for men and mammography for women); (c) positive anti-phospholipase A2 receptor 1 (PLA2R1) antibodies measured by ELISA; (d) persistent nephrotic proteinuria (i.e. urinary protein/creatinine ratio (UPCR) > 3.5g/g) after 6 months of maximal tolerated antiproteinuric treatment or early deterioration of kidney function, or complications of the nephrotic syndrome. Patients received two 1g infusion of RTX at 2-week interval.

Serum and urine samples were prospectively collected at the first infusion, at months 3 and 6 to measure RTX serum levels, anti-RTX and anti-PLA2R1 antibodies, CD19 positives cells, serum creatinine and UPCR.

Remissions were defined according to the 2012 KDIGO guidelines. Complete remission was defined by a UPCR < 0.3 g/g accompanied by a normal serum albumin concentration and a preserved kidney function. Partial remission was defined by UPCR < 3.5 g/g with over 50% reduction of proteinuria accompanied by an improvement or normalization of the serum albumin concentration and preserved kidney function (serum creatinine levels increase from baseline below 30%). Remissions were counted at month 6, and subsequently before any treatment modification. A relapse was defined by an increase of UPCR over 3.5 g/g after remission.

Measurement of anti-PLA2R1 antibodies by ELISA

Serum levels of total IgG PLA2R1 antibody were measured by an ELISA test, which was developed by EUROIMMUN AG (Lübeck, Germany). According to the manufacturer, the ELISA results were considered positive at a level > 14 RU/ml.

Measurement of rituximab by ELISA

Anti-RTX antibodies were coated according to the manufacturer's instructions (LISA-TRACKER Duo Rituximab, Theradiag[®] Croissy Beaubourg, France), and then incubated for 60 minutes at room temperature with serum samples diluted at 1:1000 or 1:10 for urine, washed and incubated with biotinylated anti-human IgG for 60 minutes at room temperature, washed and incubated with HRP Streptavidin conjugate for 30 minutes at room temperature, washed and revealed with substrate for 15 minutes at room temperature. Reaction was stopped with H₂SO₄ and plates were read at 450 nm (Sunrise Tecan[®] Männedorf, Switzerland). This assay measures only free RTX. The limit of detection for RTX was 2 µg/ml with an intra-run variability of 7.6% and inter-run variability of 9.8%.

Measurement of anti-rituximab antibodies by ELISA

Wells were coated with RTX according to the manufacturer's instructions (LISA-TRACKER Duo Rituximab, Theradiag[®] Croissy Beaubourg, France), and then incubated with samples diluted at 1:2 for 60 minutes at room temperature, washed and incubated with biotinylated RTX for 60 minutes at room temperature, washed and incubated with HRP streptavidin conjugate for 30 minutes at room temperature, washed and revealed with substrate for 15 minutes at room temperature. Reaction was stopped with H₂SO₄ and plates were read at 450 nm. Results were calculated from the generated standard curve using the right analysis.

This assay measures only free anti-RTX antibodies. The limit of detection for anti-RTX antibodies was 5 ng/ml with an intra-run variability being at 9.1% and inter-run variability at 10.6%.

Neutralization of anti-CD20 monoclonal antibodies by anti-rituximab antibodies in vitro

Drug minimal cytotoxic concentration assessment

First, we assessed *in vitro* minimal anti-CD20 mAb cytotoxic concentration. Anti-CD20 mAb (RTX, OBI, OCRE and OFA) at 6.25, 12.5, 25 and 50ng/ml were incubated with 1.5×10^3 purified B-cells (MACSprep[™] HLA B Cell Isolation Kit, Milteny Biotec) for 30 minutes at room temperature in 60-well Terasaki plates (Dutcher[®] Strasbourg, France) in duplicates of 1 µl per well. Then, 5 µl per well of standard rabbit complement (Cerdarlane[®] Ontario, Canada) were added for 45 minutes at room temperature. Dead cells were then revealed after adding 2.5 µl per well of Fluoroquencher AO/EB staining/quencher (Ingen[®] Chilly-Mazarine, France) for 10 minutes in darkness.

Two blinded independent evaluators estimated the percentage of dead cells using a fluorescent microscope (Axiovert 100 Carl Zeiss[®] Göttingen, Germany). This was confirmed with flow cytometry.

Then, serum samples incubated in the presence of anti-CD20 mAb were used to test the potential neutralizing effect of anti-RTX antibodies in a qualitative assay using complement dependent cytotoxicity assay and in a quantitative assay using flow cytometry.

Qualitative B-cell cytotoxicity assay

B-cell cytotoxicity was measured in different conditions as described by Terasaki et al. for cross-match test before kidney transplantation. Ten microliters of serum from patients with anti-RTX antibodies were incubated with ten microliters without any anti-CD20.

Same experiments were done with increasing concentrations of RTX or of new anti-CD20 mAb: OBI, OCRE and OFA, starting with the minimal drug cytotoxic concentration assessed as above (50, 150 and 250ng/ml). Serum from healthy donors were used as negative controls. Each sample was pre-incubated for two hours at room temperature, before adding 1.5×10^3 purified B-cells for a 30-minute incubation at room temperature in 60-well Terasaki plates in duplicates of 1 μ l per well. Then, 5 μ l per well of standard rabbit complement were added for 45 minutes at room temperature. Dead cells were then revealed after adding 2.5 μ l per well of Fluoroquench AO/EB staining/quench for 10 minutes in darkness. Two blinded independent evaluators estimated the percentage of dead cells using a fluorescent microscope.

Quantitative cytotoxicity assay

After a Ficoll separation, 1.8×10^6 peripheral blood mononuclear cells (PBMC) from healthy donor were incubated 30 minutes at room temperature with 20 μ l of anti-CD20 mAb (RTX, OBI, OCRE or OFA) at 50 ng/ml; pre-incubated with 20 μ l of serum from patients with anti-RTX antibodies or from healthy donor diluted at 1:2. Then, 100 μ l of standard rabbit complement were added to each tube and incubated for 45 minutes at room temperature. Cell viability was assessed using 10 μ l of trypan blue added to 90 μ l of PBMC incubated in each condition. Numbers of dead and alive cells were counted in four different fields. The cells were washed three times in 3 ml of PBS (Cell Wash BD Biosciences[®] Erembodegem, Belgium) at 4°C and incubated 30 minutes in darkness with a panel of antibodies specific for T, B and NK cells: anti-CD3, anti-CD4, anti-CD8, anti-CD45, anti-CD19, anti-CD16 et CD56

(6-color TBNK Reagent BD Biosciences). Then, lysing Solution was added and samples were incubated ten minutes in darkness.

The percentages of the T-lymphocytes (LyTS+, Ly45+, CD3+), B-lymphocytes (CD19+) and NK-cells (CD3- CD19-) were determined using Cytometer BD FACS Canto II.

Endpoints

Primary endpoint was the association of residual serum RTX concentrations and anti-RTX antibodies with response to treatment.

Secondary endpoints were: the association of anti-RTX antibodies with safety; assess whether anti-RTX antibodies were neutralizing and evaluate anti-RTX antibodies effect on new anti-CD20 mAbs.

