Cyclophosphamide followed by rituximab for aggressive multiple-relapsing antineutrophil cytoplasmic antibody-associated vasculitis

S. Lionaki[1], G.E. Fragoulis[2], A. Venetsanopoulou[2], P. Vlachoyiannopoulos[2], J.N. Boletis[1], A.G. Tzioufas[2]

[1]*Department of Nephrology, Laiko Hospital Athens, National and Kapodistrian University of Athens, Faculty of Medicine, Athens;*
[2]*Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Greece.*

Sophia Lionaki, MD
George E. Fragoulis, MD
Aliki Venetsanopoulou, MD
Panagiotis Vlachoyiannopoulos, MD
John N. Boletis, MD
Athanasios G. Tzioufas, MD

Please address correspondence to:
Dr Sophia Lionaki,
Department of Nephrology,
Laiko Hospital,
17 Agiou Thoma Street,
11527 Athens, Greece.
E-mail: sofia.lionaki@gmail.com

Received on April 29, 2016; accepted in revised form on September 2, 2016.

Clin Exp Rheumatol 2017; 35 (Suppl. 103): S155-S164.

Key words: ANCA vasculitis, aggressive, relapsing, treatment, rituximab, cyclophosphamide

Competing interests: S. Lionaki and I.N. Boletis received financial support from Roche for attending a scientific meeting.
All the other authors have declared no competing interests.

ABSTRACT

Objective. *To evaluate the long-term outcomes of patients with multi-relapsing antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), who received sequentially therapy with cyclophosphamide and rituximab, upon new onset of aggressive vasculitis.*

Methods. *We retrospectively studied patients with multiple-relapsing AAV, who were treated with the standard regimen plus rituximab, given in sequence, upon a major relapse, compared to historical patients, who had been treated with the standard regimen alone in the same setting. The main outcomes of interest were relapse rates and frequency of adverse events.*

Results. *Of 147 patients with biopsy proven AAV, 35 (23.8%) experienced at least one major relapse, of whom, 14 (9.5%) received the sequential regimen and were compared to 21 (14.3%) historic patients, who had received the standard regimen alone for the same reason. Patients in both groups achieved remission in similar rates, but those treated with the sequential regimen experienced a significant decline in the relapse rate afterwards, compared to their corresponding rate prior to study entry [0.07 episodes of relapse per patient-year (95%CI: 0.03–0.2) vs. 0.38 (95%CI: 0.35–0.60) respectively, (p=0.004)]. The need for cyclophosphamide was significantly decreased in patients in whom cyclophosphamide was followed by rituximab [3.3(0–10.5) grams vs. 14.5 (4–177) grams, (p<0.0001)] but not in controls [17.2 (0–108) grams vs. 14.5 (0–63) grams, p=0.61].*

Conclusion. *Our data show that sequential therapy with cyclophosphamide and rituximab, upon a major relapse, in patients with frequently relapsing AAV, is associated with prolonged remission, allowing minimisation of the ultimate exposure to cyclophosphamide.*

Introduction

Approximately three decades ago, the introduction of cyclophosphamide, as therapy for remission induction in antineutrophil cytoplasmic antibody-associated vasculitis (ANCA-AAV) (1-5), improved dramatically its prognosis, with approximately 85% of patients achieving remission (5). However, 11% to 57% of responders will experience one or more (6-11) disease relapses, most frequently at the same organs-systems (6). The aggressive nature of this disease demands that vasculitis involving vital organs and following a rapidly progressive course, cannot be treated otherwise but with the use of cyclophosphamide, at least initially. Treatment with repeated courses of this agent on the other hand, has been linked to substantial toxicity, fueling the search for therapeutic alternatives, to minimise its exposure (12-13). The emerging evidence that rituximab, a chimeric monoclonal anti-CD20 antibody, could abolish autoantibody production (14-19), was very interesting, in the light of the increasing evidence connecting ANCAs with the pathogenesis of AAV (20-26). Temporary depletion of B cells might also confer an opportunity for reconstitution of the immune system to restore tolerance to ANCA antigens (27-30). Eventually, rituximab was shown non inferior than cyclophosphamide for induction of remission in newly diagnosed patients with AAV (28-30) and superior than cyclophosphamide in patients with relapsing vasculitis, while it was associ-

ated with fewer relapses than azathioprine (31) when used as maintenance therapy. It evidently opened a new road in AAV therapy (28-31), but soon it became clear that it needs time to act, an apparent hurdle for a disease, which is often catastrophically aggressive and life theatening.

As the B cell autoimmune response in AAV appears to be facilitated by permissive T and B cell regulation, and by B cell-stimulating factors released by neutrophils (20, 32), targeting both simultaneously, may be crucial for achievement of clinical and immunological remission. Besides, among patients treated with rituximab, time to relapse was significantly shorter in cyclophosphamide-naïve patients (33), while adjuction of a low dose cyclophosphamide was associated with prolonged remission (34).

We aimed to assess the long term outcomes of patients with a history of multiple-relapsing AAV, after treatment with a regimen consisting of cyclophosphamide and rituximab, given in sequence, upon a new major relapse, compared to historic patients, who had received the standard regimen alone, in the same setting, focusing on the frequency of subsequent relapses and adverse events.

Patients and methods

Description of patient population and definitions

We retrospectively identified all patients of our AAV registry (1985–2014), who received therapy with the standard regimen followed by rituximab, upon disease relapse involving major organs (rituximab group). For comparison reasons, a group of historical patients was selected, from the era prior to the initiation of rituximab in the treatment of AAV (control group), which included all patients, who had been treated with the standard regimen, upon a major disease relapse (Fig. 1). The AAV registry includes patients with a biopsy showing pauci-immune small-vessel vasculitis with or without granulomatous inflammation at any tissue, positive ANCA determination by immunofluorescence microscopy and/or antigen-specific enzyme-linked immunosorbent assay (33) and signed informed consent for review of medical records. Patients might have positive cytoplasmic ANCA, anti proteinase-3 (PR3) ANCA, or both, or perinuclear ANCA, anti myeloperoxidase (MPO) ANCA, or both (35). Patients having only perinuclear ANCA were required to have a negative antinuclear antibody test.

