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ORIGINAL ARTICLE

PR3-ANCAs predict relapses in ANCA-associated vasculitis patients after rituximab

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ABSTRACT

Background. The primary challenge of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) patient care is the early detection of relapses to prevent organ damage and increase survival. Potential biomarkers for relapses are ANCA and B cells, but their predictive value is a matter of debate. Therefore this study investigated how ANCA and B-cell status related to relapses in AAV patients treated with rituximab (RTX) as remission induction (RI).

Methods. This single-centre cohort study identified 110 ANCA-positive AAV patients treated with RTX between 2006 and 2018. Serial ANCA, $CD19^+$ B-cell status and relapses were assessed >2 years.

Results. Patients (31/110) relapsed within 2 years after RTX RI treatment. Patients who achieved and maintained PR3-ANCA negativity ($n = 29$) had few relapses (3%), while persistent proteinase 3 (PR3)-ANCA positivity ($n = 49$) and reappearance of PR3-ANCAs ($n = 10$) associated significantly with more relapses (37%, $P = 0.002$ and 50%, $P = 0.002$). Patients with incomplete B-cell depletion ($n = 11$) had significantly more relapses (54%) as compared with patients with B-cell depletion [$n = 76$ (26%), $P = 0.02$]. Also, patients with repopulation of B cells ($n = 58$) had significantly more relapses (41%) as compared with patients without B-cell repopulation [$n = 27$ (15%), $P = 0.03$]. Overall, the absence of PR3- or myeloperoxidase (MPO)-ANCA positivity was highly predictive for remaining relapse-free. In PR3-ANCA-positive patients, 96% of the relapses occurred with persistent or reappearance of PR3-ANCAs and 81% with B-cell repopulation. In MPO-ANCA-positive patients, all relapses were restricted to patients with persistent MPO-ANCAs and B-cell repopulation.

Conclusions. Upon RI treatment with RTX in AAV patients, ANCA and B-cell status were predictive of the majority of relapses and specifically their absence strongly predicted a relapse-free status. Therefore the implementation of ANCA and B-cell monitoring could guide therapeutic decision-making to prevent relapses in AAV patients treated with RTX.

Keywords: ANCA, biomarkers, crescentic glomerulonephritis, immunomonitoring, rituximab, vasculitis

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) encompasses granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic GPA, which are life-threatening autoimmune diseases characterized by pauci-immune necrotizing vasculitis leading to inflammation and damage in major organs such as the kidneys, lungs, heart and neurologic system [1]. In GPA and MPA, ANCAs against proteinase 3 (PR3) and myeloperoxidase (MPO) develop and are thought to play a pathogenic role in the disease [2].

Current guidelines recommend to treat AAV patients with either cyclophosphamide (CYC) or rituximab (RTX) in combination with corticosteroids as remission induction (RI) therapy and azathioprine (AZA) or RTX as maintenance therapy [3]. RTX has been demonstrated to be as effective as CYC to achieve remission [4, 5], while generally being a more safe option with less toxicity [6, 7]. However, relapses are frequent after RTX; only 39% of patients maintained complete remission at 18 months, [8] which has also been demonstrated by others

KEY LEARNING POINTS

What is already known about this subject?

- Rituximab (RTX) is an effective remission-induction and maintenance treatment for patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).
- Maintenance treatment with RTX can be given at fixed intervals or tailored according to ANCA and/or B-cell levels, but the optimal maintenance strategy remains a matter of debate.
- Importantly, it is still unknown whether ANCA- and B cell status could predict relapses which ultimately could prevent organ damage and improve survival.

What this study adds?

- In a large cohort of AAV patient receiving remission-induction treatment with RTX, ANCA- and B-cell status were predictive of the majority of relapses and specifically their absence strongly predicted a relapse-free status.
- In PR3-ANCA-positive patients, 96% of the relapses occurred with persistent or reappearance of PR3-ANCAs and 81% with B-cell repopulation.
- In MPO-ANCA-positive patients, all relapses were restricted to patients with persistent MPO-ANCAs and B-cell repopulation.

What impact this may have on practice or policy?

- Implementation of ANCA- and B-cell monitoring could guide therapeutic decision-making to prevent relapses in AAV patients treated with RTX.

[9, 10]. Therefore it is important to effectively prevent relapses in RTX-treated AAV patients. The Efficacy Study of Two Treatments in the Remission of Vasculitis (MAINRITSAN 1) [11] and recently the Rituximab Vasculitis Maintenance Study (RITAZAREM) [12] demonstrated superior efficacy of RTX over AZA as maintenance therapy. Moreover, the Comparison Study of Two Rituximab Regimens in the Remission of ANCA Associated Vasculitis (MAINRITSAN 2) demonstrated that 'tailored RTX' maintenance (re)treatment guided by ANCA and B-cell status successfully reduced the necessary number of RTX infusions as compared with 6-monthly fixed RTX [13]. The latter study built on the assumption that ANCA and B-cell status are predictive biomarkers for relapse [14–16]. Although ANCA titres usually decrease drastically in the majority of patients after RI therapy [4, 5, 17], a subset of patients can remain ANCA-positive while in clinical remission [18] and also patients who achieved ANCA negativity can still relapse [19]. As such, the potential of ANCA levels as predictors for relapse has been a matter of continuous debate [20]. Similar debates remain on the potential of circulating $CD19^+$ B cells as predictors of relapse [21], because relapse can occur with and without the presence of circulating B cells in AAV patients [13, 22].

Altogether, the early detection and prevention of relapses is a key strategy to prevent organ damage and increase survival in AAV patients. This study addressed whether ANCA and B-cell status can predict relapses in AAV patients. Therefore we conducted a retrospective cohort study in AAV patients treated with RTX as RI in which monitoring of ANCAs and B cells was independent of therapeutic decision-making.

