

# Interstitial Immunostaining and Renal Outcomes in Antineutrophil Cytoplasmic Antibody-Associated Glomerulonephritis

Duvuru Geetha<sup>a</sup> Sanjeev Sethi<sup>b</sup> An S. De Vriese<sup>m</sup> Ulrich Specks<sup>c</sup>  
Cees G.M. Kallenberg<sup>n</sup> Noha Lim<sup>e</sup> Robert Spiera<sup>f</sup> E. William St. Clair<sup>g</sup>  
Peter A. Merkel<sup>h</sup> Philip Seo<sup>a</sup> Paul A. Monach<sup>i</sup> Nicola Lepori<sup>d</sup> Barri J. Fessler<sup>k</sup>  
Carol A. Langford<sup>l</sup> Gary S. Hoffman<sup>l</sup> Rishi Sharma<sup>d</sup> John H. Stone<sup>j</sup>  
Fernando C. Fervenza<sup>d</sup> for the RAVE-ITN Research Group

<sup>a</sup>Division of Nephrology and Division of Rheumatology, Johns Hopkins University, Baltimore, MD, <sup>b</sup>Department of Anatomic Pathology, <sup>c</sup>Division of Pulmonary and Critical Care Medicine, Mayo Clinic, and <sup>d</sup>Division of Nephrology and Hypertension, Rochester, MN, <sup>e</sup>Immune Tolerance Network, Bethesda, MD, <sup>f</sup>Rheumatology Division, Hospital for Special Surgery, New York, NY, <sup>g</sup>Division of Rheumatology and Immunology, Duke University, Durham, NC, <sup>h</sup>Division of Rheumatology, University of Pennsylvania, Philadelphia, PA, <sup>i</sup>Division of Rheumatology, Boston University Medical Center, and <sup>j</sup>Rheumatology Unit, Massachusetts General Hospital, Boston, MA, <sup>k</sup>Division of Clinical Immunology and Rheumatology, University of Alabama, Birmingham, AL, and <sup>l</sup>Center for Vasculitis Care and Research, Cleveland Clinic Foundation, Cleveland, OH, USA; <sup>m</sup>Division of Nephrology, AZ Sint-Jan Brugge, Brugge, Belgium; <sup>n</sup>Department of Rheumatology and Clinical Immunology University Medical Center, University of Groningen, Groningen, The Netherlands

## Keywords

Antineutrophil cytoplasmic antibody · B cells · T cells · Rituximab · Vasculitis

## Abstract

**Background:** Immunopathologic features predict renal function at baseline and follow-up in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis (GN). The interstitial infiltrate consists predominantly of T lymphocytes, but their pathophysiologic significance is unclear, especially in light of the success of B-cell-directed therapy. **Methods:** Renal biopsies from 33 patients treated with cyclophosphamide (CYC;  $n = 17$ ) or rituximab (RTX;  $n = 16$ ) in the RTX in ANCA-associated vasculitis (RAVE) trial were clas-

sified according to the new ANCA GN classification. T- and B-cell infiltration in the interstitium was assessed by immunostaining for CD3 and CD20. Correlations of clinical and histologic parameters with renal function at set time points were examined. **Results:** The mean (SD) baseline estimated glomerular filtration rate was 36 (20) mL/min/1.73 m<sup>2</sup>. ANCA GN class distribution was 46% focal, 33% mixed, 12% sclerotic and 9% crescentic. The interstitial infiltrate consisted of >50% CD3 positive cells in 69% of biopsies, but >50% CD20 positive cells only in 8% of biopsies. In a multiple linear regression model, only baseline glomerular filtration rate (GFR) correlated with GFR at 6, 12, and 18 months. Interstitial B-

D.G. and S.S. co-first authors.

and T-cell infiltrates had no significant impact on long-term prognosis, independent of the treatment limb. A differential effect was noted only at 6 months, where a dense CD3 positive infiltrate predicted lower GFR in the RTX group and a CD20 positive infiltrate predicted higher GFR in the CYC group. **Conclusions:** In ANCA-associated GN, the interstitial infiltrate contains mainly T lymphocytes. However, it is neither reflecting baseline renal function nor predictive of response to treatment, regardless of the immunosuppression regimen employed.