Statistical analyses

Qualitative variables were expressed as frequencies and compared using Fisher exact test. Continuous variables were expressed as median and interquartile interval and compared using Student t test. Comparisons of qualitative variables were performed using Fisher exact test. Quantitative variables were compared using Wilcoxon-Mann-Whitney test or Kruskal Wallis test for multilevel variables. Correlations were assessed by Spearman rank correlation coefficient. A *p*-value less than 0.05 indicated statistical significance. Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Residual serum rituximab levels at month 3

A total of 22 patients with primary MN were enrolled. **Table 1** shows the detailed baseline characteristics of the study cohort. All patients were on Angiotensin-Converting-Enzyme (ACE) inhibitor or Angiotensin II Receptor Blocker (ARB).

Table 1. Clinical characteristics of patients enrolled in the study (n= 22)

Characteristics	Value
Age (years)	62 ± 14
Gender (male/female)	17/5
Anti-PLA2R titer at baseline (RU/ml)	165 (67.5-318.3)
Serum creatinine at baseline (mg/dl)	1.12 (0.62-1.45)
Serum albumin at baseline (g/l)	2.10 (1.57-2.85)
UPCR ¹ at baseline (g/g)	5.70 (4.51-7.11)
UPCR ¹ at M6 (g/g)	1.95 (0.89-5.09)
CD19 count at M6 (/μl)	5 (1.75-33)
Rituximab level at M3 (μg/ml)	3.59 (1.45-9.15)
Patients with anti-rituximab antibodies	5 (23%)
Patients in remission at M6	14 (64%)
Patients treated again before M12	8 (36%)

¹UPCR: urinary protein/creatinine ratio

Compared with a cohort of comparable patients treated with same treatment regimen for *myasthenia gravis*, residual serum RTX levels were significantly lower in patients treated for MN (2.58 (1.22 – 5.42) and 14.27 (4.97-24.05) $\mu\text{g/ml}$ respectively, $p = 0.0086$) (**Figure 1A**). In the nephrotic cohort, after two weeks, RTX could be detected in the urine (assessed by ELISA) (**Figure 1B**).

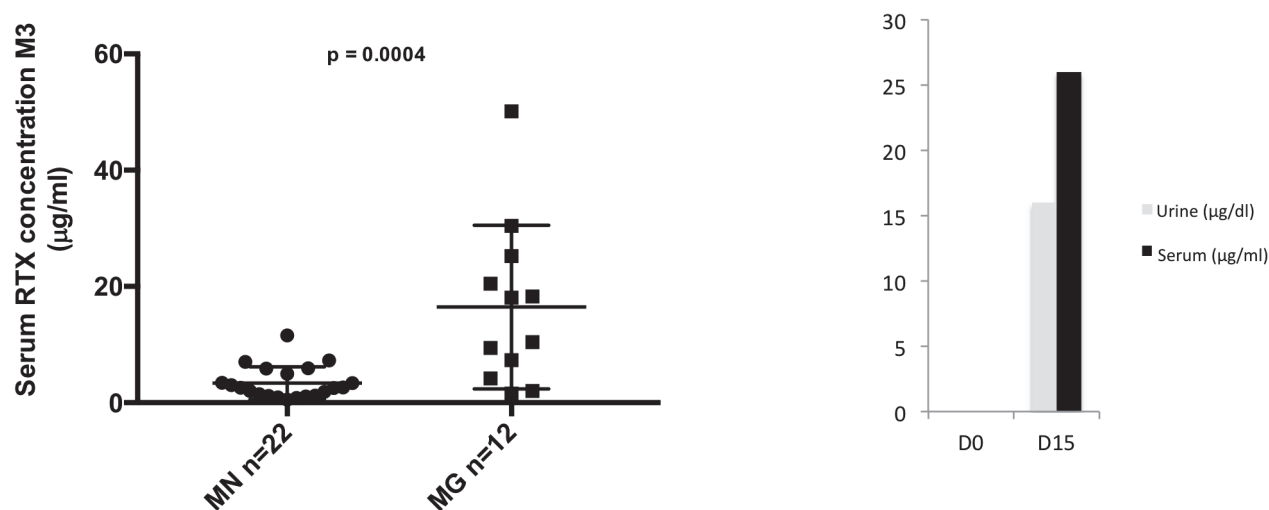


Figure 1. Pharmacokinetics of rituximab in patients with nephrotic syndrome

A. Residual serum rituximab levels at month 3 were lower in patients with membranous nephropathy comparing to patients who received the same treatment regimen for myasthenia gravis.
 B. An explanation for this could be an excessive urinary waste of rituximab in nephrotic patients.
 MN: membranous nephropathy, MG: myasthenia gravis

Complete or partial remission was obtained at month 6 in 14 of the 22 patients (63.6%). All patients achieving remission at month 6 had residual RTX concentration above 2 $\mu\text{g/ml}$. At month 6, serum RTX concentrations were undetectable in all patients. Residual serum RTX level at month 3 was significantly correlated with anti-PLA2R1 antibodies titer at month 6 ($r = -0.609$, $p = 0.0044$) (**Figure 2A**). It was also correlated with proteinuria at month 6 ($r = -0.750$, $p = 0.0001$) (**Figure 2B**) whereas there was no correlation with baseline proteinuria ($r = -0.085$, $p = 0.7136$) (**Figure 2C**). High serum RTX levels at month 3 and low anti-PLA2R1 antibodies at month 6 were associated with clinical remission at month 6 (5.705 (2.515-10.95) vs 0.66 (0.15 – 1.7) $\mu\text{g/ml}$, $p = 0.0011$ and 0 (0-3.75) vs 18 (10.25-22.75) RU/ml, $p = 0.0095$ respectively) (**Figures 2D-E**).

There was no statistical difference between serum RTX concentrations at month 3 between patients with or without anti-RTX antibodies (5.70 (3.69-10.1) and 2.7 (0.9-9.15), $p = 0.307$). High serum RTX concentrations correlated with CD19 depletion at month 3 ($r = -0.718$, $p = 0.004$) and at month 6 ($r = -0.711$, $p = 0.037$). CD19 count tended to be higher in patients with anti-RTX antibodies compared with patients without, but this result did not reach significance (47.7 (10-104.5) and 2 (1.7-41)/ μl , $p = 0.23$).