Medical records were reviewed dating back to the initial diagnosis of AAV and included information on demographics, disease activity, characteristics of AAV, clinical phenotypes, laboratory and serological measurements, and disease outcomes. We assigned as study entry; i. for the rituximab group, the date of the relapse, which led to treatment with the sequential regimen, ii. for the control group, the date of the relapse, which was determined by the criteria mentioned above. As total disease duration was considered the follow up time from the initial diagnosis of AAV to the latest visit to the outpatient rheumatology clinic. As study period was defined the time interval between entry in the present study to the latest visit to the outpatient rheumatology clinic, and disease duration at entry was considered the time from the initial diagnosis of AAV to study entry (Fig. 1). Definitions of end points were based on the Chapel Hill vasculitides nomenclature Consecus Conference (6) and the European League Against Rheumatism (EULAR) recommendations for systemic vasculitis (36). Remission was characterised by resolution of all vasculitic manifestations, including stabilisation or improvement of the renal indexes and clearing of haematuria, for more than one month (2, 6, 36). The persistence of proteinuria, in the absence of glomerular haematuria, was not considered indicative of active glomerulonephritis (2, 6, 36). Remission off therapy, or complete remission was defined as remission with no immunosuppressive therapy, or with less than 7.5 mg prednisone per day (2, 6, 36). Treatment resistance was defined as the persistence of active vasculitic manifestations in any organ, despite immunosuppressive treatment, determined at least after one month of therapy. Relapse was defined as the new onset of vasculitic signs/symptoms and could only be recorded among patients who had previously achieved remission, either on or off therapy (2, 6, 36). Major relapse was defined as the onset of new or worse vasculitic activity with involvement of at least one major organ or any life-threatening manifestation, or both (31). As minor relapse was considered the onset of new or worse vasculitic activity, not corresponding to a major relapse, but still requiring enhancement of immunosuppressive treatment (31). The relapse episode, which was chosen to determine the time of entry in the present study and follow the related analysis was the first major relapse after the initial diagnosis. The option of treating a major relapse with rituximab and glucocorticoids only, was not offered, as these cases had a history of multiple relapses, while presented with an aggressive form of the disease and thus, were considered of high risk for end stage organ disease or death.

Organ-system involvement was defined by the criteria previously reported (2, 6). Renal involvement diagnosis was made on the basis of a kidney biopsy showing pauci-immune glomerulonephritis (2, 6). Renal function was calculated using the Modification of Diet in Renal Disease equation (37) for estimation of glomerular fitration rate (eGFR). Disease activity was documented with the use of the Birmingham vasculitis activity score (BVAS) in its 2003 modification (38). The clinical phenotype was assigned according to the revised Chapel Hill vasculitides nomenclature Consensus Conference (2, 39) including granulomatosis with polyangiitis (GPA), and microscopic polyangiitis (MPA). Patients with eosinophilic granulomatosis with polyangiitis, were excluded from this study (2, 39). Analysis of adverse events data included events, which occured during the study period, namely; infusion-related reactions, infections requiring hospitalisation, malignancies, osteoporosis,new onset of diabetes mellitus, and frequency of leukopenia and hypogammaglobulinaemia during the study period. Leukopenia was defined as the reduction of white blodd cells <4000/μl (with neutropenia being <2000/μl) and hypogammaglob-

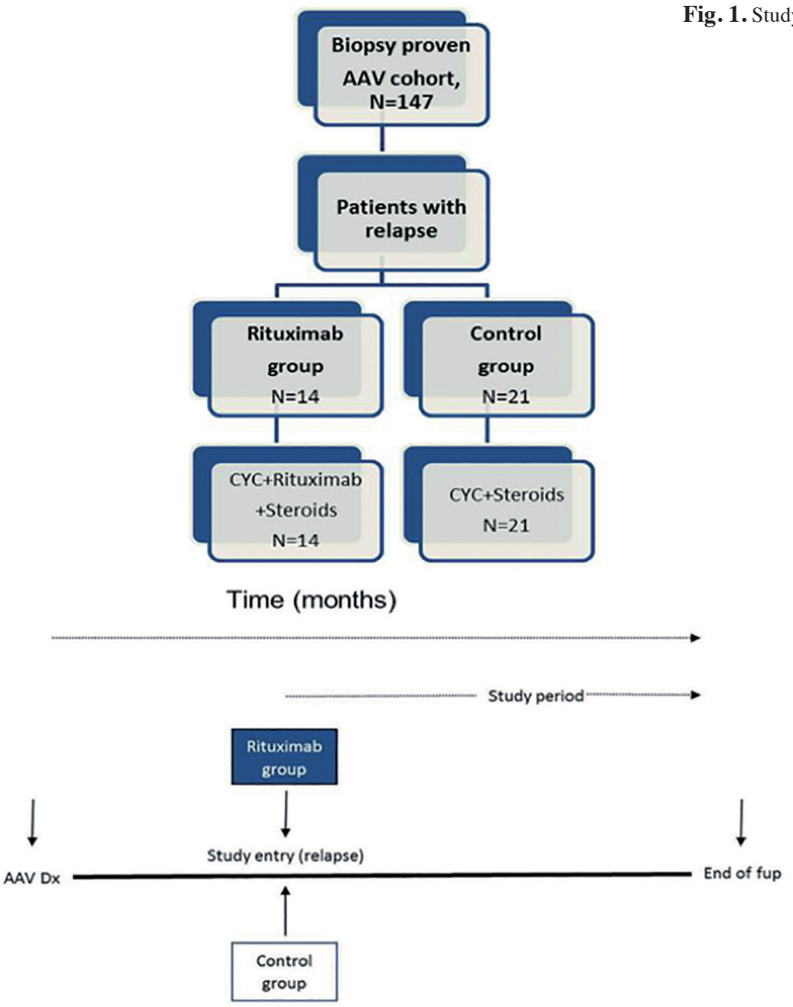

Fig. 1. Study design

ulinaemia as the reduction IgG below 11.1% of the total or below 0.71g/dl. Comparison of outcomes, in short and long term, along with the frequency and severity of adverse events, were performed between groups and within groups (before entering the study and during the study period) and included remission, treatment resistance, subsequent relapse rate, end stage renal disease, death and adverse events (Fig. 1). In addition, we tested our results after adjusting for different time periods, as our AAV registry started running in 1985. For this analysis, patients were categorised by the decade of diagnosis, *i.e.* before 2010 and after 2010 for the rituximab group, and 1990–2000 and 2001-2014 for the control group.

Therapeutic regimens

• *Sequential regimen (Rituximab group)*

Patients in this group were treated with the standard regimen (cyclophosphamide+glucocorticoids) followed by one course of rituximab (375 mg/m^2 body surface area (BSA) for 4 consecutive weeks, or 1gram every 2 weeks, given twice), which was administered intraveneously at the end of a 3-month course of cyclophosphamide (500-1000 mg/m^2 BSA depending on the current leucocyte and eGFR measurements). All patients received pulses of high dose glucocorticoids (1gram methyl-prednisolone each, intraveneously, for 3 consecutive days), followed by an oral taper of prednisone,starting from 1mg/kg body weight (BW) for the first four weeks, in a individualised manner within the following 16–20 weeks. A routine premedication scheme with paracetamol, bilusepin and hydrocortisone was used in each rituximab administration, and a course of sodium 2-sulfanylethanesulfonate before each cyclophosphamide pulse.

Standard regimen (Control group)

The control group consisted of historical patients, from the era prior to the initiation of rituximab in the treatment of AAV. They all had a major AAV relapse at the time of study entry and had been treated with cyclophosphamide, either intraveneously (500–1000 mg/m^2 BSA) or orally (1.5–2 mg/day/kg BW), corrected by the current leucocyte and eGFR measurements, for a total of 3-6 months together with glucocorticoids (3 pulses of methyl-predinisolone, one gram each, followed by oral tapering or prednisone, starting from 1 mg/kgr of BW/day). Maintenance treatment included azathioprine, or mycophenolate mofetil, or methotrexate using the dose schemes mentioned above. Patients with pulmonary haemorrhage or rapidly progressive glomerulonephritis patients in both groups were treated with plasma exchange as needed. *Pneumocystis jiroveci* pneumonia prophylaxis with sulfamethoxazole and trimethoprim was given to both groups patients (40).