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## Introduction

The antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) syndromes granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) are characterized by pauci-immune, necrotizing, small-vessel vasculitis. The large majority of patients with GPA and MPA have serum ANCA directed against neutrophil proteinase 3 (PR3) and myeloperoxidase respectively. More than 75% of the patients develop renal involvement, with rapidly progressive glomerulonephritis (GN) resulting in end-stage renal disease or death in more than 50% of patients at 5 years [1]. The development of prognostic tools allowing treatment to be tailored according to disease severity remains a major challenge.

T cells are the predominant cell type in the interstitium of the kidney of patients with AAV; their presence has been reported to correlate with serum creatinine at the time of biopsy [2, 3]. The majority of published studies on histologic predictors of renal outcome are based on cohorts receiving cyclophosphamide (CYC) for induction therapy. CYC is an alkylating agent that has profound effects on both B and T lymphocytes. Two randomized controlled trials, the rituximab (RTX) in ANCA-Associated Vasculitis Trial (RAVE) and the Randomized Trial of RTX vs. CYC in ANCA-Associated Vasculitis (RITUXVAS), have demonstrated that RTX is as effective as CYC as induction therapy [4, 5]. RTX, a B-cell depleting agent, is known to exert major quantitative and qualitative changes on T lymphocyte subsets as well [6–9]. The question thus arises whether CYC and RTX have a differential effect on the tubulointerstitial infiltrate in ANCA-associated GN and whether this has an impact on prognosis.

Thus, this study evaluates T-cell and B-cell infiltrates and ANCA GN class in patients who underwent a renal biopsy in the RAVE trial with the aim of answering 2

questions: (a) does quantification of T- and B-cell infiltrates at baseline have any value in predicting long-term renal outcome beyond that provided by traditional prognostic markers, for example, estimated glomerular filtration rate? (b) Does T- and B-cell infiltrates at baseline result in different response to RTX vs. CYC?

## Materials and Methods

### Patients

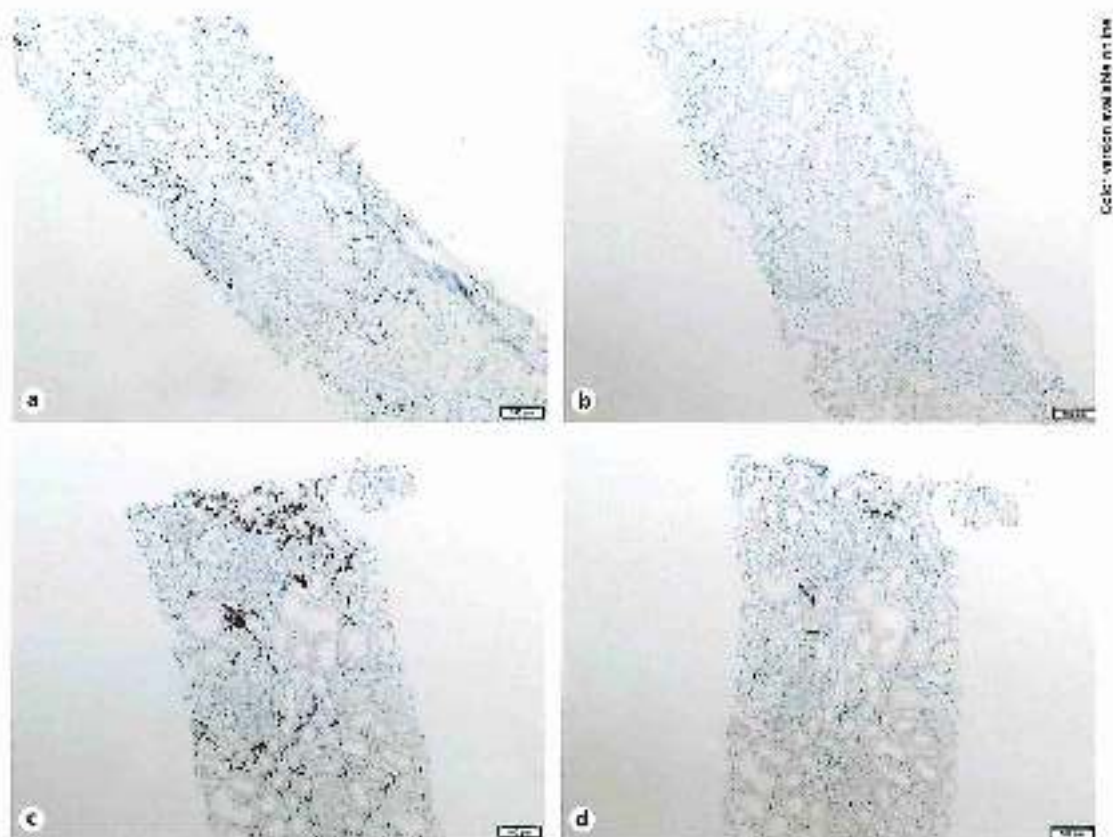
The design and results of the RAVE trial have been published previously in detail [4, 10, 11]. Patients were eligible for inclusion in the RAVE trial if they met the following criteria: (1) diagnosis of GPA or MPA, (2) positive serum PR3- or myeloperoxidase-ANCA, (3) manifestations of severe disease (defined as that which would be treated with CYC and glucocorticoids outside the context of the clinical trial), and (4) a Birmingham Vasculitis Activity Score for Wegener's Granulomatosis greater than 3. Patients with either newly diagnosed or relapsing disease were eligible for enrollment. Patients with serum creatinine >4 mg/dL, pulmonary hemorrhage requiring mechanical ventilation, and positive anti-GBM antibody were excluded. Eligible subjects were randomized to receive either 4 doses of RTX at 375 mg/m<sup>2</sup> or CYC at 2 mg/kg/day. After 3–6 months, subjects in the RTX group received no maintenance therapy, while subjects in the CYC group received azathioprine 2 mg/kg/day. Both groups received high doses of glucocorticoids during induction followed by a protocol-defined, dose-tapering regimen. In this study, we analyzed patients with renal involvement defined as biopsy-proven pauci-immune GN.