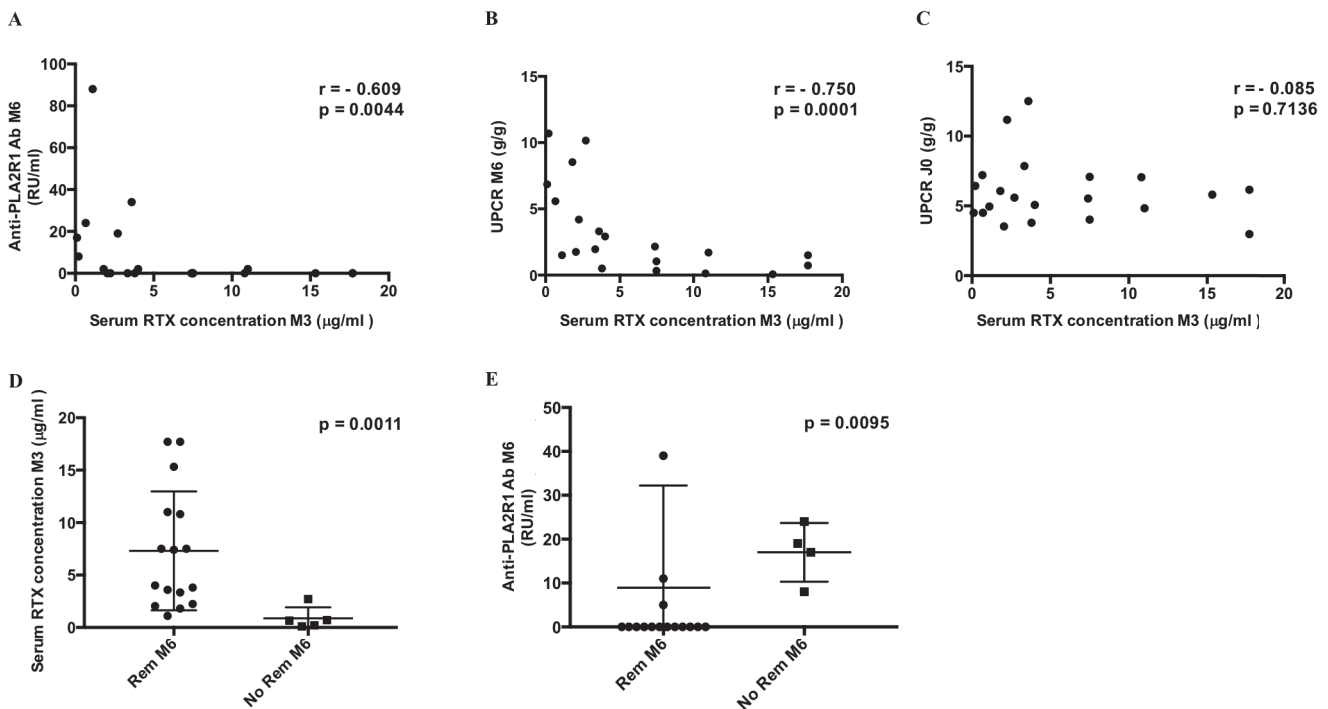


Figure 2. Residual serum rituximab concentration at month 3

- Residual serum rituximab levels at month 3 correlated with anti-PLA2R1 antibodies
 - Residual serum rituximab levels at month 3 correlated with proteinuria at month 6
 - Residual serum rituximab levels at month 3 did not correlate with baseline proteinuria
 - High serum rituximab levels at month 3 were associated with clinical remission at month 6
 - Low anti-PLA2R1 antibodies at month 6 were associated with clinical remission at month 6
- Anti-PLA2R1 Ab: anti-PLA2R1 antibodies, UPCR: urinary protein/creatinine ratio, Rem: remission.

Drug minimal cytotoxic concentration assessment

For each anti-CD20 mAb, we determined that the minimal cytotoxic concentration, defined as the lowest dose required to produce $\geq 50\%$ of B-cell cytotoxicity, was 50 ng/ml (**Figure 3**).

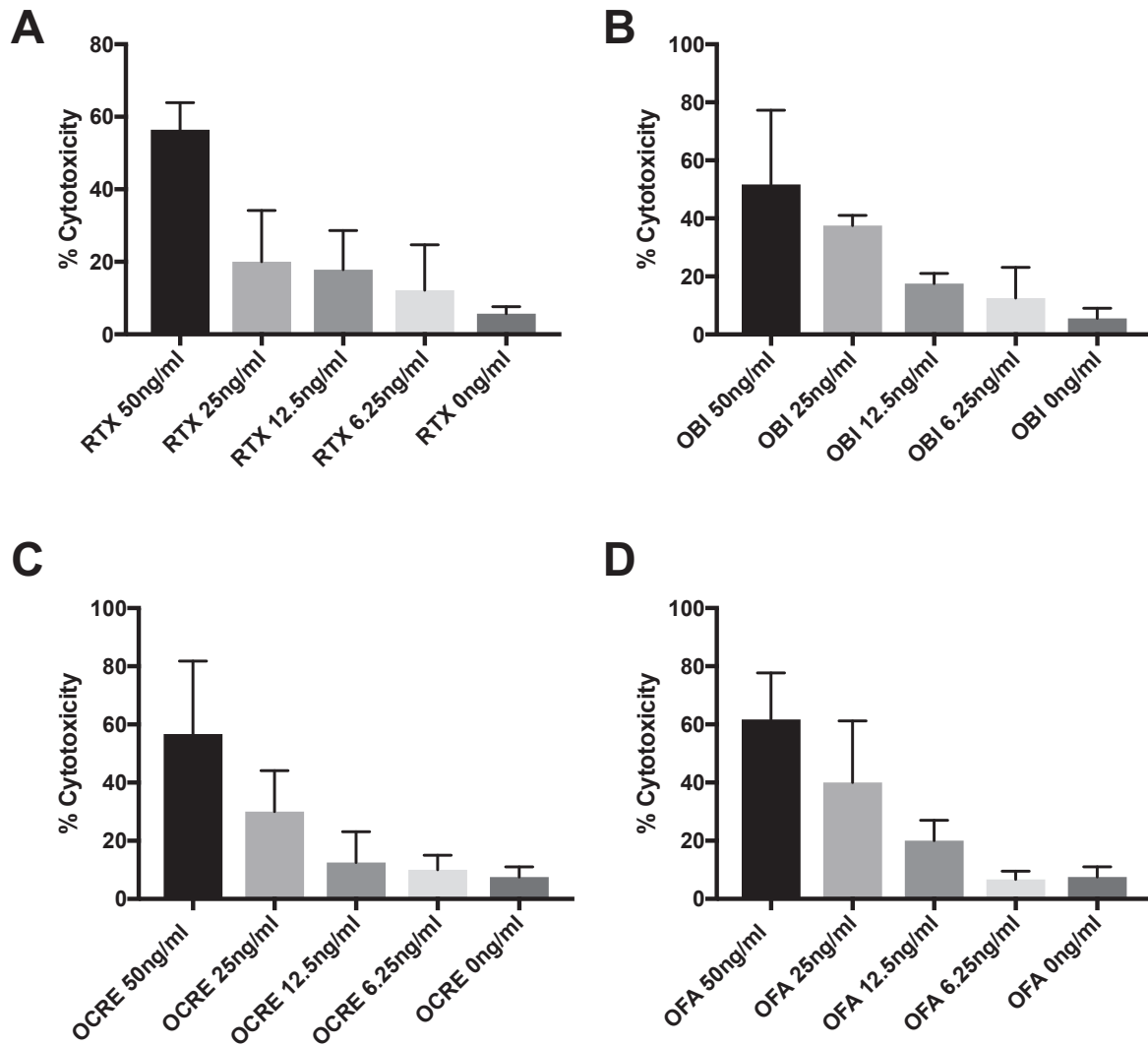


Figure 3. Determination of anti-CD20 monoclonal antibody minimal cytotoxic concentration

- A. Assessment for rituximab
- B. Assessment for obinutuzumab
- C. Assessment for ocrelizumab
- D. Assessment for ofatumumab

For both, the minimal concentration required to produce $\geq 50\%$ of cytotoxicity was 50ng/ml.
 RTX: rituximab, OBI: obinutuzumab, OCRE: ocrelizumab, OFA: ofatumumab

Detection and neutralizing effect of anti-rituximab antibodies

Anti-RTX antibodies were detected by ELISA in 5 patients (23%). We then established whether these antibodies were neutralizing with two different methods. Anti-RTX antibodies blocked RTX B-cells cytotoxicity in four patients out of five (**Table 2**).