MATERIALS AND METHODS

Study population

This retrospective single-cohort study was conducted at the Leiden University Medical Centre (LUMC) in The Netherlands. A total of 110 ANCA-positive GPA or MPA [23] patients treated with RTX as RI therapy for active disease between January 2006 and 2018 were included in this study (Supplementary data, Figure S1). Some patients did not have available B-cell depletion ($n = 26$) or repopulation data ($n = 25$). In accordance with the Dutch National Ethics Committee guidelines, ethics approval was not required for this study because it involved retrospective data and all treatment decisions were made before our evaluation.

At the Centre of Expertise for Lupus-, Vasculitis- and Complement-mediated diseases (LuVaCs), AAV patients with generalized disease were treated according to locally implemented treatment protocols. Briefly, RI was two times 1000 mg RTX intravenously (i.v.) combined with 100 mg methylprednisolone with a 2-week interval, preceded by one to three times 500–1000 mg methylprednisolone i.v. daily followed by oral corticosteroids (1 mg/kg/day, maximum 60 mg) with tapering over 3 months. Physicians deviated from the local protocol to personalize treatment based on treatment-related side effects. Maintenance treatment commenced between 3 and 6 months based on the discretion of the treating physician and consisted of AZA (1–2 mg/kg/day), RTX 500 mg every 6 months [11], mycophenolate mofetil (MMF) 1–2 g/day [aiming for an area under the curve (AUC)of 30–40 mg h/L] or methotrexate 7.5–25 mg/week with or without low-dose corticosteroids 5–7.5 mg/day (Supplementary data, Table S1). All patients received prophylactic treatment with co-trimoxazole 480 mg/day, proton pump inhibition, vitamin D, calcium supplementation and, if indicated, bisphosphonates.

Clinical data

Clinical and laboratory data were retrospectively extracted from electronic patient records. The disease extent index was used to score disease activity and organ involvement [24], which highly correlates with other disease assessment scores [25]. Briefly, each involved organ system scores two points and constitutional symptoms one point (maximum score 21). Relapse was defined as the reappearance of disease activity or worsening requiring a new RI therapy in a patient previously in remission after RI therapy as documented in the clinical records. All patients were assessed during 2 years of follow-up after the start of RI treatment (Supplementary data, Table S2). During follow-up, ANCA testing was part of standard outpatient clinical care and performed regularly, that is, the median

sample frequency was 3 [interquartile range (IQR) 2–4] times within 6 months, 5 (IQR 3–7) times within 12 months and 7 (IQR 5–11) times within 24 months. B-cell detection was performed 1 (IQR 0–2) time within 6 months, 2 (IQR 1–3) times within 12 months and 3 (IQR 1–5) times within 24 months.

ANCA status was assessed in this study as being positive or negative, because of the clinical relevance of this immunological endpoint [18, 26] and the high concordance between the assays that were used over the years [27]. Serum samples were analysed by the Department of Clinical Chemistry of the LUMC in Leiden. The indirect immunofluorescence test on immobilized granulocytes was used as an entry test. When positive, anti-PR3 and anti-MPO were quantified using the following enzyme-linked immunosorbent assay (ELISA) techniques: before 2009, a home-made anti-PR3/MPO ELISA [laboratory-developed test, (LDT)] was used; between 2009 and 2011, the Phadia anti-PR3/MPO fluorimetric enzyme-linked immunoassay on the Immunocap 250 (Thermo Fisher Scientific, Waltham, MA, USA); between 2011 and 2017, the sensitive anti-PR3/MPO assay on the Immunocap 250 (Thermo Fisher Scientific) and from 2017 onwards, the chemiluminescence immunoassay Bioflash technology (INOVA Dx, San Diego, CA, USA). As such, a positive or negative ANCA status was assessable throughout the complete study because the absolute serum levels of ANCAs could not be compared over time due to well-recognized interassay variances. The Department of Clinical Chemistry of the LUMC participates in a nation-wide external quality control programme (SKML, Nijmegan, The Netherlands) guaranteeing reliable results with the methods used. Measurements of circulating $CD19^+$ B cells were performed after RTX to confirm depletion, which was $\leq 1 \times 10^6$ cells/L, the detection limit of the method. Repopulation of $CD19^+$ B cells, defined as $\geq 2 \times 10^6$ cells/L, was assessed after the achievement of depletion or, when depletion data were unavailable, repopulation was assessed 6 months from the start of RTX onwards.

Statistics

All descriptive clinical data are expressed as mean ± standard deviation (SD) or median (IQR) for numerical data or as percentage for nominal data. Survival functions and time-to-event analyses were plotted as Kaplan–Meier curves and groups were compared by the log-rank test. Analyses of ANCA and B-cell status were censored for the last ANCA and B-cell test, respectively, and relapse analyses were censored for death or last clinical follow-up visit. All data were analysed using SPSS Statistics (IBM, Armonk, NY, USA) and graphs were constructed with SPSS version 25 or Prism 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Study population

A total of 110 AAV cases were identified that received RI therapy with RTX for either new or relapsing AAV disease (Supplementary data, Figure S1). Patient characteristics are summarized in Table 1.

Table 1. Study population

Characteristics	RTX ($N = 110$)
Male, *n* (%)	67 (61)
Age (years), mean ± SD	60 ± 17
Indication for RI, *n* (%)	
New	30 (27)
Relapse	80 (73)
Clinical subtype, *n* (%)	
GPA	85 (77)
MPA	25 (23)
ANCA immunofluorescence, *n* (%)	
c-ANCA	85 (77)
p-ANCA	25 (23)
ANCA specificity (ELISA), *n* (%)	
PR3-ANCA	88 (80)
MPO-ANCA	22 (20)
DEI, mean ± SD	4.6±2.3
Organ involvement, %	
Pulmonary	36 (33)
Renal	55 (50)
eGFR (mL/min)[a]	49 ± 24
≥60	16 (29)
30–59	26 (47)
<30	12 (22)
Previous CYC therapy, *n* (%)	70 (64)
Additional RI therapy, *n* (%)	
Methylprednisolone	37 (34)
Plasma exchange	7 (6)
High-dose oral corticosteroids with tapering, *n* (%)	98 (89)

[a]Only patients with renal involvement.
DEI: Disease Extent Index.

