Disease-activity in ANCA-associated vasculitis

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Prediction of relapses in PR3-ANCA-associated vasculitis by assessing responses of ANCA titers to treatment

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ABSTRACT

*Objective:*We performed a retrospective evaluation of whether C-ANCA titers (indirect immunofluorescence) and PR3-ANCA levels (ELISA) at diagnosis and following immunosuppressive treatment are predictive for relapse of ANCA-associated vasculitis.

Methods: Patients diagnosed with PR3-ANCA associated vasculitis between 1991 and 2002, with at least 2 years of follow-up, and treated with cyclophosphamide only (1991-1996) or switched to azathioprine after induction of remission with cyclophosphamide (1997-2002) were included in this retrospective study. ANCA were assessed by IIF and direct PR3-specific ELISA at diagnosis, and at 3, 6, 12, 18, and 24 months after diagnosis. Actuarial relapse-free survival was analysed by logrank test.

Results: We studied 87 patients positive for PR3-ANCA: 46 on cyclophosphamide maintenance, 41 switched to azathioprine. Overall actuarial relapse-free survival was 72% at 2 years and 34% at 5 years. Survival did not differ between patients on cyclophosphamide maintenance and patients switched to azathioprine maintenance (p=0.34). Patients who became and stayed negative for C-ANCA (IIF) or PR3-ANCA (ELISA) until 24 months after diagnosis had a lower risk to experience relapse (p=0.01 and p=0.02, respectively). Positive C-ANCA (IIF) titers at 3 (RR 2.0; 95% CI 1.2-3.8), 12 (RR 1.9; 95% CI 1.1-3.3), 18 (RR 2.9; 95%CI 1.3-4.6), and 24 months (RR 2.6; 95% CI 1.2-5.0) were significantly associated with relapse within 5 years after diagnosis. Also, PR3-ANCA levels >10 U/ml at 18 (RR 2.7, 95% CI 1.1-4.3) and 24 months (RR 4.6; 95% CI 1.2-6.3) were predictive for relapse within 5 years. In the azathioprine group a positive C-ANCA titer at switch to azathioprine (RR 2.2; 95% CI 1.0-5.4) was associated with relapse.

Conclusion: Positive C-ANCA (IIF) and PR3-ANCA (ELISA) titers during early follow-up identify patients at increased risk for relapse.

INTRODUCTION

Small vessel vasculitides predominantly affecting the respiratory tract and kidneys and associated with the presence of antineutrophil cytoplasmic autoantibodies (ANCA) can nowadays successfully be treated resulting in a 10-year survival of 60-90% [1-3]. Following induction of remission by immunosuppressive treatment a substantial number of patients experience relapses of disease, however. Relapses are associated with considerable morbidity and mortality. Therefore, identification of subgroups of patients with ANCA-associated vasculitis at increased risk of relapse and the subsequent adjustment of treatment are of major importance.

ANCA-specificity is an apparent risk factor for relapse, as anti-proteinase 3-ANCA (PR3-ANCA)-positive patients are more prone to experience relapse than anti-myeloperoxidase-ANCA (MPO-ANCA)-positive patients, with a relative relapse risk of around 3.7 [4, 5]. Traditionally, ANCA are detected by indirect immunofluorescence (IIF) on ethanol fixed granulocytes. A cytoplasmic-ANCA (C-ANCA) staining pattern by IIF is strongly associated with antibodies against PR3. Recently, we showed that a positive C-ANCA-titer in patients with PR3-ANCA associated vasculitis at the time of switch to azathioprine, after induction of stable remission for at least 3 months by cyclophosphamide, is associated with an increased risk for relapse [6]. As many relapses occur during tapering or shortly after stopping therapy, we hypothesize that persistent or reappearing ANCA titers following induction of remission and during tapering of immunosuppressive therapy may identify a subgroup of patients at increased risk for relapse. In order to test this hypothesis, we retrospectively analyzed serial C-ANCA titers (IIF) and PR3-ANCA levels (ELISA) until 24 months after diagnosis in patients with PR3-ANCA-associated vasculitis in relation to the occurrence of relapses during long-term follow-up. The results show that positive ANCA-tests during follow-up identify patients at increased risk for relapse.