Statistical methods

Descriptive statistics include number (N), percent (%), mean, standard deviation (sd), median and range. Demographics and clinical characteristics were compared using Fisher's exact tests for categorical measures and Mann-Whitney rank sum tests for continuous variables. Comparisons within groups (*i.e.* before and after study entry) were performed using the paired *t*-test or Wilcoxon signed-rank test. Incidence rates were calculated counting only 1st relapse post achieving of remission in order to avoid bias the data by adding multiple relapses in the same individual. The binomial principles were used to calculate 95% confidence intervals (CI). *P*-values for comparing two incidence rates were calculated by testing the hypothesis that their difference was equal to zero while we took into account the 1st relapse for each individual and censored at theta relapse in oder to avoid bias the data in cases with multiple relapsing episodes. Exact *p*-values are reported with a two sided *p*-value of 0.05 or less considered statistically significant. Kaplan–Meier

curves were used to present the comparison of the relapse-free survival in the two groups, before entering the study and during the study period, as well as within each group separately for the same time periods. Log-rank test was used to express differences between the curves. Analyses were conducted using Graph Pad 5.0.

Results

Description of the two groups

As of December 2014, 147 patients with biopsy proven AAV had been enrolled in our registry, with a mean total disease duration of 103 (±63.1) months. Among them, we identified 14 patients (9.5%), who received rituximab in sequence with cyclophosphamide (rituximab group) upon a major disease relapse, and 21(14.2%) historic patients, who had received the standard regimen alone for the same reason (control group).

As shown in Table I, demographics and baseline characteristics were similar between groups.

Characteristics related to AAV

Organ involvement and disease activity at the initial diagnosis of AAV and at study entry are presented in Table I. PR3-ANCA was the most frequent type, and GPA was the dominant clinical phenotype in both groups. Among patients with ear nose throat involvement (ENT) there were 3 (21.4%) patients with histological proof of granuloma formation in the nasal cavities in the rituximab group, and 4 (22.2%) in the control group. Four (22.2%) of the patients in the rituximab group and 6 (33.3%) of the controls had epistaxis, while otitis media was recorded in 4 (19%) cases of each group. Destructive bone disease of the upper airways, was documeneted in 1 (7.1%) patient of the rituximab group and 2 (9.5%) of the controls. 42.9% of the patients had pulmonary infiltrates in each group. Eight (57.1%) patients in the rituximab group and 12 (57.1%) of the controls had pulmonary nodules. Lung cavities were found in 6 (42.9%) and 2 (9.5%) patients respectively. None of the patients experienced respiratory failure requiring mechanical ventilation. All patients with clinical signs of glomerulonephritis except one, in both groups had a kidney biopsy perfomed. Of those, histopathology (41) revealed, 6 (85.7%) patients with focal class of ANCA-associated glomerulonephritis, and 1 (14.3%) patient with crescentic class in the rituximab group. Among controls, there had been 9 (81.8%) patients with focal class, 1 (9.1%) with crescentic class, and 1 (9.1%) with sclerotic class. Two (14.2%) of the patients in the rituximab group and 1 (4.7%) of the controls, presented with rapidly progressive glomerulonephritis and oliguria, requiring acute haemodialysis around the initial diagnosis. The median time to 1st relapse following immunosuppressive therapy after the initial diagnosis, was shorter in the rituximab group [13 (2–116) *vs*. 36.0 (13–108) months, (p=0.03)] but the the total number of relapses per patient from AAV diagnosis to study entry was similar between groups (Table I). Comparison of haematological measurements at entry, 3 and 6 months later and at the end of the study period did not reveal any significant changes between groups (supplementary Table I).

Table I. Characteristics of the two groups at AAV diagnosis and at study entry.

Characteristic	Rituximab group n=14	Control group n=21	p-value
At diagnosis of AAV			
Age (years), median (range)	51 (19–75)	47 (17–71)	0.34
Gender (males), n (%)	5 (35.7)	10 (47.6)	0.73
BVAS, median (range)	15 (5–32)	15.5 (6–33)	0.75
ANCA type, (C/PR3-ANCA), n (%)	1 (78.6)	18 (85.7)	0.66
Clinical phenotype			
Microscopic polyangiitis, n (%)	3 (21.4)	3 (14.3)	0.66
Granulomatousis with polyangiitis, n (%)	11 (78.6)	18 (85.7)	0.66
Organ involvement, n (%)			
Constitutional symptoms	13 (92.9)	18 (85.7)	0.63
Mucus	4 (28.6)	4 (19.1)	1.00
Ear nose throat	8 (57.1)	12 (57.1)	1.00
Kidney	7 (50.0)	11 (52.1)	1.00
Lung	7 (50.0)	12 (57.1)	0.74
Skin	9 (64.3)	7 (33.3)	0.09
Neurological	5 (35.7)	6 (28.6)	0.72
Gastrointestinal	3 (21.4)	0 (0.0)	0.26
From AAV diagnosis to study entry			
Time to 1st relapse (months), mean (±SD)	34.1 ± 40.2	47.2 ± 30.7	0.03
Cyclophosphamide cumulative dose (g), median (range)	14.5 (4–177)	11.0 (0–63)	0.31
Cumulative treatment with steroids (months), median (range)	18.0 (1–168)	25.0 (6–98)	0.64
Plasma-exchange, ever, n (%)	4.7	3 (14.3)	0.64
Relapses per patient, episodes, median (range)	2 (1–4)	1 (1–4)	0.08
BVAS, median (range)	10.5 (4–17)	12.0 (4–27)	0.36
Organ involvement at study entry, n (%)			
Kidney	5 (35.7)	9 (42.9)	1.00
Lung	5 (35.7)	7 (33.3)	1.00
Ear nose throat	5 (35.7)	13 (61.9)	0.18
Skin	4 (28.6)	4 (19.0)	0.68
Neurological	5 (35.7)	3 (14.3)	0.22
Constitutional symptoms	7 (50.0)	9 (42.9)	0.74
Mucus membrane	3 (21.4)	2 (9.5)	0.37
Gastrointestinal	2 (14.3)	0 (0.0)	0.15
BVAS (total group), median (range)	9.5 (4-17)	10 (4-27)	0.96
BVAS (patients with major relapse), (mean±SD)	8.7 (±8.79) 1	15.3 ± 6.71	0.77
Total disease duration (months), median (range)[§]	63.5 (10–228)	130 (15–240)	0.15

[‡§]Time from disease diagnosis to the last visit to the rheumatology clinic.

Table II. Comparison of outcomes between groups during the study period.