For these patients, in a first set of experiments, RTX (50 ng/ml) added to healthy donor serum in the presence of rabbit complement induced 80 to 100% B-cell death (**Figure 4A**). It was blocked when RTX was pre-incubated with serum containing anti-RTX antibodies: B-cells cytotoxicity fell to less than 20% (**Figure 4A**). For increasing concentrations of RTX, we observed a dose-response effect in RTX B-cell cytotoxicity. In presence of anti-RTX antibodies, RTX appeared to be effective for high doses (> 50% B-cells cytotoxicity for RTX at 250ng/ml) (**Figure 4A**).

In a second set of experiments, RTX incubated with whole lymphocyte population induced B-cell apoptosis assessed by CD19+ cell count which dropped to 8 (9%) with 50ng/ml of RTX (**Figure 4B**). After pre-incubation with serum containing anti-RTX antibodies, the proportion of CD19+ cells increased to 51 (18%) (**Figure 4B**).

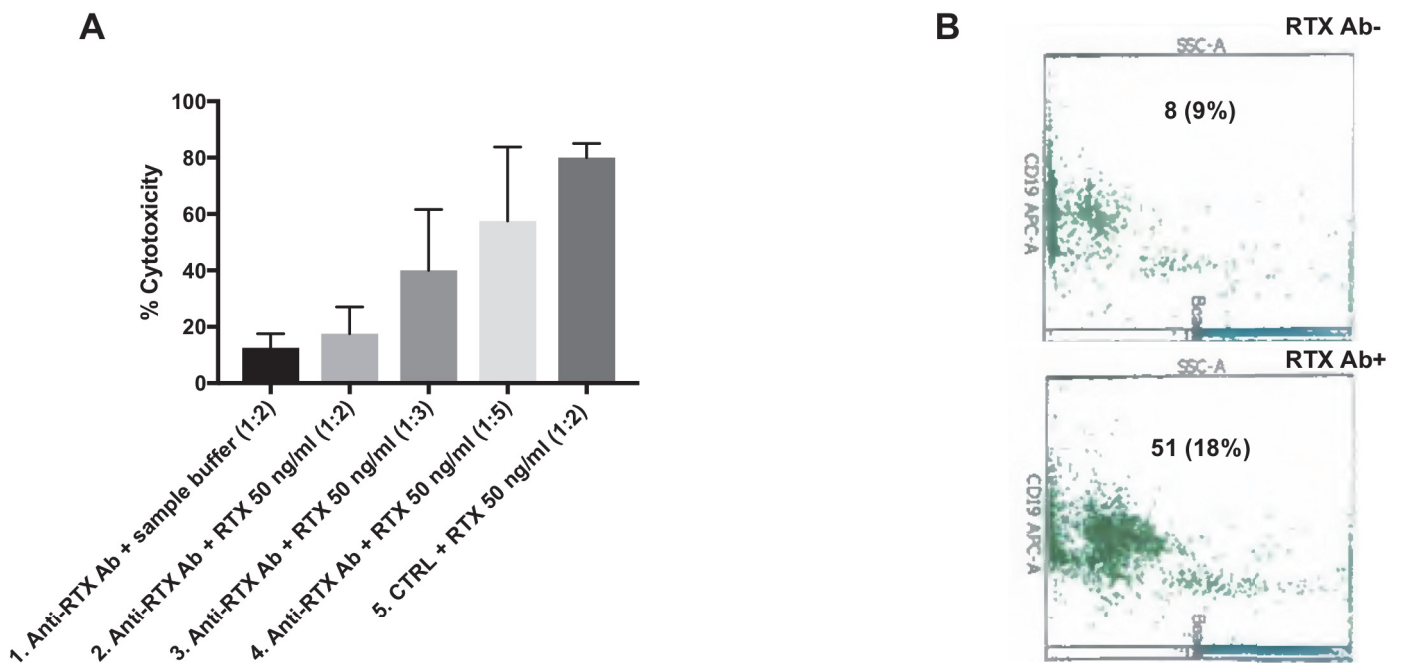


Figure 4. Cytotoxicity assays for rituximab

A. Qualitative assay using cross-match test: In presence of anti-rituximab antibodies in serum, rituximab B-cells cytotoxicity is inhibited demonstrating the neutralizing character of these antibodies. We observed a dose-response effect in RTX cytotoxicity, related to anti-RTX antibodies saturation

B. Quantitative assay using flow cytometry

Anti-RTX Ab: serum from patient with anti-rituximab antibodies, RTX: rituximab, CTRL: negative control

Because neutralizing antibodies may affect treatment efficacy, we analyzed their impact on patient outcome at month 6. Patients in whom anti-RTX antibodies developed required more frequently a second course of treatment before month 12 (four patients out of five with anti-RTX antibodies and four patients out of 17 without antibodies, $p = 0.0036$) (**Figure 5**).

With respect to an association between safety and anti-RTX antibodies, only one patient in each group presented a drug infusion reaction. This reaction did not require treatment discontinuation.

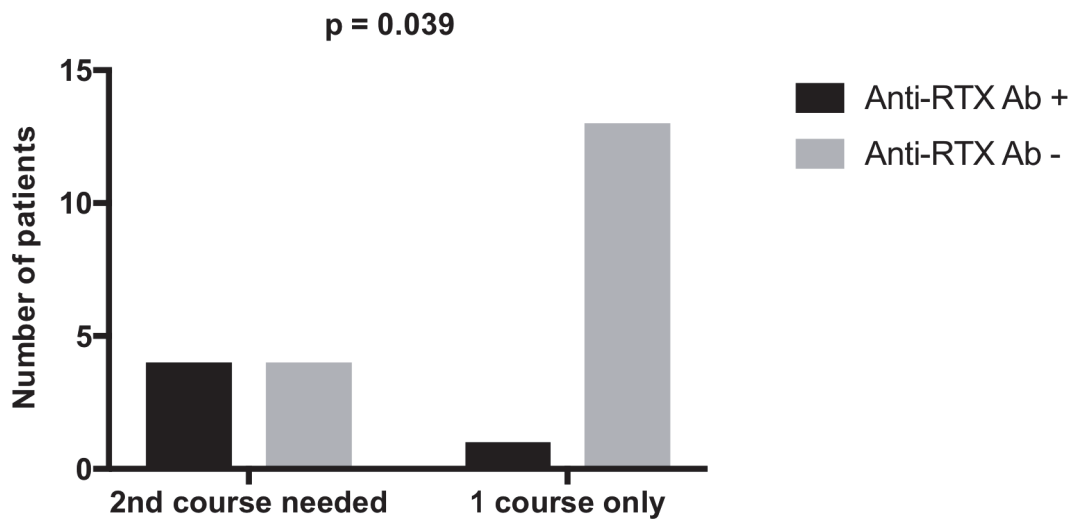


Figure 5. Neutralizing anti-rituximab antibodies impact on treatment

Patients with anti-rituximab antibodies did not respond or relapse more frequently than patients without antibodies. They needed to receive a second course of treatment before month 12.