PATIENTS AND METHODS

Patients and treatment

Eighty-seven patients diagnosed with PR3-ANCA-associated vasculitis who received induction treatment with cyclophosphamide and were followed for at least two years, were included in this retrospective study. Charts were reviewed from all patients diagnosed with PR3-ANCA associated vasculitis between January 1991 and March 2002. During this period

122 patients were newly diagnosed with PR3-ANCA-associated vasculitis at our center. 35 patients could not be included: 18 patients with less than 2 years of follow-up (10 died, 8 were transferred to another center), 16 patients receiving a different treatment regimen, and one patient because of missing samples. Charts of the included patients were reviewed for relapse up to 5 years after diagnosis (n=65) and for those diagnosed after March 1999 up to March 2004 (n=22). Out of 87 patients, 68 had histologically proven granulomatous disease and consequently fulfilled the Chapel Hill consensus criteria for Wegener's granulomatosis. Of the other 19 patients, three were classified as having necrotizing crescentic glomerulonephritis and 16 as having microscopic polyangiitis.

Induction treatment consisted of oral cyclophosphamide (2 mg/kg) and prednisolone (1 mg/kg; maximum dose 60 mg) daily. Doses of cyclophosphamide were adjusted to maintain the white blood cell count above 4×10^9/l. After 4 to 6 weeks, the daily prednisolone dose was tapered by 10 mg every 2 weeks until the dose reached 30 mg, and thereafter by 5 mg every 2 to 4 weeks. Once remission was achieved the daily dose of oral cyclophosphamide was tapered by 25 mg every 3 months. Patients were seen and evaluated for signs and symptoms of active disease at our outpatient clinic at least once a month during the first year and once every three months thereafter. From 1997 on, patients were increasingly switched to azathioprine (1.5-2 mg/kg daily), following three months of stable remission, with tapering of azathioprine by 25 mg every 3 months. Cumulative dosages of cyclophosphamide, corticosteroids and azathioprine were calculated and corrected for weight. Additionally, cumulative dosages of cyclophosphamide at 3, 6, and 12 months after diagnosis were calculated.

At diagnosis, at 3, 6, 12, 18, and 24 months, and at relapse, serum ANCA titers were determined by indirect immunofluorescence and EDTA-plasma was collected for testing ANCA levels by direct PR3-ANCA ELISA. Plasma samples were spun at 3000 rpm for 10 minutes, and supernatants were stored at -80°C until use. At all time points, disease activity was scored using the Birmingham Vasculitis Activity Score (BVAS) [7].

Definitions

Remission was defined as the absence of clinical signs and symptoms of active vasculitis (BVAS=0) in combination with a normal C-reactive protein level (<3 mg/l). A relapse was defined as clinical signs of vasculitic activity in combination with biopsy proven active vasculitis, or the occurrence of nodular pulmonary lesions after exclusion of infectious or malignant diseases. Renal vasculitic disease was defined as biopsy proven necrotizing glomerulonephritis or a combination of microscopic glomerular erythrocyturia, erythrocyte cell casts, proteinuria, and a decrease in creatinine clearance [8].

Detection of C-ANCA by IIF

C-ANCA titers during follow-up were collected from the patient records. C-ANCA had been detected by indirect immunofluorescence (IIF) as described previously [9]. ANCA titers had been analysed by serial dilution of sera starting at 1:20. When a cytoplamic staining pattern (C-ANCA) was present in at least a 1:40 dilution, sera were considered positive for C-ANCA. A perinuclear or atypical staining pattern was considered negative for C-ANCA.

Detection of PR3-ANCA

In all patients, the presence of PR3-ANCA at diagnosis was confirmed by antigen specific capture enzyme-linked immunosorbent assay (ELISA) [10]. PR3-ANCA levels in all stored plasma samples were quantitatively assessed by direct PR3-ANCA ELISA, as described previously with minor modifications [11]. Plasma samples from individual patients were tested within one assay to prevent inter-assay variation. In short, Nunc Maxisorp 96-well microtiter plates were coated at 37°C for 1.5 hours with PR3 (1 μg/ml) in coating buffer (0.1 M sodium carbonate, pH 9.6) which was inactivated by 5 mM phenylmethylsulfonyl fluoride. Blocking buffer (0.1 M Tris HCl, 0.3 M NaCl, 1% bovine serum albumin (BSA)) was subsequently added and left for 1 hour. Plasma samples were diluted in incubation buffer (0.1 M Tris HCl, 0.3 M NaCl, 1% BSA, 0.05% Tween 20 (Sigma)) and subsequently applied at a dilution of 1:100 and 1:300. A standard curve was made using a reference plasma sample that was included in each test. Values were expressed as arbitrary units/ml. Values of ≥10 units/ml (mean + 3 SD of 65 normal controls) were considered positive.