Characteristic	Rituximab group n=14	Control group n=21	*p*-value
Study period (months) median (range)†	30.5 (5–68)	36 (3–228)	0.19
Immunosuppressive regimen			
Cyclophosphamide, n (%)	12 (85.7)	18 (85.7)	1.00
Steroids, n (%)	18 (100)	18 (100)	1.00
Plasma-exchange, n (%)	0 (0.0)	1 (5.6)	1.00
Rituximab, n (%)	14 (100)	0	<0.0001
Outcome post treatment at study entry			
Complete remission, n (%)	3 (21.4)	6 (28.6)	0.71
Remission on therapy, n (%)	11 (78.6)	15 (71.4)	0.71
Remission (complete or on therapy), n (%)	13 (92.8)	21 (100)	0.49
Relapses per patient (episodes), median, (range)	0 (0–1)	1 (0–2)	0.02
Relapses per patient among PR3-ANCA (episodes), median (range)	1 (0–1)	2 (0–2)	0.02
Time to 1st relapse (months), mean (±SD)	45.5 ± 13.4	48.3 ± 45.8	0.8
Proportions of patients with remission at 1-year	14/14 (100)	18/21 (85.7)	0.26
3-years	13/14 (92.8)	17/21 (80.9)	0.63
5-years	12/14 (85.7)	15/21 (71.4)	0.43
Patients (%) with subsequent major relapse	2/14 (14.3)	6/19 (31.6)	0.25
Cyclophosphamide cumulative dose, median (range) (grams)	3.3 (0–10.5)	17.6 (0–108)	0.001
Death from any cause, n (%)	1 (7.1)	0	0.4
End stage renal disease, n (%)	1 (7.1)	1 (4.8)	1.00
BVAS at end of follow-up (mean±SD)	0.6 ± 1.5	0.4 ± 0.9	0.83
Adverse events			
Infections (events), n (%)	6 (42.9)	18 (85.7)	0.01
Diabetes mellitus, n (%)	0 (0.0)	0 (0.0)	1.00
Osteoporosis, n (%)	2 (14.3)	5 (23.8)	0.66
Malignancy, n (%)	1 (7.4)	3 (14.3)	0.63
Hypogamamglobulinaemia, n (%)	5/11 (45.5)	2/10 (20)	1.00
Serum IgG (mean±SD)	11.35 ± 4.5	14.5 ± 3.02	0.06
Leukopenia, n (%) by the end of 6th month of study period	3/21.4)	1 (4.8)	0.13
Neutropenia, n (%) by the end of 6th month of study period	1 (7.1)	1 (4.8)	0.77
Leukopenia, n (%) by the end of study period	3 (21.4)	1 (4.8)	0.13
Neutropenia, n (%) by the end of study period	1 (7.1)	1 (4.8)	0.77

†Time from study entry to the last visit to the rheumatology clinic.

Table III. Comparison of relapse rates and cyclophosphamide requirements between groups before and after study entry.

Parameter	Prior to study entry	Study period	*p*-value
Rituximab group, n=14			
Follow up time (months), (median, range)	33 (2–160)	35 (6–68)	0.24
Relapse rate, (episodes/patient-year)	0.38	0.07	0.004
Relapses per patient, (episodes), median (range)	2 (1–4)	0 (0–1)	<0.001
Relapses per patient among PR3-ANCA, (episodes), median (range)	2 (1–4)	0 (0–1)	0.0001
Relapse rate among PR3-ANCA (episodes per patient-year)	0.38	0.10	0.02
Cyclophosphamide need (g), median (range)	14.5 (4.0–177)	3.3 (0.0–10.5)	<0.0001
Control group, n=21			
Follow up time (months), median (range)	35 (6–165)	53 (3–228)	0.23
Relapses rate, (episodes per patient-year)	0.26	0.31	0.67
Relapses per patient, (episodes), median (range)	1 (1–4)	1 (0–2)	0.26
Relapses per patient among PR3-ANCA, (episodes), median (range)	1 (1–4)	1 (0–2)	0.61
Relapse rate among PR3-ANCA (episodes per patient-year)	0.30	0.36	0.66
Cyclophosphamide need (g), median (range)	14.5 (0–63)	17.2 (0–108)	0.31

AAV relapses before and after study entry

Relapse rates of AAV were estimated before and after study entry in both groups (Tables II, III). Prior to study entry, time to relapse was significantly shorter in those patients, who subsequently received the sequential regimen, compared to the time to relapse which was recorded for the control group (p=0.03) (Table I) and the relapse free survival was shorter (Fig. 2-1A). In contrast, these patients showed no difference in the time to relapse after they had received the sequential regimen (during the study peiod) compared to the controls (Table II) (Fig. 2-1B). However, a significant decline in the median number of relapses per patient was observed in the rituximab group during the study period, compared to the number of relapses per patient prior to study entry for the same group (Table III), while analysis of the respective data for the control group, did not reveal any difference. Accordingly, the rate of AAV relapse in the rituximab group, post study entry, was significantly lower, than the one prior to study entry (p=0.004) for the same group (Table III) (Fig. 2B). Also, although not statistical significant (p=0.08), the follow up time for the patients, who received the combined regimen was slightly shorter compared to that of the patients who received cyclophosphamide only.

Patients with PR3-ANCA, who received rituximab in sequence with cyclophosphamide, experienced a significant decline in the number of relapses per patient post study entry, while this change was not observed among the PR3-ANCA patients of the control group (Table III). Adjusting for the time-period of treatment did not reveal any changes in our findings (data not shown).

Cyclophosphamide exposure before and after study entry

The cumulative dose of cyclophosphamide was not different between groups at study entry (Table I). However, during the study period, the need for cyclophosphamide was significantly decreased in the rituximab group, compared to need of cyclophosphamide