Relapses and ANCA monitoring after RI treatment with RTX

During 2 years of follow-up, 110 patients treated with RTX suffered 31 relapses (28%) (Figure 1A): 24 were major relapses involving renal ($n = 15$), pulmonary ($n = 9$) and/or neurologic involvement ($n = 4$). Of note, all minor relapses occurred in PR3-ANCA-positive patients. The number of relapses was not significantly different in PR3-ANCA- versus MPO-ANCA-positive patients [24/88 (27%) versus 7/22 (32%), P = 0.6; Figure 1B] nor in newly diagnosed versus relapsing patients [12/30 (40%) versus 19/80 (24%), P = 0.2; Supplementary data, Figure S2A]. No differences were observed when comparing these subgroups for the number of major relapses. In accordance with previously published controlled trials [11, 28–30], the number of relapses was significantly different per maintenance regimen after RI with RTX (P = 0.004; Supplementary data, Figure S2B). Relapses occurred in 5/7 (71%) patients with MMF, 5/14 (36%) with no immunosuppression, 10/29 (34%) with monotherapy low-dose corticosteroids, 2/8 (25%) with other maintenance regimens, 6/25 (24%) with AZA and 3/25 (12%) patients with RTX as maintenance therapy. Of note, the choice of maintenance therapy was not different for newly diagnosed versus relapsing patients.

ANCA negativity after treatment with RTX was achieved in 47/110 patients (43%) within 2 years after a median of 22 (IQR 15–40) weeks (Figure 1C). ANCA negativity was achieved significantly more frequently in PR3-ANCA-positive as compared with MPO-ANCA-positive patients [42/88 (48%) versus 5/22 (23%), P = 0.05; Figure 1D]. Achievement of ANCA negativity