Statistical analysis

For comparison of paired data the Wilcoxon signed rank test was used, for unpaired data the Mann-Whitney U-test was used. Proportions between groups were compared with the χ^2 test. For multiple comparisons the Kruskal-Wallis analysis of variance was used, followed by Dunn's test to determine differences between groups. Correlation coefficients were calculated by Spearman's test. Relapse-free survival curves were calculated using Kaplan-Meier estimates for survival distribution. Differences in actuarial relapse-free survival between groups were analysed by log rank test with relapse free survival as dependent variable. The endpoint for survival analysis was the occurrence of relapse. Patients without relapse were censored at five years after diagnosis, March 2004 or at death, whichever came first. Positive and negative predictive values of ANCA-positivity to experience relapse were calculated. Analyses were performed with GraphPad Prism version 3.00 (GraphPad Software Inc., San Diego, CA). A two-sided p-value <0.05 was considered to indicate statistical significance.

RESULTS

Clinical characteristics

In Table 1 clinical characteristics at diagnosis are given for the included patients. Apart from age (median age in the group on cyclophosphamide maintenance 58 years versus 47 years in the group on azathioprine maintenance, p=0.003), there were no significant differences in clinical characteristics at diagnosis between patients receiving cyclophosphamide maintenance treatment and patients switched to azathioprine maintenance. Patients on cyclophosphamide maintenance received a mean dosage of 0.46 ± 0.21 gram/kg cyclophosphamide during 16 ± 6 months; patients in the azathioprine group first received a mean dosage of 0.23 ± 0.13 gram/kg cyclophosphamide during 6±4 months, and subsequently 0.51 ± 0.32 gram/kg azathioprine during 14 ± 9 months.

Table 1. Clinical characteristics at diagnosis

	All patients (n=87)
At diagnosis	
male/female (n (%))	55 (63%)/32 (37%)
age (years)#	55 (14-80)
C-ANCA titer (IIF)#	1:320
CRP (mg/l)*	126.3 ± 96.7
Creatinine (μmol/l)#	117 (40-918)
BVAS#	25 (7-48)
Organ Involvement	
Ear, nose and throat: n (%)	77 (89%)
Lung: n (%)	46 (53%)
Kidney: n (%)	67 (77%)

* mean ± SD, # median (range)

In total, 52 patients (60%) experienced one or more relapses of disease within 60 months; 26 patients (63%) in the group switched to azathioprine (n=41) and 26 patients (57%) in the cyclophosphamide group (n=46). Clinical characteristics at the time of relapse are shown in table 2. Clinical characteristics at relapse did not differ between patients on cyclophosphamide maintenance and patients switched to azathioprine maintenance treatment. Four patients died within 5 years of follow-up, all after having reached stable remission, and experienced one or more relapses of disease. None of these patients died due

to disease exacerbation. Overall actuarial relapse free survival was 72% at 24 months and 34% at 60 months after diagnosis. Relapse-free survival did not differ between patients on cyclophosphamide maintenance and those switched to azathioprine (relative risk (RR) 1.4; 95% confidence interval (CI) 0.8-2.0, p=0.34).

Table 2. Clinical Characteristics at relapse

	Patients experiencing relapse (n=52)
At relapse	
C-ANCA titer (IIF)#	1:320
CRP (mg/l)*	50.8 ± 53.0
Creatinine (µmol/l)#	118 (71-1243)
BVAS#	12.5 (3-24)
Organ Involvement	
Ear, nose and throat: n (%)	34 (65%)
Lung: n (%)	5 (10%)
Kidney: n (%)	33 (63%)

* mean ± SD, # median (range)

Relapse-free survival did also not differ between patients diagnosed as having Wegener's granulomatosis (n=68) and those diagnosed as having microscopic polyangiitis (n=16). Clinical characteristics at diagnosis did not differ between patients experiencing a relapse (n=52) and those not experiencing a relapse (n=35).