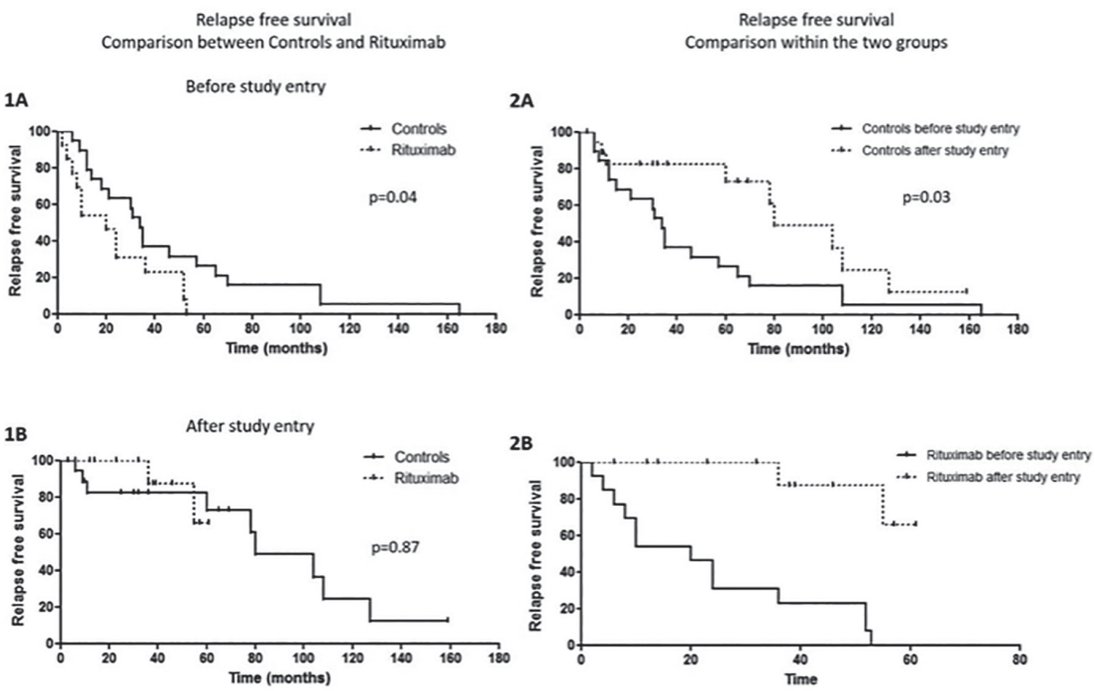

Fig. 2. Kaplan-Meier curves showing comparison of the relapse free survival (**A**) between the two groups before and after entering the study, and also (**B**) within the same group before and after entering the study.

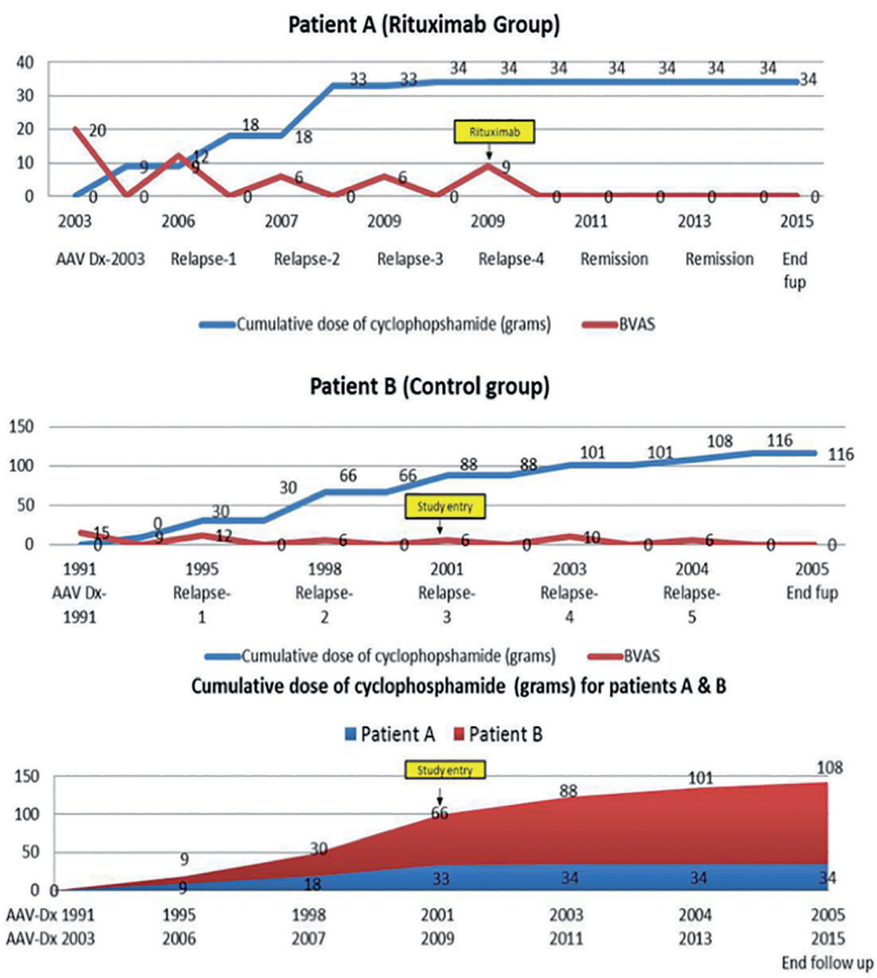

Fig. 3. The clinical course of AAV, in two patients (A & B) from the rituximab and the control group respectively, as depicted by the vasculitic activity score (BVAS) score before and after study entry and the corresponding cumulative dose of cyclophosphamide (grams).

of the control group (mean additional cumulative dose of cyclophosphamide 3.3 grams *vs*. 17.6 grams, p=0.001) (Table II). This finding was attributed to the fact that the need for cyclophosphamide, was significantly reduced in the rituximab group during the study period, compared to the cyclophosphamide requirement from AAV diagnosis to study entry [3.3 (0–10.5) *vs*. 14.5 (4–177) grams, respectively, (p<0.0001)] (Table III), a phenomenon, which was not observed in the control group (Table III). Duration of treatment with glucocorticoids was not different between groups.

Adverse events

Comparison of the frequency of adverse events between the two groups is shown in Table II. There was only one patient, who developed a mild reaction related to the infusion of rituximab, which ended up uneventfully and the patient was able to receive the next dose properly. We observed a higher frequency of infective episodes in the control group during the study period than in the the rituximab group (p=0.01) (Table II). One death was recorded during the study period, which occurred in a patient in the rituximab group, who experienced another disease relapse after achieving of remission with the se-

quential therapy. It occurred 14 months post therapy with the sequential regimen, with severe involvement of the lungs and the central nervous system. The death was attributed to resistant to therapy central nervous system vasculitis, despite aggressive therapy with cyclophosphamide and rituximab. The patient was repeatedly found to be positive for PR3-ANCA.