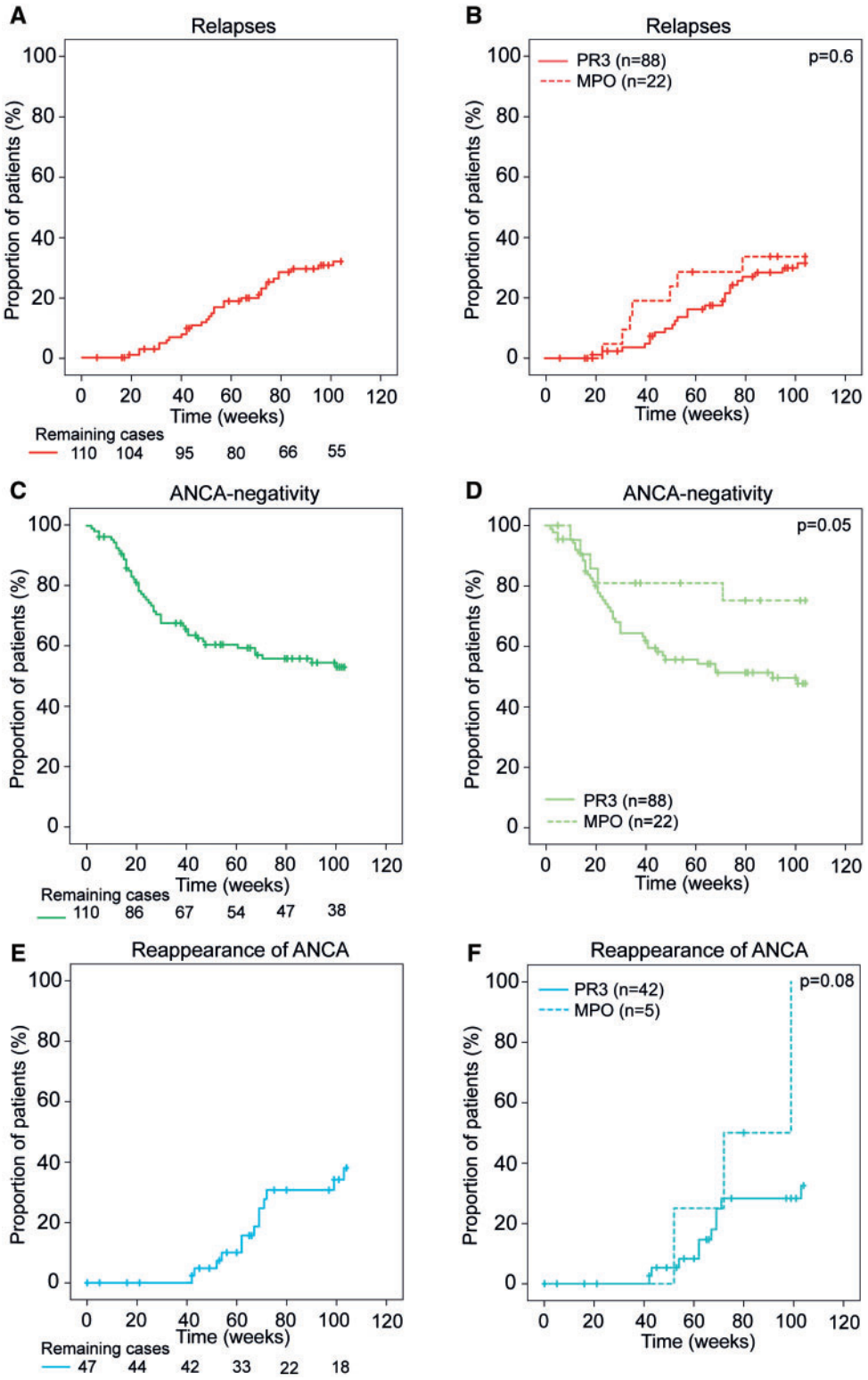

FIGURE 1: Relapses and ANCA monitoring after RI treatment with RTX. (**A**) Time to relapse (red lines) after RI therapy with RTX in AAV patients ($n = 110$) depicted as Kaplan–Meier plots. (**B**) Stratified analyses of time to relapse for PR3- ($n = 88$) versus MPO-ANCA-positive ($n = 22$) patients (red lines). (**C**) Time to ANCA negativity after RI therapy with RTX depicted as Kaplan–Meier plots. (**D**) Stratified analyses of time to ANCA negativity for PR3- versus MPO-ANCA-positive patients. (**E**) Kaplan–Meier plots of time to reappearance of ANCA ($n = 47$, blue lines) after RI therapy with RTX. (**F**) Stratified analyses of time to reappearance of ANCA for PR3- versus MPO-ANCA-positive patients who achieved ANCA negativity. The footnote indicates the number of the remaining patients in each arm with available data.

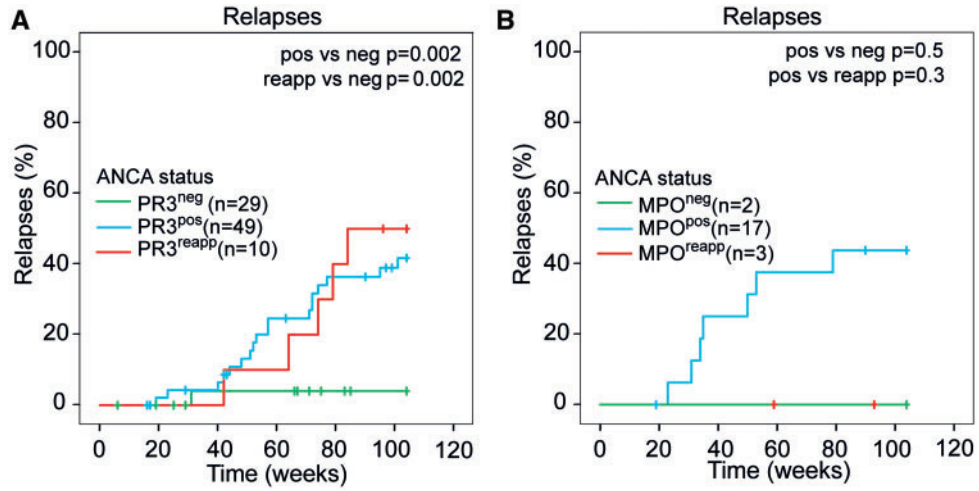

FIGURE 2: PR3-ANCA status associated with relapses after RTX. (**A**) Kaplan–Meier plots of relapses after RI therapy with RTX in PR3-ANCA-positive patients who achieved and maintained PR3 negativity (green lines, $n = 29$), patients who remained PR3-ANCA positive (blue lines, $n = 49$) or patients who initially achieved PR3 negativity but experienced reappearance of PR3-ANCA positivity (red lines, $n = 10$). (**B**) Kaplan–Meier plots of relapses after RI therapy with RTX in MPO-ANCA-positive patients who achieved and maintained MPO-ANCA negativity (green lines, $n = 2$), patients who remained MPO-ANCA positive (blue lines, $n = 17$) and patients who initially achieved MPO-ANCA negativity but experienced reappearance of MPO-ANCA positivity (red lines, $n = 3$). To test statistical differences between the groups, a log-rank test was performed.

was not statistically different for newly diagnosed versus relapsing patients [17/30 (57%) versus 30/80 (37%), P = 0.2; Supplementary data, Figure S3A]. Achieving ANCA negativity was affected by the choice of maintenance therapy (Supplementary data, Figure S3B; overall P = 0.03). ANCA negativity was achieved in 0/7 patients (0%) with MMF, 2/8 (25%) with other maintenance regimens, 3/14 (21%) with no immunosuppression, 12/25 (48%) with AZA, 12/25 (48%) with RTX and 17/29 (59%) with low-dose corticosteroid monotherapy as maintenance treatment.

In patients who achieved ANCA negativity within 2 years ($n = 47$), the subsequent reappearance of ANCA was observed in 13/47 (28%; Figure 1E), which happened a median of 67 (IQR 53–72) weeks after RTX. Reappearance of ANCA was neither statistically different in PR3-ANCA- versus MPO-ANCA-positive patients [10/42 (28%) versus 3/5 (60%), P = 0.08; Figure 1F] nor in newly diagnosed versus relapsing patients [7/17 (43%) and 6/30 (23%), P = 0.1; Supplementary data, Figure S3C]. Also, the choice of maintenance therapy did not significantly affect the reappearance of ANCAs (P = 0.2; Supplementary data, Figure S3D].

PR3-ANCA status associated with relapses after RTX

The consequence of PR3-ANCA and MPO-ANCA status after RTX was studied in regard to relapses by stratifying patients into three groups: persistent ANCA negative, persistent ANCA positive or reappearance of ANCAs after the achievement of negativity (Figure 2). Only 1/29 patients (3%) who achieved and maintained PR3-ANCA negativity after treatment relapsed (which was a major relapse with pulmonary involvement; Figure 2A), while patients with persistent PR3-ANCA positivity had significantly more relapses [18/49 (37%), P = 0.002]. Also, patients who had reappearance of PR3-ANCA positivity after the achievement of negativity and had significantly more relapses, that is, 5/10 (50%, P = 0.002; Figure 2A). Of note, the reappearance of PR3-ANCA positivity preceded relapses with a median interval period of 10 (IQR 1–15) weeks. In MPO-ANCA-positive patients, all relapses were observed in patients who had persistent MPO-ANCA positivity as compared with patients who achieved MPO-ANCA negativity with or without subsequent reappearance [7/17 (41%) versus 0/5 (0%), P = 0.3; Figure 2B]. The predictive test characteristics of ANCA status for relapses are summarized in Table 2.