### Histologic Evaluation and Immunohistochemistry Staining for CD3 and CD20

Paraffin sections stained with silver, periodic acid-Schiff, hematoxylin and eosin, and trichrome were forwarded from study sites to a single pathologist (S.S.) for central review. The biopsies were first classified into focal, crescentic, mixed, and sclerotic class according to the recently developed classification schema of ANCA-associated GN [12]. Chronic changes were scored and graded according to the newly proposed classification system [13]. Briefly, global and segmental glomerulosclerosis was scored from 0 to 3, tubular atrophy from 0 to 3, interstitial fibrosis from 0 to 3, and arteriosclerosis from 0 to 1. The scores were then added (total renal chronicity score) to grade the overall severity of the chronic lesions into minimal (0–1 total score), mild (2–4 total score), moderate (5–7 total score), and severe (≥8 total score) [13].

Renal biopsy sections were then stained for CD3, a marker for the whole T-cell population and for CD20, the B-cell target of RTX, using immunohistochemical (IHC) methods. The extent of the interstitial infiltrate in the renal parenchyma on the IHC slides was semi-quantitatively scored on a scale of 0–3: score 0, no infiltrate; score 1, when an infiltrate was present but in less than 25% of the parenchyma; score 2, when the infiltrate was present in 25–50% of the parenchyma; and score 3, when the infiltrate was present in more than 50% of the parenchyma (Fig. 1).

Tissue sectioning and IHC staining was performed at the Pathology Research Core (Mayo Clinic, Rochester, MN, USA) using



Cells: version available on line

**Fig. 1.** Representative immunohistochemistry staining for CD3 and CD20 positive cells in 2 cases. CD3 staining is shown on the left column and CD20 on the right column. Each panel represents one case. **a** The top panel shows CD3 positive cells (3+, greater than 50% parenchyma involved) in the interstitium and almost

complete absence (0 score) of CD20 positive cells in the interstitium (**b**). The bottom panel shows CD3 positive cells (3+, greater than 50% parenchyma involved) in the interstitium (**c**) and few CD20 positive cells (1+, CD20 positive in less than 25% of the parenchyma; **d**).

the Leica Bond RX stainer (Leica). Formalin fixed, paraffin-embedded tissue was sectioned at 5 microns and IHC staining was performed online. Slides for CD20 stain were retrieved for 20 min using Epitope Retrieval 2 (EDTA; Leica). The CD20 primary antibody (Clone L26; Dako) was diluted to 1:400 in Background Reducing Diluent (Dako) and incubated for 15 min. Slides for CD3 stain were retrieved for 30 min using Epitope Retrieval 2 (EDTA; Leica). The CD3 primary antibody (Clone P7.2.38; Dako) was diluted to 1:200 in Bond Antibody Diluent (Leica) and incubated for 15 min. The detection system used was Polymer Refine Detection System (Leica). This system includes the hydrogen peroxidase block, post primary and polymer reagent, DAB, and Hematoxylin. Immunostaining visualization was achieved by incubating slides 10 min in DAB and DAB buffer (1:19 mixture) from the Bond Polymer Refine Detection System. To this point, slides were rinsed between steps with 1X Bond Wash Buffer (Leica). Slides were counterstained for 5 min using Schmidt hematoxylin and molecular biology grade water (1:1 mixture), followed by several rinses in 1X Bond wash buffer and distilled water. Once the immunohistochemistry process was completed, slides were removed from the stainer and rinsed in tap water for 5 min. Slides were dehydrated