Anti-RTX Ab: anti-rituximab antibodies, RTX: rituximab.

Cross-reactivity of neutralizing anti-rituximab antibodies and new anti-CD20 monoclonal antibodies

We then tested whether humanized (OBI and ORE) and fully human anti-CD20 mAbs (OBI) were inhibited or not by anti-RTX antibodies. The same experiments conducted with RTX were repeated with each anti-CD20 mAb. Results are summarized in **table 2**.

Table 2. Anti-CD20 monoclonal antibodies B-cell cytotoxicity for patients with anti-rituximab antibodies

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Anti-RTX ¹ antibodies titer at M6 (ng/ml)	40	130	47	30	> 150
Anti-RTX antibodies neutralization on RTX ¹ 50ng/ml	++	++	+	++	++
Anti-RTX antibodies neutralization on OBI ² 50ng/ml	++	++	0	+	0
Anti-RTX antibodies neutralization on OCRE ³ 50ng/ml	++	++	0	0	0
Anti-RTX antibodies neutralization on OFA ⁴ 50ng/ml	++	++	0	+	0
Response to treatment	No	Yes	No	No	No

¹RTX: rituximab

²OBI: obinutuzumab

³OCRE: ocrelizumab

⁴OFA: ofatumumab

0: no neutralizing effect of anti-rituximab antibodies on anti-CD20 mAb

+: mild neutralizing effect

++: strong neutralizing effect

Two patients' profiles were observed (Figure 6). For two patients, anti-RTX antibodies blocked B-cell cytotoxicity for both OBI, OCRE and OFA (**Figure 6A**) whereas anti-CD20 mAb efficacy was not impaired for three patients (**Figure 6B**). Anti-RTX antibodies titer did not correlate with therapeutic response.

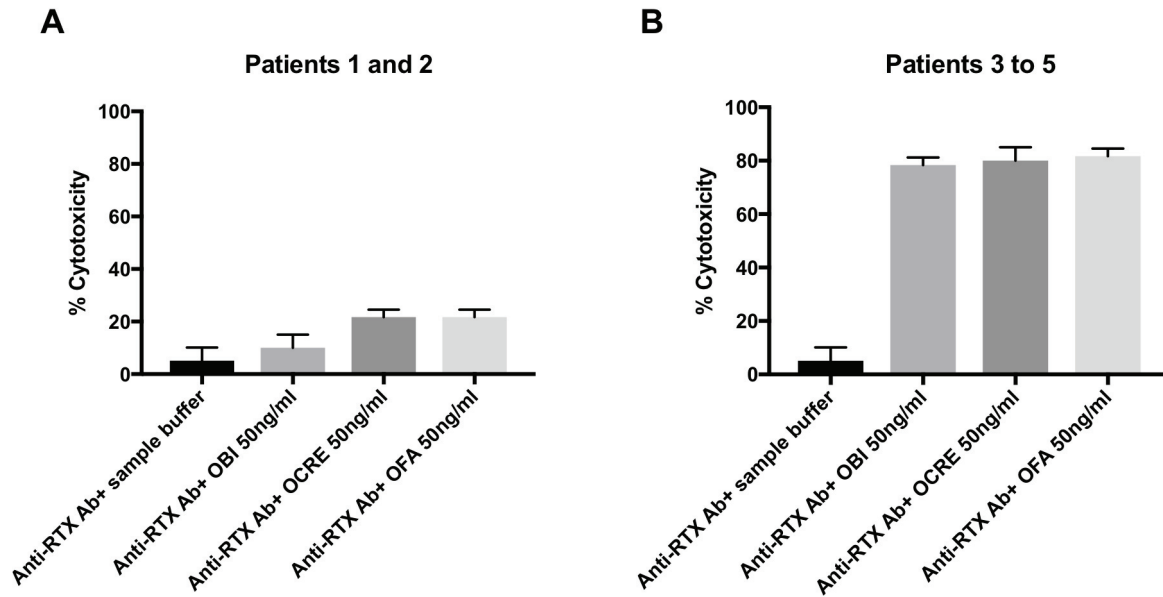


Figure 6. Cross reactivity for humanized and fully human anti-CD20 monoclonal antibodies in patients with anti-rituximab antibodies
 A. For two patients, anti-rituximab antibodies neutralized both obinutuzumab, ocrelizumab and ofatumumab cytotoxicity
 B. For three patients, there was no cross reactivity between new anti-CD20 therapies and anti-rituximab antibodies
 RTX: rituximab, OBI: obinutuzumab, OCRE: ocrelizumab, OFA: ofatumumab

In those two patients, in the first set of experiments, each anti-CD20 mAb at 50ng/ml added to healthy donor serum in the presence of rabbit complement induced 80 to 100% B-cell death. It was blocked when anti-CD20 mAb was pre-incubated with serum containing neutralizing anti-RTX antibodies: B-cells cytotoxicity decreased to 20% (**Figure 7**). With increasing concentrations of anti-CD20 mAb, we did not observe a relevant dose-response effect in B-cells cytotoxicity for OBI, OCRE or OFA. In presence of anti-RTX antibodies, none of the anti-CD20 mAbs tested demonstrated cytotoxicity (for OBI, OCRE and OFA, B-cell cytotoxicity was always less than 50% even even at anti-CD20 Ab concentration of 250ng/ml) (**Figure 7**).

In the second set of experiments, OBI, OCRE or OFA incubated with whole lymphocyte population induced B-cell apoptosis assessed by CD19+ cell count which dropped to 7/ μ l (14%) with 50ng/ml of OBI (**Figure 7A**), 7/ μ l (14%) with 50ng/ml of OCRE (**Figure 7B**) and 12/ μ l (14%) with OFA (**Figure 7C**). After pre-incubation with serum containing neutralizing anti-RTX antibodies, the proportion of CD19+ cells increased to 50/ μ l (18%) with OBI (**Figure 7A**), 51/ μ l (18%) with OCRE (**Figure 7B**) and 48/ μ l (17%) with OFA (**Figure 7C**). **Figure 8** provides a schematic summary of the flow cytometry data.