$CD19^+$ B-cell status associated with relapses after RTX

The assessment of $CD19^+$ B-cell status included depletion ($\leq 1 \times 10^6$ cells/L) and repopulation ($\geq 2 \times 10^6$ cells/L), which demonstrated that 74/84 (87%) of the RTX-treated patients achieved depletion of $CD19^+$ B cells after a median of 5 (IQR 3–10) weeks (Figure 3A). Depletion of B cells was not significantly different for PR3-ANCA- versus MPO-ANCA-positive patients nor between newly diagnosed versus relapsing patients (respectively, 88% versus 87%, P = 0.8 and 100% versus 83%, P = 0.2; Supplementary data, Figure S4A and B). Subsequent repopulation of $CD19^+$ B cells was observed in 58/85 patients (68%) after a median of 47 (IQR 31–68) weeks (Figure 3B). B-cell repopulation occurred significantly more frequently in MPO-ANCA-positive patients [12/16 (75%)] as compared with PR3-ANCA-positive patients [46/69 (67%), P = 0.04; Supplementary data, Figure S4C]. There was no significant difference between newly diagnosed versus relapsing patients [17/26 (65%) versus 41/59 (69%), P = 0.9; Supplementary data, Figure S4D). Of note, B-cell repopulation was not significantly affected by the choice of maintenance treatment (P = 0.6; Supplementary data, Figure S4E).

Patients who did not achieve B-cell depletion after RTX had significantly more relapses than patients who did [6/10 (60%) versus 20/74 (27%), P = 0.01; Figure 3C). All six relapses in

Table 2. Test characteristics of ANCA and B-cell status to predict relapses after RTX

ANCA and/or B-cell status	Sensitivity	Specificity	PPV	NPV
PR3-ANCA positivity[a]	96 (79–100)	44 (31–57)	39 (34–45)	97 (80–99)
Persistent PR3-ANCA positivity	75 (53–90)	52 (39–64)	37 (29–45)	85 (73–92)
Reappearance of PR3-ANCA positivity	21 (7–42)	92 (83–97)	50 (24–76)	76 (71–79)
MPO-ANCA positivity[a]	100 (59–100)	13 (1–41)	35 (31–40)	100
Persistent MPO-ANCA positivity	100 (59–100)	33 (12–62)	41 (33–50)	100
Reappearance of MPO-ANCA positivity	0 (0–41)	80 (52–96)	0	63 (57–69)
No B-cell depletion	23 (9–44)	93 (83–98)	60 (32–83)	73 (68–77)
PR3-ANCA-positive patients	20 (6–44)	92 (80–98)	50 (22–78)	73 (68–78)
MPO-ANCA-positive patients	33 (4–78)	100 (69–100)	100	71 (59–81)
Repopulation of B cells	86 (67–96)	40 (28–54)	41 (35–48)	85 (72–95)
PR3-ANCA-positive patients	81 (58–95)	44 (30–59)	37 (30–44)	83 (65–92)
MPO-ANCA-positive patients	100 (59–100)	44 (14–79)	58 (44–72)	100
B-cell repopulation and:				
PR3-ANCA positivity[a]	81 (58–95)	67 (52–80)	52 (40–63)	89 (77–95)
Persistent PR3-ANCA positivity	57 (34–78)	67 (50–81)	48 (38–58)	92 (61–99)
Reappearance of PR3-ANCA positivity	24 (8–47)	94 (83–99)	63 (30–86)	74 (69–79)
B-cell repopulation and:				
Persistent MPO-ANCA positivity	100 (59–100)	44 (14–79)	58 (44–72)	100
Reappearance of MPO-ANCA positivity[b]	NA	NA	NA	NA

Data are shown as percentage (95% CI). NPV: negative predictive value, PPV: positive predictive value.
[a]PR3/MPO-ANCA positivity was defined as either persistent PR3/MPO-ANCA positive or reappearance of PR3/MPO-ANCA positivity.
[b]B-cell repopulation and reappearance of MPO-ANCA positivity did not occur in any patient and is therefore not specified.

patients with inadequate B-cell depletion were major, with renal ($n = 2$), renal and pulmonary ($n = 1$), renal and neurologic ($n = 1$), pulmonary ($n = 1$) and neurologic ($n = 1$) involvement.

Specifically, MPO-ANCA-positive patients with incomplete B-cell depletion seemed to have more relapses [2/2 (100%) versus 4/14 (29%), P = 0.02], whereas in PR3-positive patients no significant difference was observed [4/8 (50%) versus 16/60 (27%), P = 0.2; Figure 3E]. Second, patients with $CD19^+$ B-cell repopulation had significantly more relapses as compared with patients who remained B-cell depleted [24/58 (41%) versus 4/27 (15%), P = 0.02; Figure 3D]. Of 24 relapses in patients with B-cell repopulation, 20 were major (11 with renal, 3 with renal and pulmonary, 3 with pulmonary, 2 neurologic and 1 with renal and neurologic involvement) and 4 were minor. Of 4 relapses in B-cell-depleted patients, 3 were major (2 pulmonary and 1 neurologic involvement) and 1 was minor. The association of B-cell repopulation with relapses was not significantly different in PR3-ANCA-positive patients [17/46 (37%) versus 4/23 (17%), P = 0.2], while there was a trend in MPO-ANCA-positive patients [7/12 (58%) versus 0/4 (0%), P = 0.07; Figure 3F]. The predictive test characteristics of B-cell status for relapses are summarized in Table 2.