in increasing concentrations of ethyl alcohol and cleared in 3 changes of xylene prior to permanent cover slipping in xylene-based medium.

#### Statistical Analyses

The candidate predictors of outcome were age, ANCA type, treatment arm, estimated glomerular filtration rate at baseline ( $GFR_0$ ) calculated by the 4 variable MDRD formula [14], biopsy class, and the interstitial staining score for CD3 and CD20. The outcome parameters were GFR at 6 months ( $GFR_6$ ), 12 months ( $GFR_{12}$ ), and 18 months ( $GFR_{18}$ ).

Comparisons of categorical variables were performed using a chi-square test or a Fisher exact test depending on the cell sizes. Comparisons of continuous variables were performed using a Wilcoxon rank sum test. Pearson's correlation coefficient was calculated to determine associations of clinical and histologic parameters with outcome parameters. Multiple linear regression analyses were performed to assess the predictive value of clinical and histologic variables to renal outcome. All statistical tests were 2 sided and a  $p$  value of 0.05 was considered to indicate statistical significance. The R 3.2.2 was used for all statistical analyses.

**Table 1.** Baseline clinical characteristics

	RTX (n = 16)	CYC (n = 17)	p value
Age, years, mean (SD)	56 (15)	59 (10)	0.87
Gender, %			>0.99
Female	50	47	
Male	50	53	
Race, %			0.48
Caucasian	94	100	
African American	6	0	
Body mass index, kg/m <sup>2</sup> , mean (SD)	30 (5)	29 (4)	0.38
Type of AAV, %			0.3
MPA	38	59	
GPA	62	41	
ANCA type, %			0.3
MPO	44	65	
PR3	56	35	
BVAS/WG (0–67), mean (SD)	7.5 (2.07)	8.2 (2.97)	0.76
Serum creatinine at entry, mg/dL, mean (SD)	2.5 (0.86)	1.9 (0.74)	0.05
MDRD eGFR at entry, mL/min/1.73 m <sup>2</sup> , mean (SD)	29 (11)	43 (25)	0.06
VDI (0–64), mean (SD)	3.1 (2.59)	1.3 (1.85)	0.04
C-reactive protein, mg/dL, mean (SD)	0.9 (0.77)	3.2 (4.37)	0.1
Erythrocyte sedimentation rate, mm/h, mean (SD)	38 (19)	56 (32)	0.13
Serum albumin, g/dL, mean (SD)	5.8 (8.64)	3.3 (0.47)	0.06
Number of organs involved, mean (SD)	2.8 (1.17)	3.1 (1.34)	0.76
Alveolar hemorrhage, %	31	18	0.44

BVAS/WG, Birmingham Vasculitis Activity Score for Wegener's Granulomatosis; VDI, Vasculitis Damage Index.

## Results

Of the 102 patients who had renal involvement in the RAVE trial, 45 patients had biopsy-proven pauci-immune GN, and the remaining 57 patients had a clinical diagnosis of ANCA-associated GN. Thirty three of the 45 renal biopsies were available for evaluation. Because biopsy blocks were not available for further cuts or because sections contained no glomeruli, only 25 of these patients had unstained slides available for CD3 and CD20 immunohistochemical staining. Four patients had unstained slides available only for CD3 staining, and the remaining 4 patients did not have unstained slides left.

Of the patients included in this study, 21 were newly diagnosed and 12 had relapsing disease. ANCA GN class distribution was 46% focal, 33% mixed, 12% sclerotic, and 9% crescentic. Baseline renal function and the vasculitis damage index differed according to treatment arm (CYC, *n* = 17 and RTX, *n* = 16). Compared to patients treated with RTX, the CYC group had a lower serum creatinine ( $1.9 \pm 0.74$  vs.  $2.5 \pm 0.86$  mg/dL) and a lower vasculitis damage index ( $1.3 \pm 1.85$  vs.  $3.1 \pm 2.59$ ; Table 1). Other

baseline characteristics were not significantly different between treatment arms. Overall, 31 patients achieved remission with a median (range) time to remission of 60 (26–177) days. There was no difference in time to remission between the treatment arms.