Serial C-ANCA measurement by IIF

At diagnosis, all but 5 patients were positive for C-ANCA. These 5 patients showed either a perinuclear (n=1) or an atypical staining pattern (n=4). All patients included were positive for PR3-ANCA by capture ELISA. C-ANCA titers at diagnosis and at relapse were significantly higher than at 3, 6, 12, 18, and 24 months after diagnosis (p<0.001). Sixty-nine out of the 87 included patients became at least once negative for C-ANCA during follow-up whereas 18 patients were persistently positive for C-ANCA. At 3 months, 51 patients (59%) were already negative for C-ANCA increasing to 57 patients (66%) at six months after diagnosis (Figure 1). Only 16 out of the 57 patients (28%) who were negative for C-ANCA at 6 months after diagnosis, stayed negative for C-ANCA until 24 months after diagnosis. At 12, 18, and 24 months after diagnosis an increasing number of patients became again positive for C-ANCA (Figure 1), and C-ANCA titers at these time points were significantly higher than at 3 and

6 months after diagnosis (C-ANCA titers at 12 months versus 3 months, p = 0.024; at all other time points, p < 0.001). Patients switched to azathioprine did not differ from those on cyclophosphamide maintenance treatment with respect to being positive for C-ANCA at any time point. In addition, we found no correlation between cumulative cyclophosphamide dosages at 3, 6 and 12 months after diagnosis on the one hand and ANCA-titers, changes in titers or occurrence of relapse at the other hand.

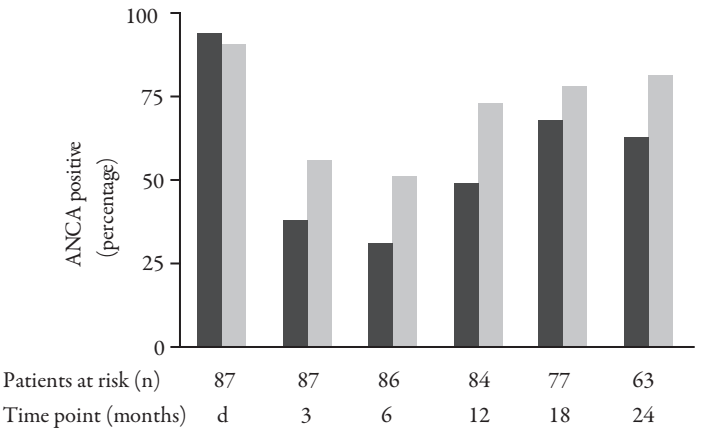

Figure 1. Percentage of patients who were positive for C-ANCA by IIF (≥1:40) (solid squares) or positive for PR3-ANCA by direct ELISA (≥10 U/ml) (grey squares) at diagnosis (d) and during follow-up 3, 6, 12, 18 and 24 months after diagnosis. At diagnosis all patients (n=87) were positive by capture PR3-ANCA ELISA. Patients who experienced a relapse (n=24) during 24 months follow-up withdrew from the study group.

C-ANCA titers and risk of relapse

Compared to patients who stayed positive for C-ANCA from 6 months after diagnosis onwards (n=22) or became positive for C-ANCA again within 24 months (n=41), the 16 patients who were negative for C-ANCA at 6 months after diagnosis and stayed negative for C-ANCA had a significantly reduced risk for relapse (p=0.012) (Figure 2). Only four of these 16 patients (25%) experienced a relapse within 60 months after diagnosis. In contrast, 14 out of the 22 patients (64%) who were positive for C-ANCA from 6 months after diagnosis onwards, experienced a relapse during follow-up (median time to relapse, 20 months). Twenty-nine out of the 41 (71%) patients who were negative for C-ANCA 6 months after diagnosis and became positive for C-ANCA during follow-up, experienced

a relapse (median time to relapse:, 28 months). The time point of becoming positive for C-ANCA, either at 6, 12, 18, or 24 months, did not influence relapse rate.

Positive C-ANCA titers 3, 12, 18, and 24 months after diagnosis were associated with a significantly increased risk for relapse (Figure 3a). A positive C-ANCA titer at switch to azathioprine was significantly associated with increased risk for relapse (RR 2.2; 95% CI 1.0-5.6).