Discussion

This study reports results of long term outcomes in patients with a history of multiple-relapsing AAV, who received sequentially, cyclophosphamide and rituximab, upon a new, major disease relapse, in comparison with a historical control group, treated with the standard regimen alone in the same setting. Exposure to cyclophosphamide cannot be avoided in aggressive forms of AAV (1, 4, 42). We added rituximab in the standard regimen, as a "potential" cyclophosphamide sparing agent, in patients who were known to be "frequent relapsers" (14) and already had a significant exposure to cyclophosphamide. Remission was achieved in both groups in similar rates, but importantly, in patients, who received the sequential regimen, time to relapse, after treatment with rituximab, was comparable to the one of the patients who received the standard regimen. In addition, the former experienced a significant decline in the number of relapses per patient during the study period, compared to the number of relapses per patient prior to study entry. In a study, which included only newly diagnosed AAV cases, for whom the actual "relapsing propensity" was unknown, Jones *et al*. (43) found that the frequency of relapses was not different between patients who received rituximab, and those who did not. Based on our findings, the sequential regimen was associated with a more protective effect against disease relapse, than the repeated use of the standard regimen alone. McGregor *et al*. (33) have shown that time to first relapse, following treatment with rituximab, was shorter in patients who had never been exposed to cyclophosphamide previously. We speculated that accomplishment of immunological remission is probably a matter of targeting the immune system at multiple sites. Treatment with cyclophosphamide followed by rituximab broadens the disease modifying immunosuppression, by adding the effect of the reorganisation of the B cell repertoire. If the propensity for relapse is being modified following temporary, eradication of B lymphocytes, the option of sequential therapy should be considered for cases with previous relapses, in order to restore tolerance to ANCA autoantigens and avoid repetitive relapses. The rationale for the use of rituximab in sequence with cyclophosphamide in cases with major relapses was based on the relatively slow action of rituximab, which necessitates simultaneous employment of rapid immune suppressors, to limitate systemic inflammation as soon as possible, to prevent irreversible tissue injury or death. Glucocorticoids and cyclophosphamide are established, prompt and efficient immune regulators (1, 4) providing strong anti-inflammatory and immunomodulatory effect instantaneously. Cyclophosphamide and its metabolites induce elimination of aberrant T regulatory cells ($CD4^+CD25^+$ T cells) permiting withhold of the autoimmune phenomenon (15) which has been proven life saving. The accumulating knowledge regarding AAV pathogenesis (44) supports the notion that the B cell autoimmune response is facilitated by impaired T cell and B cell suppression and by B cell stimulation by activated neutrophils (20). Multiple studies have shown that patients with AAV have a deficiency and dysfunction of T regulatory cells that probably contribute to the loss of tolerance, that allows the emergence and persistence of a pathogenic ANCA autoimmune response (20).

An increased proportion of $CD4^+$ $CD25^+$ T cells, but with decreased percentatge of Foxp3-positive cells along with several abnormalities in the suppressive Tregs network have been reported in patients with active AAV (45-47). The RAVE group measured the absolute and relative number of $CD5^+$ B cells longitudinally in their trial participants (28, 48). The authors concluded that the percentage of peripheral $CD5^+$ B cells might reflect disease activity in rituximab-treated patients, although sole staining for CD5 as a putative surrogate marker for Breg cells did not identify a subpopulation of B cells with clear potential for meaningful clinical use (Unizony 2015) (48). The Chapel Hill group has shown that patients with active AAV have lower percentages of peripheral blood $CD5^+$ B cells, whereas patients in remission had a percentage of $CD5^+$ B cells no different from healthy controls (49). Moreover, normalisation of peripheral blood $CD5^+$ B cells cells after targeted B cell therapy with rituximab, correlated with more effective remission (49), while low % of $CD5^+$ B cells at the time of repopulation portends to a shorter time to relapse following rituximab therapy regardless of the immunosuprressive dose (49). Furthermore, a low percentage of $CD5^+$ B cells at B cell repopulation was associated with a shorter time to relapse following rituximab therapy irrespective of additional immunosuppressive therapy (50). Discrepancy of results in such studies probably reflect the fact they were designed differently with patients who were treated with rituximab only or rituximab plus additional agents (48-50).

Perhaps, inclusion of rituximab in the initial phase of therapy should be also considered for all patients, who are at increased risk for relapse, such as the patients with PR-3 ANCA or specific organ involvement (51-52). Patients with PR-3 ANCA are genetically different from patients with MPO-ANCA, have different clinical phenotype and are at prone to relapse (51-52), while rituximab has been shown superior to oral cyclophosphamide for the induction of remission in relapsing disease (31).

Not surprisingly, the reduction of AAV relapses in the rituximab group was also associated with a critical decrease in the need for cyclophosphamide subsequently. A paradigm of this is patient A (Fig. 2A), who did not experience any further relapse, post treatment with the sequential regimen, and thus, the cumulative dose of cyclophosphamide remained unaltered during the follow-

ing 6 years, till the end of follow up. In contrast, patient B (Fig. 2B) from the control group, received multiple courses of cyclophosphamide, post study entry, to be able to face all subsequent diseases relapses (Fig. 3). Minimisation of cyclophosphamide exposure is critical for patients with AAV, as its long term use has been shown to increase substantially the risk for malignancies (12-13) in a dose dependent manner. Studies with extended follow up duration, revealed that the incidence of malignancy is at least double that of the background population in patients exposed to cyclophosphamide (1, 12, 13, 53-57), while immunosuppressive regimens with reduced doses of cyclophosphamide have been associated with decreased risk of malignancy. Most important of all, the CYCLOPS study, showed that a significantly reduced dosing regimen of cyclophosphamide is less toxic in short term as well (53).

Notably, in our study the frequency of infections was lower in the patients, who received rituximab in sequence with cyclophosphamide. One possible explanation might be the total dosage reduction in cyclophosphamide, as it was given for only three months, while prophylaxis with cotrimoxazole was given in both groups. However, in the rituxvas study, which used rituximab in combination with cyclophosphamide, for induction of remission, there was a difference, although not statistically significant, in the total number of adverse events, which occurred between the two groups (42% *vs*. 36%) (58). A major difference between our study and the rituxvas one was that it was a prospective study, which recruited patients with newly diagnosed AAV, while we included patients with a past history of multiple-relapsing AAV. Thus, in our case patients in the control group experienced multiple relapses post study entry, which lead to the repeated use of cyclophosphamide with a significant cumulative immunosuppressive effect thereafter. The burden of morbidity related to infections is well known to depent on the cumulative dose of cyclophosphamide and the cumulative incidence of other adverse events related to this agent like leukopenia (59). Limitations pertaining to this study, include the small number of patients, which is mostly attributed to the fact that the disease is rare, while studying disease relapses requires long periods of follow up. Moreover, its retrospective design cannot exclude the possibility that some bias have been introduced during patient selection and the likelihood that the frequency of adverse events, has been seriously affected by earlier therapies, mainly by the use of cyclophosphamide or other immunosuppressive agents, which were given for maintenance therapy did not allow specific estimations regarding this issue.

In our experience, sequential therapy with cyclophosphamide and rituximab in patients with AAV, known to be "frequent" relapsers, in whom the disease involved severely vital organs, was associated with a significant change in the time to the first relapse and achievement of prolonged remission. In aggresive forms of AAV, avoidance of cyclophosphamide is not realistic. Treatment with rituximab after the standard regimen was shown more protective against disease relapse, than the repeated use of the standard regimen alone. It allowed us to minimise the ultimate exposure to cyclophosphamide, as the number of relapses per patient was significantly decreased afterwards. A better adverse event profile was recorded, probably related to the shorter exposure to cyclophosphamide. Undouptebly, further research is required (60) to ensure if patients with multiple-relapsing AAV, or those with certain risk factors for relapse, might benefit from administration of rituximab after the standard inductive regimen, upon a major relapse, as a cyclophosphamide-sparing intervention. Perhaps, these findings might serve as a background to generate a randomised control trial in this field.

Acknowledgments

We would like to express our deep gratitute and sincere thanks to our mentor, Professor Haralampos M. Moutsopoulos, for the kind patronage and invaluable guidance in this effort.