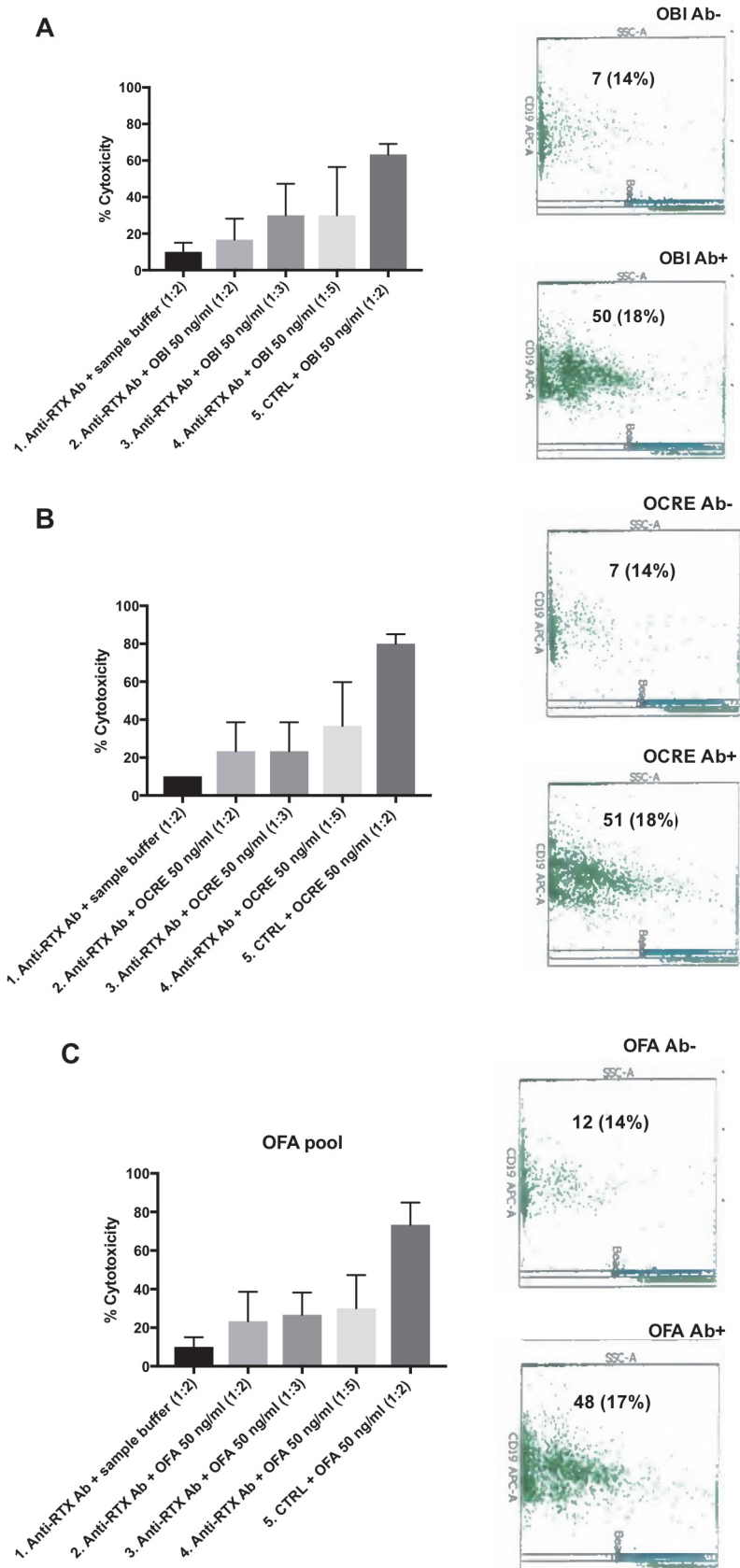


Figure 7. Cross-reactivity for new anti-CD20 monoclonal antibodies in two patients with anti-rituximab antibodies

A. Results for obinutuzumab from cross-match test and fluorescence activated cell sorting (FACS)

B. Results for ocrelizumab from cross-match test and FACS

C. Results for obitumumab from cross-match test and FACS

RTX: rituximab, OBI: obinutuzumab, OCRE: ocrelizumab, OFA: obitumumab

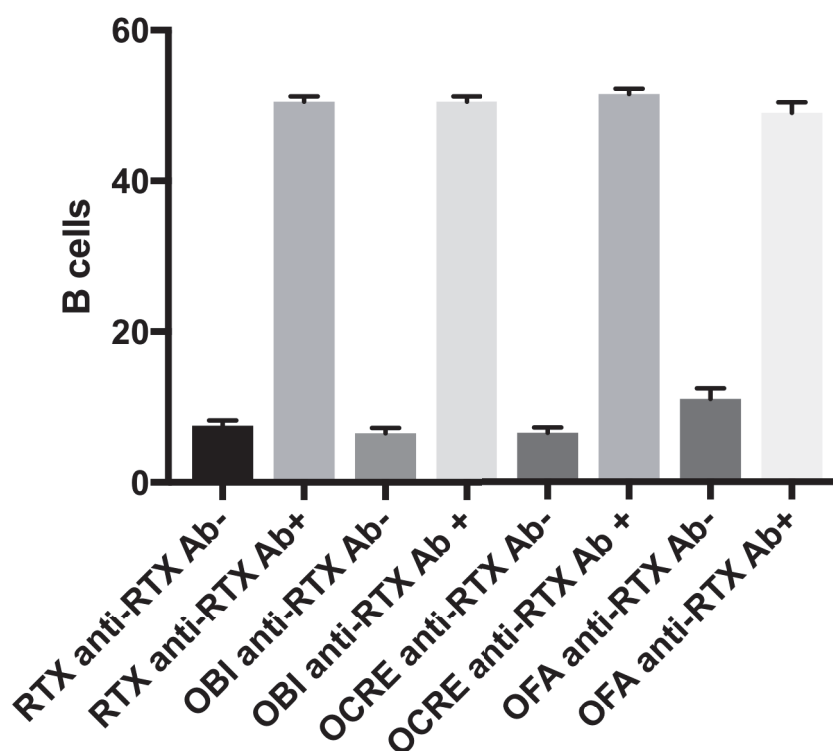


Figure 8. In two patients, anti-rituximab antibodies neutralized rituximab and could also inhibit both obinutuzumab, ocrelizumab and ofatumumab
 RTX: rituximab, OBI: obinutuzumab, OCRE: ocrelizumab, OFA: ofatumumab, Ab: antibodies

Anti-rituximab antibodies specific blockade

To confirm that the observed neutralizing effect was due to anti-RTX antibodies, sera from patients who developed neutralizing antibodies were diluted from 1:1 to 1:1000 and added to RTX 50ng/ml. Then, B-cell cytotoxicity was assessed as described previously. For each anti-CD20 mAb, inhibition effect was lost for a 1:10 dilution (**Figure 9**).