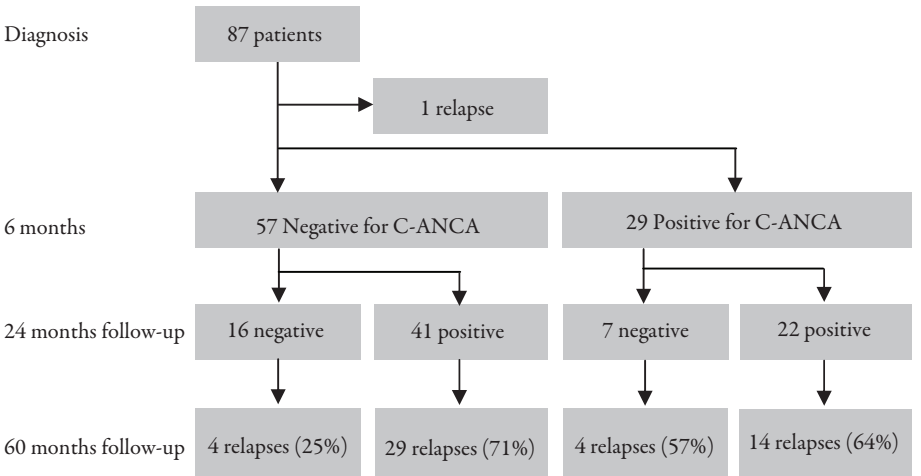

Figure 2. Relapse-rate within 60 months according to C-ANCA status at 6 months after diagnosis and during 24 months follow-up in the respective groups.

PR3-ANCA

At diagnosis PR3-ANCA levels as measured by direct ELISA were less than 10 units/ml in 9 patients. One of these patients had an atypical ANCA staining pattern by IIF; the others all had strong cytoplasmic staining patterns. By definition, all were positive by PR3-capture ELISA. Like C-ANCA titers, PR3-ANCA levels were significantly higher at diagnosis than at 3, 6, and 12 months after diagnosis. In total, 52 patients became negative for PR3-ANCA at some moment during 24 months of follow-up. At 3 months after diagnosis, 38 patients were negative for PR3-ANCA, and at 6 months after diagnosis 42 patients were negative for PR3-ANCA (Figure 1). An increasing number of patients was positive for PR3-ANCA at 12, 18, and 24 months after diagnosis (Figure 1). PR3-ANCA levels were significantly higher at these time points than 6 months after diagnosis ($p<0.001$). Cumulative cyclophosphamide

dosages 3, 6, and 12 months after diagnosis did not correlate with PR3-ANCA-titers and changes in titers.