Combined ANCA and B-cell status predicted relapses after RTX

The relevance of combining ANCA and B-cell status to predict relapses after RI treatment with RTX was investigated in both PR3-ANCA- and MPO-ANCA-positive patients (Figure 4). As demonstrated earlier, patients who achieved and maintained PR3-ANCA negativity had the lowest relapse frequency [1/26 (4%)], irrespective of whether patients had B-cell repopulation or not [0/14 (0%) versus 1/12 (8%), P = 0.2; Figure 4A]. Patients with a persistent PR3-ANCA-positive status had an increased relapse frequency (37%), which was not significantly different with B-cell repopulation or not [12/25 (48%) versus 3/10 (30%), P = 0.5]. Patients with the reappearance of PR3-ANCA positivity had an increased relapses rate (50%) that was restricted to patients with B-cell repopulation and not without [5/8 (63%) versus 0/1 (0%), P = 0.4; Figure 4A].

In MPO-ANCA-positive patients, all relapses were confined to patients with persisting MPO-ANCA positivity that experienced B-cell repopulation, whereas no relapses were observed in patients without B-cell repopulation [7/12 (58%) versus 0/2 (0%), P = 0.2; Figure 4B]. Patients with the reappearance of MPO-ANCA without B-cell repopulation ($n = 2$) did not have any relapses.

Overall, PR3-ANCA positivity predicted 96% [95% confidence interval (CI) 79–100] of the relapses, while PR3-ANCA negativity predicted a relapse-free status [97% (95% CI 80–99); Table 2]. With respect to B-cell status, achieving B-cell depletion was specifically predictive of a relapse-free status [92% (95% CI 80–98)] and 81% (95% CI 58–95) of relapses occurred with B-cell repopulation. Combining PR3-ANCA and B-cell status increased the specificity and positive predictive value. Although MPO-ANCA-positive patients' numbers were relatively low, 100% of the relapses were restricted to patients with persistent MPO-ANCA positivity and B-cell repopulation.

DISCUSSION

The use of RTX as an RI regimen in AAV patients is associated with a relatively high rate of relapses, emphasizing the need for early biomarkers that can predict and thereby potentially prevent relapses. The current study demonstrates that monitoring of ANCA and B-cell status could guide therapeutic decisions to prevent relapses in AAV patients after RTX as an RI regimen. In PR3-ANCA-positive patients, 96% of the relapses occurred with persistent or reappearance of PR3-ANCA positivity,