Key messages

- Minimisation of cyclophosphamide exposure is critical for patients with a multiple relapsing course of ANCA-associated vasculitis.
- Induction therapy with cyclophosphamide followed by rituximab in patients with a major relapse and a history of multiple-relapsing ANCA-associated vasculitis, was associated with significantly reduced relapse rates and fewer adverse events subsequently, compared to therapy with cyclophosphamide alone.
- Cyclophosphamide requirement was significantly reduced in patients with a history of multiple-relapsing ANCA-associated vasculitis, who received rituximab in sequence with cyclophosphamide compared, to those who received cyclophosphamide and glucocorticoids alone.
- Broadening the disease-modifying immunosuppression may help patients with ANCA-associated vasculitis with a propensity to relapse, in achieving clinical and immunological remission.

References

1. HOFFMAN GS, KERR GS, LEAVITT RY *et al*.: Wegener granulomatosis: an analysis of 158 patients. *Ann Intern Med* 1992; 116: 488-98.
2. FALK RJ, HOGAN S, CAREY TS *et al*.: Clinical course of anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and systemic vasculitis. The Glomerular Disease Collaborative Network. *Ann Intern Med* 1990; 113: 656-63.
3. HOGAN SL, NACHMAN PH, WILKMAN AS *et al*.: Prognostic markers in patients with anti-neutrophil cytoplasmic autoantibody-associated microscopic polyangiitis and glomerulonephritis. *J Am Soc Nephrol* 1996; 7: 23-32.
4. DE GROOT K, ADU D, SAVAGE CO, EUVAS (EUROPEAN VASCULITIS STUDY GROUP): The value of pulse cyclophosphamide in ANCA-associated vasculitis: meta-analysis and critical review. *Nephrol Dial Transplant* 2001; 16: 2018-27.
5. BRIEDIGKEIT L, ULMER M, GÖBEL U *et al*.: Treatment of Wegener's granulomatosis. *Adv Exp Med Biol* 1993; 336: 491-5.
6. NACHMAN PH, HOGAN SL, JENNETTE JC *et al*.: Treatment response and relapse in anti-neutrophil cytoplasmic autoantibody-associated microscopic polyangiitis and glomerulonephritis. *J Am Soc Nephrol* 1996; 7: 33-9.
7. SEO P, MIN YI, HOLBROOK JT *et al*.: WGET Research Group. Damage caused by Wegener's granulomatosis and its treatment: prospective data from the Wegener's Granulomatosis Etanercept Trial (WGET). *Arthritis Rheum* 2005; 52: 2168-78.