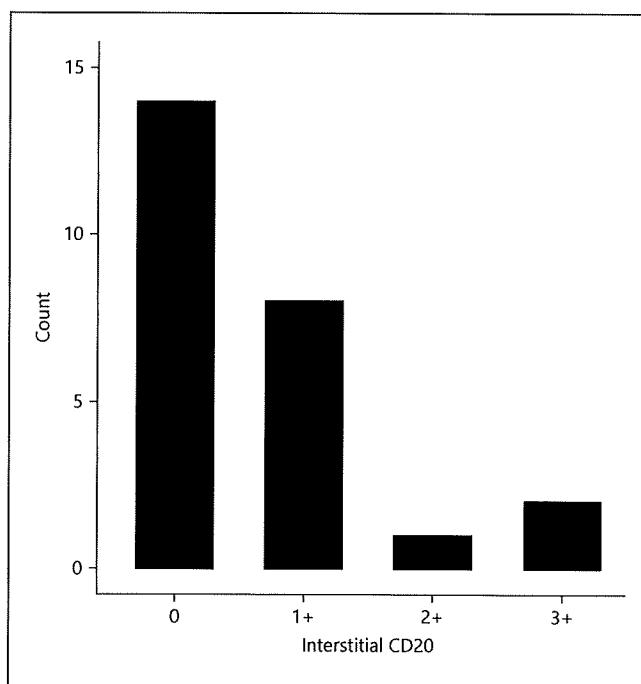
All of the 29 biopsies that were evaluated had some degree of CD3 positive interstitial infiltration (Fig. 2). In a large majority of cases, the CD3 positive infiltrate was prominent (>25% in 93%, >50% in 69%). In contrast, 14 of the 25 biopsies had no demonstrable infiltration with CD20 positive cells (Fig. 3). Only a small minority featured a dense CD20 positive infiltrate (>25 in 12%, >50 in 8%).

In a univariate analysis (data not shown), there was no correlation of interstitial CD3 and CD20 scores with GFR<sub>0</sub>, GFR<sub>6</sub>, GFR<sub>12</sub>, or GFR<sub>18</sub> in the entire cohort and in the 2 treatment arms. There was no correlation of CD3 and CD20 scores with GN class, ANCA type, and new versus relapsing disease.

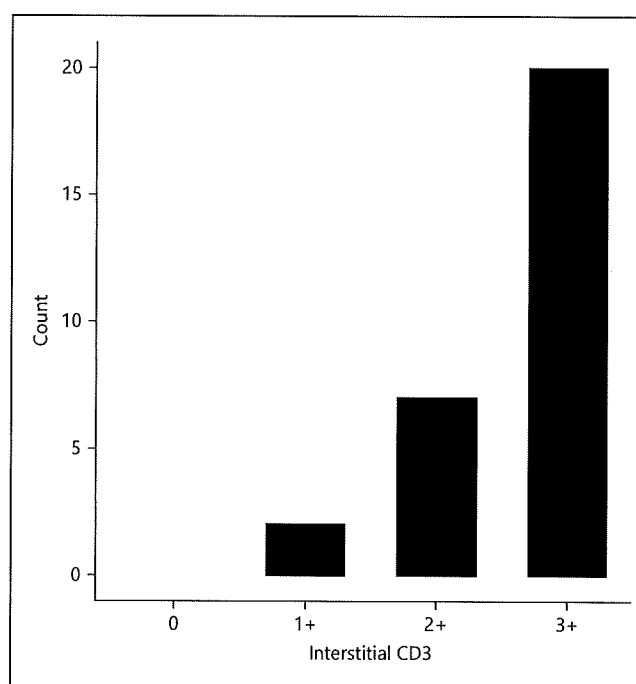
In a multiple linear regression analysis (Table 2), GFR<sub>0</sub> was predictive of GFR<sub>6</sub>, GFR<sub>12</sub>, and GFR<sub>18</sub>. CD3 positive interstitial infiltrates were predictive of GFR<sub>6</sub>

**Table 2.** Multiple linear regression model of clinical and pathological parameters with outcome in the entire study group ( $n = 33$ )