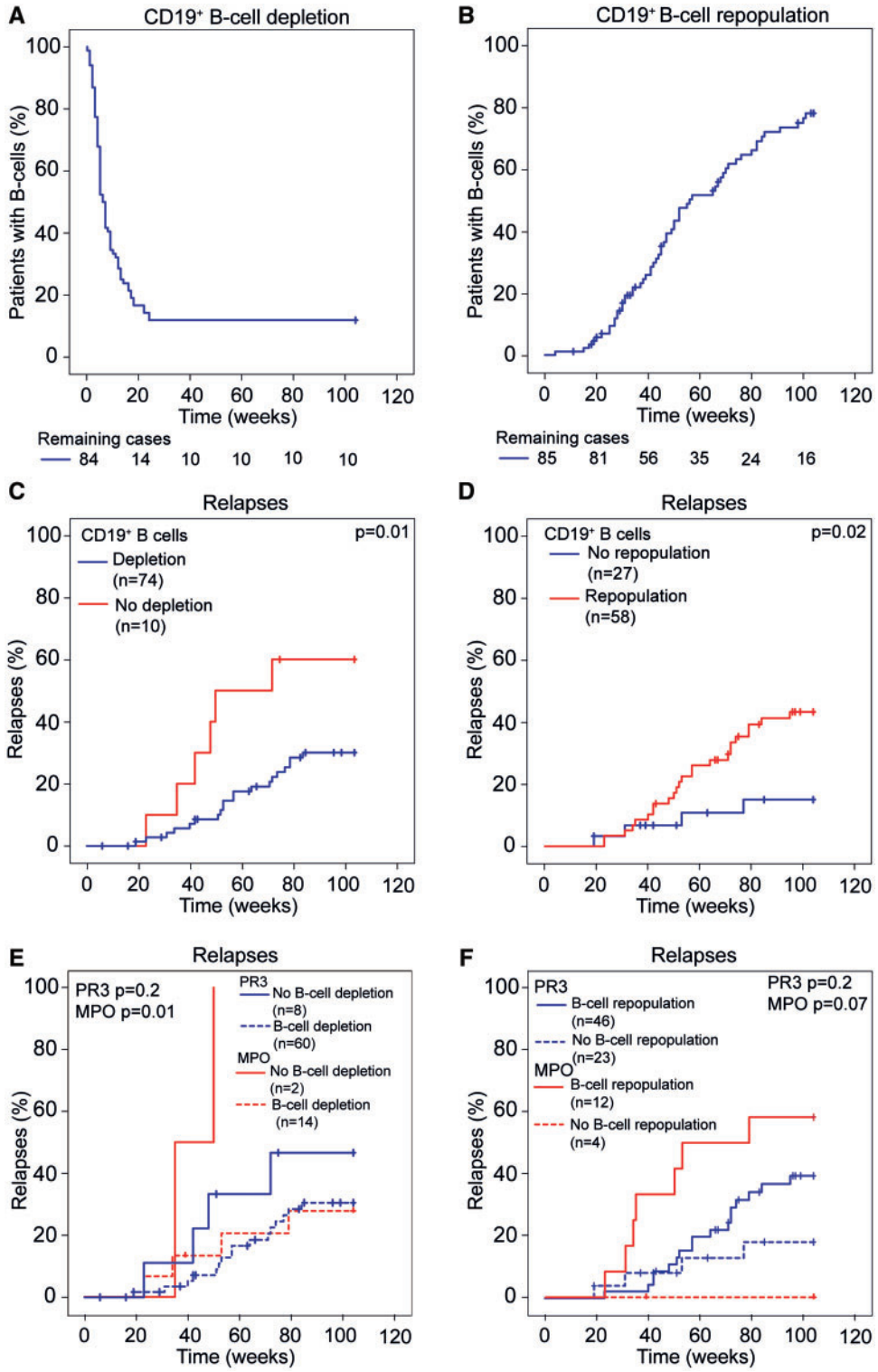

FIGURE 3: CD19$^+$ B-cell status associated with relapses after RTX. (**A**) Kaplan–Meier plots of time to B-cell depletion in AAV patients after RTX as RI ($n = 84$). (**B**) Kaplan–Meier plots of time to B-cell repopulation within 2 years after RTX as RI in AAV patients ($n = 85$). (**C**) Kaplan–Meier plot of time to relapse in patients who had no CD19$^+$ B-cell depletion ($<1 \times 10^6$/L; ($n = 10$, red lines) after RTX compared with patients with CD19$^+$ B-cell depletion ($n = 74$) (blue lines). (**D**) Kaplan–Meier plot of time to relapse in patients who had CD19$^+$ B-cell repopulation ($>1 \times 10^6$/L; $n = 58$, red line) after RTX compared with patients without CD19$^+$ B-cell repopulation ($n = 27$, blue line). (**E**) Stratified analyses of time to relapse in PR3-ANCA-positive (blue lines) versus MPO-ANCA-positive patients (red lines) without B-cell depletion (solid) or with B-cell depletion (dashed) depicted as Kaplan–Meier curves. (**F**) Stratified analyses of relapses in PR3-ANCA-positive (blue lines) versus MPO-ANCA-positive patients (red lines) with (solid) or without (dashed) B-cell repopulation depicted as Kaplan–Meier curves. To test statistical differences between the groups, the log-rank test was used to assess time-to-event data. The footnote indicates the number of the remaining patients in each arm with available data.

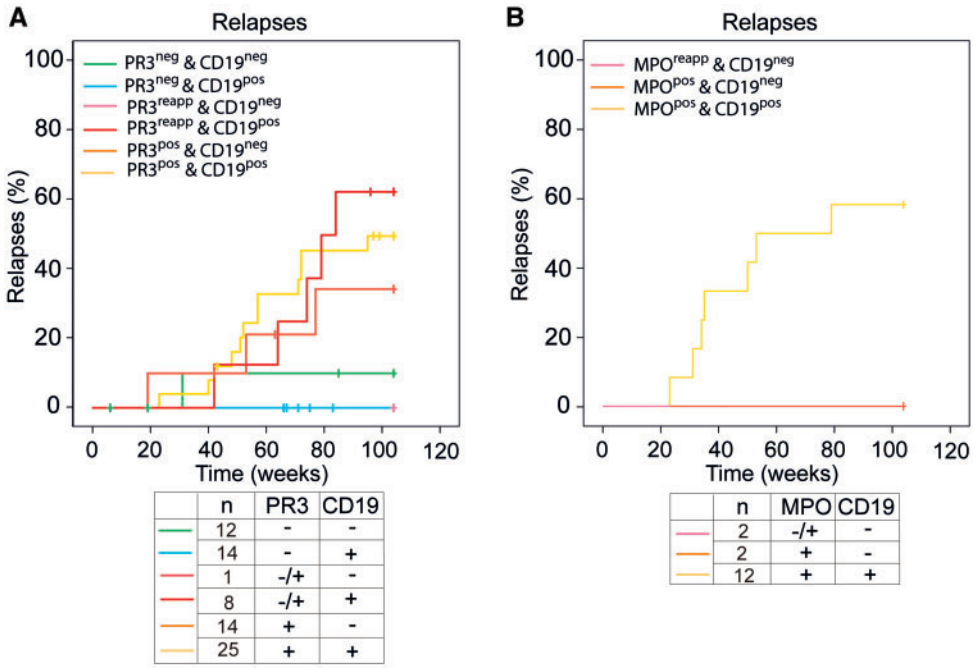

FIGURE 4: Combined ANCA and B-cell status predicted relapses after RTX. (**A**) Kaplan–Meier plots of time to relapse in PR3-ANCA-positive patients after RI treatment with RTX comparing patients who achieved PR3-ANCA negativity or not in combination with or without the presence of $CD19^{+}$ B cells. Six subgroups of patients were identified: achieving PR3-ANCA negativity without B-cell repopulation (green, $n = 12$), achieving PR3-ANCA negativity with B-cell repopulation (blue, $n = 14$), reappearance of PR3-ANCA positivity after achieving PR3-ANCA negativity without B-cell repopulation (pink, $n = 1$), reappearance of PR3-ANCA positivity after achieving PR3-ANCA negativity with B-cell repopulation (red, $n = 8$), persistent PR3-ANCA positivity without B-cell repopulation (orange, $n = 14$) and persistent PR3-ANCA positivity with B-cell repopulation (yellow, $n = 25$). (**B**) Kaplan–Meier plots of time to relapse in MPO-ANCA-positive patients after RI treatment with RTX comparing three subgroups of patients: reappearance of MPO-ANCA positivity after achieving MPO-ANCA negativity without B-cell repopulation ($n = 2$, pink), persistent MPO-ANCA positivity without B-cell repopulation ($n = 2$, orange) and persistent MPO-ANCA positivity with B-cell repopulation ($n = 12$, yellow). To test statistical differences between the groups, a log-rank test was performed.

often in conjunction with B-cell repopulation. The absence of PR3-ANCA positivity and B cells after RTX was highly predictive of a relapse-free status. Although the MPO-ANCA-positive patients were a relatively small group, all relapses occurred in patients with persistent MPO-ANCA positivity and B-cell repopulation.