8. SAVAGE CO, WINEARLS CG, EVANS DJ *et al*.: Microscopic polyarteritis: presentation, pathology and prognosis. *Q J Med* 1985; 56: 467-83.
9. REINHOLD-KELLER E: [How long should cyclophosphamide therapy of Wegener's granulomatosis continue?]. *Dtsch Med Wochenschr* 2002; 127 (37).
10. BACON PA: The spectrum of Wegener's granulomatosis and disease relapse. *N Engl J Med* 2005; 352: 330-2.
11. HOGAN SL, FALK RJ, CHIN H *et al*.: Predictors of relapse and treatment resistance in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis. *Ann Intern Med* 2005; 143: 621-31.
12. FAURSCHOU M, SORENSEN IJ, MELLEMKJAER L *et al*.: Malignancies in Wegener's granulomatosis: incidence and relation to cyclophosphamide therapy in a cohort of 293 patients. *J Rheumatol* 2008; 35: 100-5.
13. TALAR-WILLIAMS C, HIJAZI YM, WALTHER MM *et al*.: Cyclophosphamide-induced cystitis and bladder cancer in patients with Wegener granulomatosis. *Ann Intern Med* 1996; 124: 477-84.
14. SPECKS U, FERVENZA FC, MCDONALD TJ *et al*.: Response of Wegener's granulomatosis to anti-CD20 chimeric monoclonal antibody therapy. *Arthritis Rheum* 2001; 44: 2836-40.
15. POPA ER, STEGEMAN CA, BOS NA *et al*.: Differential B- and T-cell activation in Wegener's granulomatosis. *J Allergy Clin Immunol* 199; 103: 885-94.
16. STEVENSON HC, FAUCI AS: Activation of human B lymphocytes. XII. Differential effects of *in vitro* cyclophosphamide on human lymphocyte subpopulations involved in B-cell activation. *Immunology* 1980; 39: 391-7.
17. CUPPS TR, EDGAR LC, FAUCI AS: Suppression of human B lymphocyte function by cyclophosphamide. *J Immunol* 1982; 128: 2453-7.
18. ZAJA F, RUSSO D, FUGA G *et al*.: Rituximab for myasthenia gravis developing after bone marrow transplant. *Neurology* 2000; 55: 1062-3.
19. RATANATHARATHORN V, CARSON E, REYNOLDS C *et al*.: Anti-CD20 chimeric monoclonal antibody treatment of refractory immune-mediated thrombocytopenia in a patient with chronic graft-versus-host disease. *Ann Intern Med* 2000; 133: 275-9.
20. JENNETTE JC, FALK RJ: B cell-mediated pathogenesis of ANCA-mediated vasculitis. *Semin Immunopathol* 2014; 36: 327-38.
21. KALLENBERG CG, STEGEMAN CA, ABDULAHAD WH *et al*.: Pathogenesis of ANCA-associated vasculitis: new possibilities for intervention. *Am J Kidney Dis* 2013; 62: 1176-87.
22. FALK RJ, TERRELL RS, CHARLES LA *et al*.: Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci USA* 1990; 87: 4115-9.
23. XIAO H, HEERINGA P, HU P *et al*.: Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002; 110: 955-63.
24. FALK RJ, JENNETTE JC: ANCA are pathogenic--oh yes they are! *J Am Soc Nephrol* 2002; 13: 1977-9.
25. JENNETTE JC, FALK RJ: New insights into the pathogenesis of vasculitis associated with antineutrophil cytoplasmic autoantibodies. *Curr Opin Rheumatol* 2008; 20: 55-60.
26. KLEMMER PJ, CHALERMSKULRAT W, REIF MS *et al*.: Plasmapheresis therapy for diffuse alveolar hemorrhage in patients with small-vessel vasculitis. *Am J Kidney Dis* 2003; 42: 1149-53.
27. JAYNE DR, GASKIN G, RASMUSSEN N *et al*.; EUROPEAN VASCULITIS STUDY GROUP: Randomized trial of plasma exchange or high-dosage methylprednisolone as adjunctive therapy for severe renal vasculitis. *J Am Soc Nephrol* 2007; 18: 2180-8.
28. STONE JH, MERKEL PA, SPIERA R *et al*.: RAVE-ITN RESEARCH GROUP: Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N Engl J Med* 2010; 363: 221-32.
29. JONES RB, TERVAERT JW, HAUSER T *et al*.; EUROPEAN VASCULITIS STUDY GROUP: Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N Engl J Med* 2010; 363: 211-20.
30. SPECKS U, MERKEL PA, SEO P *et al*.; RAVE-ITN RESEARCH GROUP: Efficacy of remission-induction regimens for ANCA-associated vasculitis. *N Engl J Med* 2013; 369: 417-27.
31. GUILLEVIN L, PAGNOUX C, KARRAS A *et al*.; FRENCH VASCULITIS STUDY GROUP: Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N Engl J Med* 2014; 371: 1771-80.
32. JENNETTE JC, WILKMAN AS, FALK RJ: Anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and vasculitis. *Am J Pathol* 1989; 135: 921-30.
33. MCGREGOR JG, HOGAN SL, KOTZEN ES *et al*.: Rituximab as an immunosuppressant in antineutrophil cytoplasmic antibody-associated vasculitis. *Nephrol Dial Transplant* 2015; 30 (Suppl. 1): i123-31.
34. MANSFIELD N, HAMOUR S, HABIB AM *et al*.: Prolonged disease-free remission following rituximab and low-dose cyclophosphamide therapy for renal ANCA-associated vasculitis. *Nephrol Dial Transplant* 2011; 26: 3280-6.
35. HAGEN EC, DAHA MR, HERMANS J *et al*.: Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. EC/BCR Project for ANCA Assay Standardization. *Kidney Int* 1998; 53: 743-53.
36. MUKHTYAR C, FLOSSMANN O, HELLMICH B *et al*.; EUROPEAN VASCULITIS STUDY GROUP (EUVAS): Outcomes from studies of antineutrophil cytoplasm antibody associated vasculitis: a systematic review by the European League Against Rheumatism systemic vasculitis task force. *Ann Rheum Dis* 2008; 67: 1004-10.
37. LEVEY AS, GREENE T, BECK GJ *et al*.: Dietary protein restriction and the progression of chronic renal disease: what have all of the results of the MDRD study shown? Modification of Diet in Renal Disease Study group. *J Am Soc Nephrol* 1999; 10: 2426-39.
38. LUQMANI RA, BACON PA, MOOTS RJ *et al*.: Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. *QJM* 1994; 87: 671-8.
39. JENNETTE JC, FALK RJ, BACON PA *et al*.: 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum* 2013; 65: 1-11.
40. STEGEMAN CA, TERVAERT JW, DE JONG PE *et al*.: Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med* 1996; 335: 16-20.
41. BERDEN AE, FERRARIO F, HAGEN EC *et al*.: Histopathologic classification of ANCA-associated glomerulonephritis. *J Am Soc Nephrol* 2010; 21: 1628-36.
42. KRISTENSEN T, GREGERSEN JW, KRAG SR *et al*.: The relation between histopathological classification and renal outcome, ANCA subtype and treatment regimens in ANCA-associated vasculitis. *Clin Exp Rheumatol* 2016; 34 (Suppl. 97): 105-10.
43. JONES RB, FURUTA S, TERVAERT JW *et al*.: European Vasculitis Society (EUVAS). Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis: 2-year results of a randomised trial. *Ann Rheum Dis* 2015; 74: 1178-82.
44. ELEFANTE E, TRIPOLI A, FERRO F *et al*.: One year in review: systemic vasculitis. *Clin Exp Rheumatol* 2016; 34 (Suppl. 97): S1-6.
45. MORGAN MD, DAY CJ, PIPER KP *et al*.: Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology* 2010; 130: 64-73.
46. RIMBERT M, HAMIDOU M, BRAUDEAU C *et al*.: Decreased numbers of blood dendritic cells and defective function of regulatory T cells in antineutrophil cytoplasmic antibody-associated vasculitis. *PLoS One* 2011; 6: e18734.
47. FREE ME, BUNCH DO, MCGREGOR JA *et al*.: Patients with antineutrophil cytoplasmic antibody-associated vasculitis have defective Treg cell function exacerbated by the presence of a suppression-resistant effector cell population. *Arthritis Rheum* 2013; 65: 1922-33.
48. UNIZONY S, LIM N, PHIPPARD DJ *et al*.: Peripheral $CD5^+$ B cells in antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheumatol* 2015; 67: 535-44.
49. BUNCH DO, MCGREGOR JG, KHANDOOBHAI NB,*et al*.: Decreased $CD5^+$ B cells in active ANCA vasculitis and relapse after rituximab. *Clin J Am Soc Nephrol* 2013; 8: 382-91.
50. BUNCH DO, MENDOZA CE, AYBAR LT *et al*.: Gleaning relapse risk from B cell phenotype: decreased $CD5^+$ B cells portend a shorter time to relapse after B cell depletion in patients with ANCA-associated vasculitis. *Ann Rheum Dis* 2015; 74: 1784-6.
51. LYONS PA, RAYNER TF, TRIVEDI S *et al*.: Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med* 2012; 367: 214-23.
52. LIONAKI S, BLYTH ER, HOGAN SL *et al*.: Classification of antineutrophil cytoplasmic autoantibody vasculitides:the role of antineutrophil cytoplasmic autoantibody speci-

ficity for myeloperoxidase or proteinase 3 in disease recognition and prognosis. *Arthritis Rheum* 2012; 64: 3452-62.
53. BOOTH AD, ALMOND MK, BURNS A *et al*.; PAN-THAMES RENAL RESEARCH GROUP: Outcome of ANCA-associated renal vasculitis: a 5-year retrospective study. *Am J Kidney Dis* 2003; 41: 776-84.
54. DE GROOT K, HARPER L, JAYNE DR *et al*.: Pulse versus daily oral cyclophosphamide for induction of remission in antineutrophil cytoplasmic antibody-associated vasculitis: a randomized trial. *Ann Intern Med* 2009; 150: 670-80.
55. GUILLEVIN L, CORDIER JF, LHOTE F *et al*.: A prospective, multicenter, randomized trial comparing steroids and pulse cyclophosphamide versus steroids and oral cyclophosphamide in the treatment of generalized Wegener's granulomatosis. *Arthritis Rheum* 1997; 40: 2187-98.
56. GODEAU B, MAINARDI JL, ROUDOT-THORAVAL F *et al*.: Factors associated with Pneumocystis carinii pneumonia in Wegener's granulomatosis. *Ann Rheum Dis* 1995; 54: 991-4.
57. PUGNET G, PAGNOUX C, TERRIER B *et al*.: Rituximab versus azathioprine for ANCA-associated vasculitis maintenance therapy: impact on global disability and health-related quality of life. *Clin Exp Rheumatol* 2016; 34 (Suppl. 97): 54-9.
58. JONES RB, TERVAERT JW, HAUSER T *et al*.; EUROPEAN VASCULITIS STUDY GROUP: Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N Engl J Med* 2010; 363: 211-20.
59. LITTLE MA, NIGHTINGALE P, VERBURGH CA *et al*.; EUROPEAN VASCULITIS STUDY GROUP (EUVAS): Early mortality in systemicvasculitis: relative contribution of adverse events and active vasculitis. *Ann Rheum Dis* 2010; 69: 1036-43.
60. CHAIGNE B, GUILLEVIN L: Unsolved questions and concerns about treatment of antineutrophil cytoplasm antibody-associated vasculitides. *Clin Exp Rheumatol* 2016; 34 (Suppl. 97): S121-8.