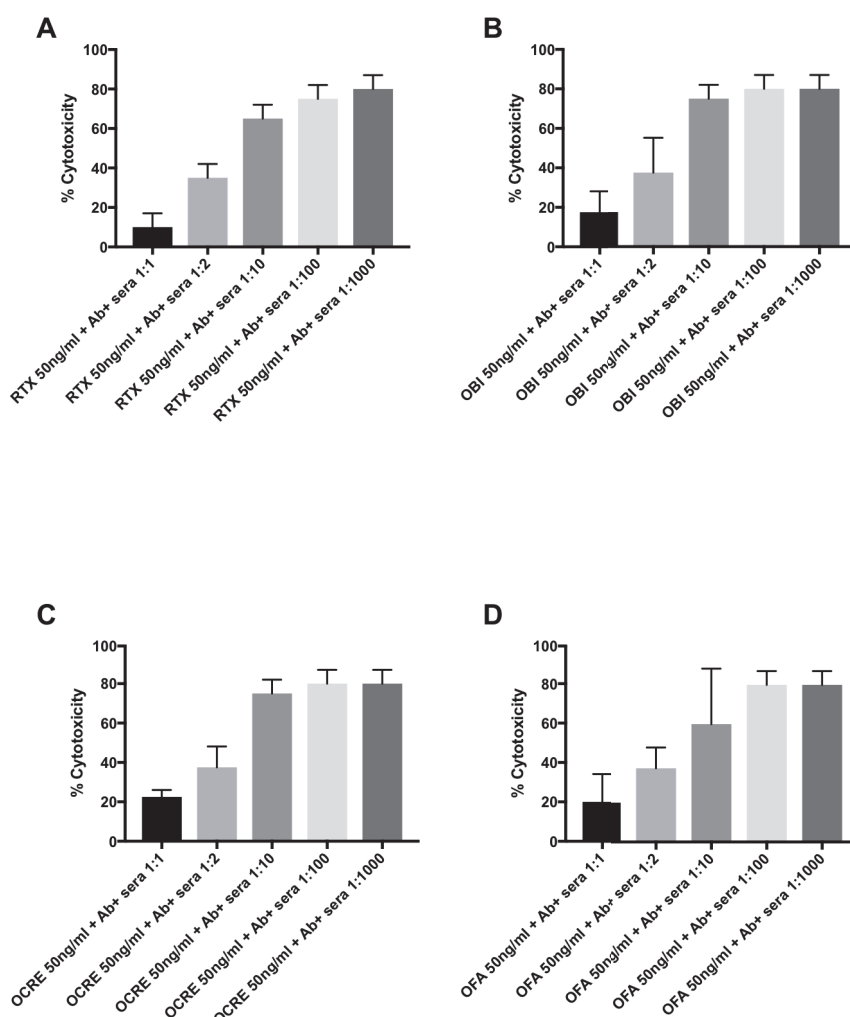


Figure 9. Neutralizing anti-rituximab antibodies serial dilutions confirm specific inhibition

- A. Assay for rituximab
- B. Assay for obinutuzumab
- C. Assay for ocrelizumab
- D. Assay for ofatumumab

For both, minimal dilution to produce < 50% of cytotoxicity was 1:10.

RTX: rituximab, OBI: obinutuzumab, OCRE: ocrelizumab, OFA: ofatumumab

Relevant observation for one patient

One patient treated by 4 courses of RTX for MN relapses, developed primary biliary cirrhosis associated with anti-M2 and anti-gp120 antibodies. These antibodies were detected after his first relapse before the onset of symptoms and were negative at diagnosis.

DISCUSSION

The number of therapeutic mAbs targeting CD20 is increasing and they are more broadly and frequently used in auto-immune diseases. Drug monitoring is not routinely performed but could guide personalized treatment in a large patients population treated with anti-CD20 mAbs.

Residual serum RTX levels were significantly lower in our MN cohort compared with a non-nephrotic population. Clinical observations suggested that RTX treatment might be less sustained in patients with nephrotic range proteinuria when compared to non-nephrotic patients and undetectable from month 3 (27). It is conceivable that a reason is that significant amounts of RTX might be lost in the urine in nephrotic patients (28). We observed an early excretion of RTX in the urine of our nephrotic cohort. Counsilman et al. reported an urinary clearance of RTX as about 25% of the total clearance in heavy nephrotic patients. It was suggested that the amount of RTX excretion into the urine roughly correlated with the degree of proteinuria in their patients (29). Conversely, Fervenza and al. were unable to observe a correlation between RTX levels and the degree of proteinuria (11). Stahl et al. better referred to a close correlation of the excretion of RTX to the excretion of IgG molecules suggesting a loss of filter selectivity as the primary determinant of RTX urine excretion (30). Residual RTX serum levels could be detected in some patients six to nine months after the first infusion due to recycling from endothelial cells via neonatal Fc receptors (FcRn) (31). In patients with MN, RTX half-life was calculated as 11.5 days, as compared to 18 days in patients with rheumatoid arthritis (12,32). Heavy proteinuria and factors such as reduced serum half-life of IgG, impaired recycling of IgG through FcRn and internalization and destruction of RTX by target B cells could contribute to low RTX levels. Pharmacokinetic studies demonstrated an intra-individual and a large inter-individual variability, related either to disease or genetic factors, which could also explain differences in clinical response (32,33).

We demonstrated that higher residual serum RTX concentrations at month 3 were significantly associated with higher remission rate at month 6 with lower proteinuria and lower anti-PLA2R1 antibodies at month 6. We could not reach significance for correlating low residual serum RTX levels with seropositivity for anti-RTX antibodies. As found in other immune diseases, residual serum RTX concentration at month 3 correlated with CD19 count. We did not reach significance for correlation with anti-RTX antibodies positivity and poor B-cell depletion, but we were limited by the small number of patients in whom anti-RTX

antibodies were detectable. Recent evidence suggested that an inadequate depletion is associated with poor clinical response in both rheumatoid arthritis (32,34) and SLE (35). It was shown that clinical response depended more on the degree of depletion than the dose of RTX in rheumatoid arthritis. Our results support Ferverza et al. who found no correlation between RTX levels and the degree of B cells depletion in a MN cohort. Although serum RTX concentration may not faithfully reflect the amount of drug delivered to lymphoid tissue, measurement of residual serum RTX concentration at 3 months could be a useful adjunct to monitoring of MN patients, while early measurements at day 15 were shown not to be predictive (11). Undetectable serum RTX at month 3 could impact treatment as encouraging a third RTX infusion in patients with persistent anti-PLA2R1 activity.

We found that anti-RTX antibodies were present in 23% of patients treated with RTX for primary MN. Ferverza and al. reported that 40% of patients with MN developed ADAs to RTX after one year of follow-up. With longer follow-up, we found an higher incidence of anti-RTX. ADAs rates varied widely among biologics across diseases (36). We showed that anti-RTX antibodies blocked B-cell cytotoxicity, suggesting that they are clinically relevant as described for other monoclonal antibodies. We reported that 80% of anti-RTX-positive patients experienced a second course of treatment before month 12 compared with 23.5% of anti-RTX-negative patients. Reasons for a third infusion were relapse or incomplete response. In a meta-analysis of RA, spondyloarthritis, psoriasis and inflammatory bowel disease studies, the ADAs against infliximab and adalimumab decreased response to therapy by 68% (37). Antibodies to the anti-TNF α antibody infliximab developed in 61% of patients with Crohn's disease blunting treatment response and correlated with low infliximab concentrations (22). Studies of infliximab (37–39) and adalimumab (39,40) in patients with rheumatoid arthritis showed that immunogenicity was strongly linked to sub-therapeutic serum drug levels and a lack of clinical response. Anti-RTX antibodies were reported in 4.3% of rheumatoid arthritis patients in the Randomized Evaluation of Long-term Efficacy of Rituximab trial, although no direct relationship with clinical response was observed (41). Approximately 11% of those patients developed ADAs to RTX, but the clinical relevance is unknown. In pemphigus vulgaris and ANCA vasculitis, patients with anti-RTX antibodies presented disease relapses (23,24). In SLE, antibodies to RTX were detected in one-third of patients and correlated with poor B-cell depletion and poor clinical outcome (24,25). In multiple sclerosis, anti-RTX antibodies concerned 37% and were significantly associated with a higher number of RTX infusions and correlated with poor B-cell depletion (42).

The presence of ADAs was significantly associated with a requirement for dose increases (41,43). Anti-RTX titer did not correlate with therapeutic response due to their delayed emergence.