The present study was conducted in a retrospective, real-life cohort where immunomonitoring was part of standard clinical practice but not related to therapeutic decision-making. As such, the cohort offered the unique possibility to assess the relation between relapses and ANCA and B-cell status. The relapse frequency observed in the present study population resembled previously published large, randomized trials: approximately one-third relapsed after RTX as RI [8–10], RTX maintenance had superior efficacy to AZA maintenance [11] and AZA seemed to be superior to MMF maintenance [30] (Supplementary data, Figure S2B). Additionally, the effects of RTX on ANCAs observed in our cohort were in line with a cohort study on RI with RTX followed by 6-months of RTX maintenance, which showed that <50% of the patients achieved PR3-ANCA negativity within 14 months [18], supporting that the present study investigated a representative cohort of AAV patients suitable for studying the predictive value of ANCA and/or B-cell status for relapses in AAV patients. Of note, we did not observe more relapses in PR3-ANCA-positive as compared with MPO-ANCA-positive patients nor more in relapsing versus newly diagnosed patients, which both were the case in the Rituximab for ANCA-associated Vasculitis (RAVE) trial [8]. This discrepancy could be explained by the setting of our retrospective, real-life cohort: there was no up front patient selection and therefore this study also included AAV patients with severe renal insufficiency, with the need for plasma exchange and/or limited disease activity, in contrast to the inclusion criteria of the RAVE trial. Still, the rate of kidney involvement was comparable, although the average estimated glomerular filtration rate at baseline was 77 mL/min in the RAVE trial versus 49 mL/min in our cohort. Additionally, only RTX-naïve patients were included in the RAVE trial, which was not the case in our study. Lastly, this study was conducted in an academic referral centre, which can result in a bias towards more severe and more relapsing patients. Conflicting studies exist on the association of serial ANCA measurements over time with disease activity and their potential to predict relapses [14, 15, 18, 19, 31–34]. The heterogeneity in ANCA testing, timing and frequency of testing and data analyses among the studies limit the comparability of these studies [35]. A meta-analysis demonstrated that increasing ANCAs and persistent positive ANCAs were modestly associated with future relapses [36]. Importantly, no studies using RTX for induction or maintenance therapy were included in this meta-analysis. Also, increased immunosuppression with corticosteroids or AZA upon strong increasing ANCA titres was associated with a reduced

risk of relapses [37]. An important limitation of biomarker studies is the observation that ANCA responses vary depending on the choice of RI regimen [5, 15]. An important finding in our study was that PR3-ANCA positivity (persistent or reappearance) predicted relapses after RTX RI treatment, which was in line with other previous studies [9, 15, 18].

Thus far the potential of $CD19^+$ B cells as predictors of relapses in RTX-treated AAV patients has not always been evident [21]. In the MAINRITSAN 2 study, approximately half of the patients who experienced a relapse had undetectable B cells [13], in contrast to another study where B-cell return preceded relapses in 52% of the patients after RTX as RI therapy [22] while one-third of the patients had B-cell repopulation without subsequent relapse. Of note, these studies used different definitions for B-cell depletion, respectively, $<1 \times 10^6$/L and $<10 \times 10^6$/L, which strongly affected their interpretation [38]. In the RITUXVAS trial, 71% of the patients had returning B cells after RTX (+CYC), in whom only 30% experienced a relapse, and no relapse was observed in patients with persistent B-cell depletion [22]. Our study suggested that circulating B cells have a predictive role for relapses in MPO-ANCA-positive patients, whereas the absence of repopulating B cells was predictive of remaining relapse free.

Several limitations of our study should be mentioned, including the retrospective and uncontrolled design of our single-centre study with data collection over a long period of time. To overcome this, we only analysed robust and objective endpoints, including relapses requiring RI treatment, the reversal of ANCA positivity to negativity and vice versa and the presence or absence of $CD19^+$ B cells [27]. Due to the real-life setting of this retrospective cohort, there were a few inevitable, unevenly distributed baseline characteristics, such as the distribution of newly diagnosed versus relapsing patients and the type of maintenance treatment. However, stratified subgroup analyses demonstrated minor differences in subgroups that did not substantially affect the main conclusions of this study (Supplementary data, Figures S2–4). Also, some of our analyses lacked statistical power due to small numbers of patients. Additionally, it needs to be acknowledged that not all patients had a complete 2-year follow-up and in some patients, B cells were not monitored. Lastly, our study results are relevant in the light of the most recent randomized clinical trials demonstrating the superior efficacy of RTX as maintenance treatment [11–13], which is now adopted as the recommended treatment choice in the most recent American College of Rheumatology guidelines. There is at present no consensus on the frequency of RTX infusions for maintenance therapy, as studies have used different intervals and dosing (i.e. every 4 or 6 months with dosing ranges from 500 to 1000 mg). Importantly, our study indicates that a fixed RTX strategy will lead to the overtreatment of ANCA-negative AAV patients. Nevertheless, it should be taken into account that ANCA- and B-cell-tailored RTX maintenance could also lead to overtreatment because not all patients with ANCA positivity and/or B-cell repopulation relapsed.

In conclusion, this study demonstrates that monitoring of ANCA and B-cell status could guide therapeutic decision-making after RI therapy with RTX in AAV patients. The absence of ANCAs with/without B cells strongly predicted a relapse-free status. Persistent or the reappearance of PR3-ANCA positivity predicted almost all relapses, while relapses in MPO-ANCA-positive patients were all restricted to patients with persistent MPO positivity and B-cell repopulation. Based on our study, a tailored ANCA/B-cell-guided RTX approach can be envisioned that should be evaluated in prospective studies to establish whether ANCA and B-cell immunomonitoring actually reduce overtreatment and damage accrual in AAV patients.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

L.S.D, E.D., C.K., T.J.R. and Y.K.O.T. designed the study. L.S.D, E.D., C.K. O.W.B., A.R., J.A.B. and Y.K.O.T performed acquisition and interpretation of the data. L.S.D. and E.D. analysed the data. L.S.D. drafted the article. All authors revised the article, provided intellectual content to the work, approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

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