Variable	GFR <sub>6</sub>		GFR <sub>12</sub>		GFR <sub>18</sub>	
	B (SE)	<i>p</i> value	B (SE)	<i>p</i> value	B (SE)	<i>p</i> value
GFR <sub>0</sub>	0.8 (0.1)	<0.001	0.7 (0.2)	0.018	1.4 (0.4)	0.012
CD3 infiltration	-10.1 (4.4)	0.041	-6.4 (9)	0.5	-4.7 (15.9)	0.781
CD20 infiltration	5.3 (3)	0.107	3.1 (5.6)	0.594	-3.8 (8.9)	0.686
ANCA GN class (=focal)	5.9 (10.8)	0.596	12.4 (18.3)	0.521	15.4 (30.6)	0.635
ANCA GN class (=mixed)	-16.3 (13.5)	0.254	-1.3 (24.4)	0.96	4.2 (43.2)	0.926
ANCA GN class (=sclerotic)	-26.6 (13.2)	0.069	-10 (26.7)	0.72	1.3 (38.6)	0.975
Chronicity score	0.8 (1.3)	0.579	1.5 (1.9)	0.454	1.4 (2.6)	0.629
Age	-0.4 (0.2)	0.152	-0.4 (0.4)	0.327	-0.4 (0.7)	0.648
BVAS/WG at entry	-1.5 (0.9)	0.134	-1.6 (1.3)	0.258	1.7 (2.2)	0.484
ANCA type (=PR3)	-9.9 (5.6)	0.107	-2.6 (11)	0.821	-1.1 (19.7)	0.956
Alveolar hemorrhage (=yes)	7.9 (5.7)	0.193	8.2 (9)	0.39	1.4 (15.4)	0.929
Treatment arm (=CYC)	-1.8 (6.4)	0.788	6.9 (13.1)	0.613	7.7 (21.8)	0.74



**Fig. 2.** Distribution of the biopsies according to the intensity of interstitial infiltration with CD20 positive cells.



**Fig. 3.** Distribution of the biopsies according to the intensity of interstitial infiltration with CD3 positive cells.

but not GFR<sub>12</sub> or GFR<sub>18</sub>. CD20 positive interstitial infiltrates were not predictive of GFR<sub>6</sub>, GFR<sub>12</sub>, or GFR<sub>18</sub>. Chronicity score was not predictive of GFR<sub>6</sub>, GFR<sub>12</sub>, or GFR<sub>18</sub>. When stratified by treatment arm (Table 3), CD3 positive infiltrates correlated with a lower GFR<sub>6</sub> but did not predict GFR<sub>12</sub> or GFR<sub>18</sub> in the patients

treated with RTX, while they did not correlate with GFR at any time point in the CYC arm. CD20 positive infiltrates correlated with a better GFR<sub>6</sub>, but this did only reach the limit of statistical significance in the CYC-treated patients. There was no impact on GFR at later time points.

**Table 3.** Multiple linear regression model of CD3 and CD20 immunostaining with outcome in the 2 treatment arms

	GFR <sub>6</sub>		GFR <sub>12</sub>		GFR <sub>18</sub>	
	B (SE)	<i>p</i> value	B (SE)	<i>p</i> value	B (SE)	<i>p</i> value
RTX ( <i>n</i> = 16)						
CD3	-11.7 (4.2)	0.038	3.2 (11.7)	0.803	-5.8 (16.1)	0.779
CD20	14.8 (6.7)	0.079	20.9 (12.6)	0.195	8.8 (18.2)	0.712
CYC ( <i>n</i> = 17)						
CD3	-22.1 (11.4)	0.102	-31.2 (17.0)	0.126	-19.1 (16.2)	0.291
CD20	11.9 (4.6)	0.042	6.9 (5.7)	0.281	-6.1 (5.4)	0.309

## Discussion

The present study provides a detailed characterization of the tubulointerstitial infiltrate in patients with renal disease enrolled in the RAVE trial. The standardized treatment and follow-up in the RAVE trial allowed the determination of the predictive value of several clinical and immunopathological characteristics and the examination of a potential differential effect according to treatment limb.