Not all ADAs interfere with the activity of the biologic agent. Distinctions have been made between binding ADAs that do not disrupt the clinical effect of a drug (i.e., non-neutralizing antibodies) and neutralizing ADAs, which have been shown to reduce a drug's bioactivity. The presence of neutralizing ADAs does not necessarily preclude a therapeutic effect. Pharmacological efficacy will depend on the balance between drug concentrations and antibody levels and whether the resulting drug levels are high enough to achieve the desired outcome. Non-neutralizing ADAs, by contrast, bind to a portion of the drug molecule that is not essential to its therapeutic activity (e.g., to the allotope). In such cases, the formation of antibodies is triggered, even when the structure of the molecule is fully human, by polymorphisms expressed in the constant portion of the light and heavy chains, which vary between individuals. The biologic effect of non-neutralizing ADAs is less well understood than that of neutralizing ADAs, but this does not mean that it does not exist (44). The therapeutic molecule can be eliminated by mechanisms other than the direct blockage of the idiotype by antibodies. For instance, the formation of immune complexes, which are eliminated by the reticuloendothelial system, accelerates clearance of the drug (45). This will impact whether the drug is present in sufficient levels to maintain its activity.

We established with two different methods, that anti-RTX antibodies were neutralizing in 80% of cases and could affected clinical response. According to Wolbink et al. and Krishna et al., also non-neutralizing ADAs should be considered (45,46). Inhibition of RTX activity could favor the persistence of pathogenic memory B-cells and induce disease relapse, with an earlier reconstitution of the B-cell compartment (47).

It is reported that patients who develop ADAs are more likely to show anaphylactic infusion reactions, which include cytokine release syndrome and non-acute immune reactions such as immune complex disease (48). Increased rates of infusion reactions had been reported in patients with ADAs to infliximab (49,50). Regarding safety, studies on anti-TNF reported that infusion-related reactions were more common in patients with ADAs than in those without. This finding has not been reproduced for anti-CD20 infusions (51). Rituximab infusion reactions were rare in our cohort: only two patients presented non-severe manifestation related to RTX-infusion and treatment did not need to be discontinued.

If we consider that immunogenicity, given its potential to interfere with treatment, is an important factor that should be considered in the overall treatment strategy, our goal as physicians should be to take actions to reduce the likelihood of ADAs formation. There are essentially three ways to improve this: modifying the administration or management of the drug, increasing the dose and therefore the number of available molecules; interfering with the ability of the immune system to produce ADAs, generally by adding immunosuppressive agents to the regimen or using new drugs which are supposed to be less immunogenic such as humanized or fully human mAbs. In this perspective, we evaluated *in vitro* whether three new B-cell depleting molecules were affected by neutralizing anti-RTX antibodies developed in MN patients. To our knowledge, this is the first study looking at cross-reactivity between ADAs developed in patients treated with RTX and new anti-CD20 mAbs. This has not been described for RTX and even other mAb therapies (e.g., anti-TNF α). In two patients, anti-RTX antibodies appeared to block OCRE, OBI and OFA B-cell cytotoxicity. That fact could impact therapeutic strategies. Those results suggested that patients who develop ADAs for a specific molecule might also experience lack of therapeutic response using a same class drug. In those patients, switching RTX for a new anti-CD20 therapy would not be the most appropriate choice. Regarding ADAs target and according to what have been previously described (52), we hypothesized that neutralizing ADAs might bind to murine epitopes and idiotopes located at the antigen-binding site and prevent fixation of the therapeutic agent to its target. In contrast, non-neutralizing ADAs might bind to allotopes and human neo-antigens at the hinge of fusion proteins.

One patient with anti-RTX antibodies developed primary biliary cirrhosis associated with anti-M2 and anti-gp120 antibodies after his last relapse. Four patients of the eight ones who relapsed developed neutralizing anti-RTX antibodies at month 6. Those RTX immunized patients seemed to have a good response to additional course of RTX but the repeated courses might have favored the development of autoimmune disease, added to the underlying autoimmune propensity of the patient.

The main limitation of our study is that it is a monocentric retrospective study: we have analyzed a relatively small number of patients, and our results could be difficult to generalize. Prospective studies with longer follow-up and following memory B-cell reconstitution are necessary. Our study remains original and innovative. Drug monitoring and development of ADAs have been well studied in anti-TNF α . Studies on RTX are recent and rare in nephrology field. This is the first one to demonstrate that ADAs developed against a specific mAb could blocked molecules from the same therapeutic class. We can notice that recently proposed treatment algorithms for anti-TNF α therapy utilize the results of therapeutic drug monitoring and immunogenicity testing to guide appropriate selection and tailoring of biologic therapy for patients with rheumatoid arthritis (53–55). In the future, for MN treated with RTX, drug monitoring and immunogenicity screening could be part of a personalized strategy. This will need to be validated with prospective interventional studies.

CONCLUSION

Our study provides new insight into RTX monitoring and ADAs screening in primary MN. First, high residual serum RTX levels at month 3 are associated with higher remission rate at month 6. Then, neutralizing anti-RTX antibodies are not rare and their presence at month 6 is associated with subsequent MN relapse. Finally, anti-RTX antibodies can neutralize other anti-CD20 mAbs. Further studies are needed to develop personalized therapeutic strategies in primary MN based on drug monitoring and immunogenicity testing. Personalized care is not only essential for improving therapeutic outcomes and safety, but also for promoting cost-effective use.

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SERMENT D'HIPPOCRATE

Au moment d'être admise à exercer la Médecine, je promets et je jure d'être fidèle aux lois de l'honneur et de la probité.

Mon premier souci sera de rétablir, de préserver ou de promouvoir la santé dans tous ses éléments, physiques et mentaux, individuels et sociaux.

Je respecterai toutes les personnes, leur autonomie et leur volonté, sans aucune discrimination selon leur état ou leurs convictions. J'interviendrai pour les protéger si elles sont affaiblies, vulnérables ou menacées dans leur intégrité ou leur dignité. Même sous la contrainte, je ne ferai pas usage de mes connaissances contre les lois de l'Humanité.

J'informerai les patients des décisions envisagées, de leurs raisons et de leurs conséquences.

Je ne tromperai jamais leur confiance et n'exploiterai pas le pouvoir hérité des circonstances pour forcer les consciences.

Je donnerai mes soins à l'indigent et à quiconque me les demandera. Je ne me laisserai pas influencer par la soif du gain ou la recherche de la gloire.

Admise dans l'intimité des personnes, je tairai les secrets qui me seront confiés. Reçue à l'intérieur des maisons, je respecterai les secrets des foyers et ma conduite ne servira pas à corrompre les mœurs.

Je ferai tout pour soulager les souffrances. Je ne prolongerai pas abusivement les agonies. Je ne provoquerai jamais la mort délibérément.

Je préserverai l'indépendance nécessaire à l'accomplissement de ma mission. Je n'entreprendrai rien qui dépasse mes compétences. Je les entretiendrai et les perfectionnerai pour assurer au mieux les services qui me seront demandés.

J'apporterai mon aide à mes confrères ainsi qu'à leurs familles dans l'adversité.

Que les hommes et mes confrères m'accordent leur estime si je suis fidèle à mes promesses ; que je sois couverte d'opprobre et méprisée si j'y manque.