In accordance with previous data [15], renal function at the time of biopsy predicted renal outcomes in our study cohort. The combination of baseline renal function and histology has been shown to be a better predictor of renal function compared to baseline renal function alone [16–18]. Across several validation studies, the new histologic classification of ANCA GN proposed by the international working group of renal pathologists has consistently correlated with long-term renal outcome in focal and sclerotic class, but has yielded variable results in crescentic and mixed class depending on the study cohort [19]. However, in one study, GN class alone did not improve prognostication after adjustment for established prognostic factors such as age, baseline renal function, degree of tubular atrophy, and percentage of normal glomeruli [20]. Our data also show that GN class does not independently predict long-term renal prognosis. Interstitial fibrosis and tubular atrophy have been shown to be predictors of long-term renal function in prior studies [21, 22]. In contrast, we did not find any correlation of chronicity score to long-term renal function and this may be attributed to our small sample size. However, the study by Hauer et al. [21] demonstrated that the relationship between interstitial fibrosis and tubular atrophy with GFR at 18 months was not independent of GFR at entry, suggesting that these changes reflect damage that was

present before the initiation of treatment and this may explain our findings as well.

A pronounced interstitial infiltrate correlates with poor renal function at baseline [23–25], but the effect on 1-year renal function is variable. A positive correlation between interstitial inflammation and renal function at 1 year was noted in a multicenter study by the European vasculitis study group [24]. However, the extent of the interstitial infiltrate did not appear to predict long-term renal function in a single-center study of 22 patients with GPA [23] as well as in a biopsy study of 28 patients included in the RTX arm of the RITUXVAS study [25].

Further characterization of the interstitial infiltrates revealed CD3 positive T cells to be the predominant cell type [2, 3]. Our study confirms the predominance of CD3 positive T cells in the interstitium. There was a noteworthy CD 20 positive B cell interstitial infiltrate in only a minority of biopsies. These findings suggest that the therapeutic effects of RTX on the kidney disease may not be mediated by depletion of B cells in the kidney, but rather in the secondary lymphoid tissue where T-B cell interactions may drive the pathogenic immune response.

We found no correlation between interstitial T-cell infiltrates in the diagnostic biopsy and GFR at 12 and 18 months. At first sight, these results may seem at odds with the findings of the biopsy study of the RITUXVAS trial, where T-cell-mediated lesions did predict renal outcome [25]. However, a closer look reveals that the negative impact of T-cell tubulitis was limited to GFR at 6 months and 1 year but no longer significant at 2 years [25].

The strength of our analysis is that biopsies from patients on both CYC and RTX arms, who received similar glucocorticoid dosing and tapering protocols, were evaluated, allowing the study to tease out a potential differential effect of the type of immunosuppressive regimen on

immunopathological processes and outcome. More in particular, the issue whether B-cell-directed therapy may be associated with under-treatment in the light of the T-cell dominance in the inflammatory infiltrate could be raised. When stratified by the treatment arm, a dense CD3 positive infiltrate predicted lower GFR at 6 months, only in the RTX group. There was no differential effect at later time points. Whether these results can be explained by the play of chance or truly represent a transient phase of under-treatment by RTX is difficult to assess. At any event, the absence of a long-term negative impact in the RTX arm is what is clinically most relevant, commensurate with the outcome of RAVE that demonstrated non-inferiority of RTX vs. CYC.

At 6 months, a CD20 positive infiltrate predicted a higher GFR, but this reached the level of statistical significance only in the CYC group. Similarly, there was no differential effect at later time points. These results are in accordance with the absence of a significant impact of B cells in the renal tissue on long-term renal outcome in the RTX arm of RITUXVAS [25].

Interestingly, a study of repeat renal biopsies, before and 12 months after the start of induction therapy with CYC found a decrease in the number of CD 20 positive cells, while the CD3 infiltrate remained unchanged [26].

Regrettably, no data on repeat renal biopsies exist following RTX induction therapy.

A limitation of our study is the small sample size, which may have failed to uncover an association of T- or B-cell infiltrates with long-term renal function. However, given the absence of any evidence in this study that staining renal biopsies for T and B cells is crucial in the selection of treatment regimens involving either RTX or CYC, even a much larger study (impractical to perform) would be unlikely to demonstrate a clinically important use of such evaluations.

In conclusion, a majority of patients with ANCA-associated GN have an interstitial infiltrate consisting mainly of T lymphocytes, independent of ANCA type or GN class. Our data do not support a prognostic value of either T-cell or B-cell infiltrates. Importantly, there is no long-term differential effect according to the treatment arm, consistent with the clinical observation that remission rates are similar in both CYC- and RTX-treated patients.

#### Disclosure Statement

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