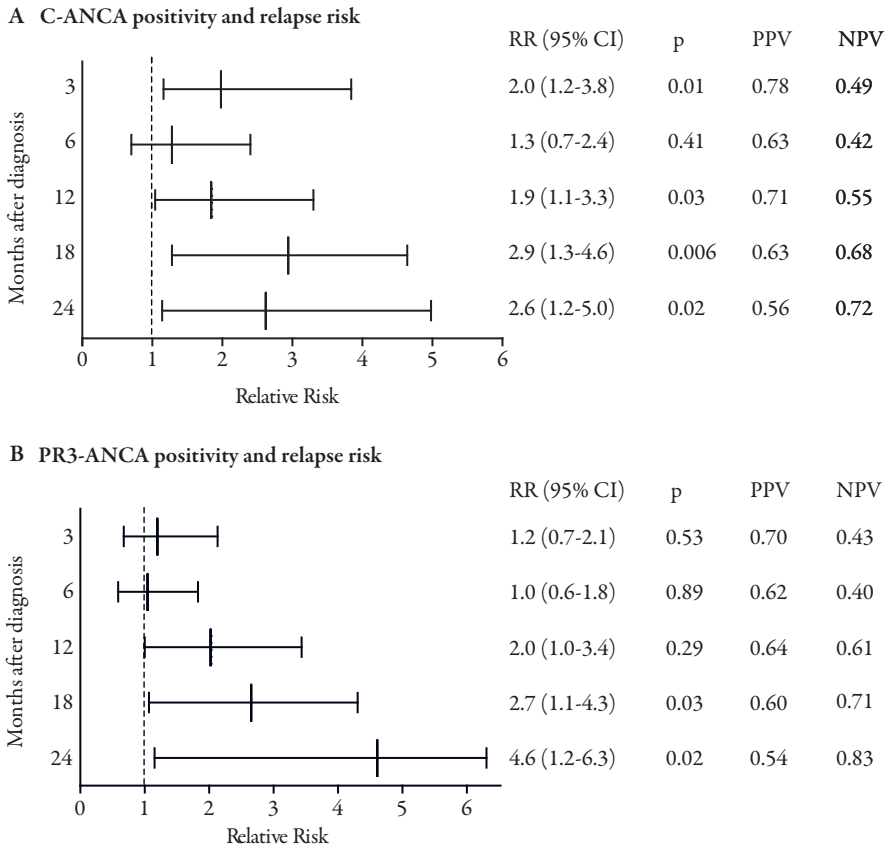

Figure 3. (**A**) Relative risk of relapse of patients positive for C-ANCA at 3, 6, 12, 18, and 24 months after diagnosis compared with patients negative for C-ANCA at these time points. (**B**) Relative risk of relapse of patients positive for PR3-ANCA at 3, 6, 12, 18, and 24 months as compared with patients negative for PR3-ANCA at these time points. Lines indicate relative risk and 95% confidence interval. Relative risk (95% confidence interval). *P*, p-value; PPV, positive predictive value; NPV, negative predictive value.

PR3-ANCA levels and risk of relapse

Patients who were negative for PR3-ANCA 6 months after diagnosis and stayed negative for PR3-ANCA until 24 months after diagnosis (n=7) did not experience a relapse during

follow-up (p=0.021 compared with the remaining patients). Twenty-one of the 35 patients (60%) who stayed positive for PR3-ANCA during 24 months of follow-up, experienced a relapse compared to 32 (71%) of 45 patients who became positive for PR3-ANCA again within 24 months (p=0.38).

Patients who became positive for PR3-ANCA again 6 or 12 months after diagnosis (n=27) relapsed earlier (median time to relapse 27 months) than patients who became positive for PR3-ANCA at 18 or 24 months after diagnosis (n=18; median time to relapse 40 months) (p=0.020).

At 3, 6, and 12 months after diagnosis PR3-ANCA positive patients did not have a significantly increased risk to experience a relapse (Figure 3b). However, patients positive for PR3-ANCA 18 months and 24 months after diagnosis had a significantly increased risk to experience relapse (Figure 3b).

DISCUSSION

Most patients with ANCA-asssociated vasculitis can be brought into remission with current immunosuppressive induction therapy. Following induction of remission immunosuppressive therapy is tapered with reductions in corticosteroid and cyclophosphamide dose, or cyclophosphamide is switched to azathioprine to reduce treatment related toxicity. This latter approach leads to rates of relapse-free survival during short and medium-term follow-up of more than 85% at 18 months [12]. With longer follow-up, however, disease relapses are frequent and occur in 50% or more of patients at least once within 5 years, especially in patients with ANCA with specificity for proteinase 3 [2, 12, 13]. The data of the current study indicate that the course of C-ANCA- (IIF) and PR3-ANCA titers during follow-up contributes to identify patients at increased risk for relapse.

In our group of 87 patients with PR3-ANCA-associated vasculitis 48% became negative for PR3-ANCA and 66% negative for C-ANCA (IIF) within 6 months following start of treatment. In particular, the limited number of patients who were C-ANCA/PR3-ANCA negative at 18 and 24 months after diagnosis had a strongly reduced risk to experience relapse. Apparently, immunosuppressive therapy can be tapered safely in these patients. However, other studies with a limited follow-up and including both PR3- and MPO-ANCA positive patients have also found a positive relation between disappearance of C-ANCA during the first year after diagnosis and subsequent relapse-free survival [4, 14-17]. In our study, the majority of patients who initially became negative for C-ANCA and/or

PR3-ANCA, gradually became ANCA positive during the second year of follow-up when immunosuppressive therapy was tapered and stopped. Seventy-three per cent of patients from this latter group relapsed within 60 months, comparable to the group of patients who were persistently positive for C-ANCA or PR3-ANCA. In addition, patients who became positive for PR3-ANCA again within the first year after diagnosis had a significantly shorter time to relapse than patients who became positive for PR3-ANCA at a later time point during follow-up.

It is not clear how ANCA influence the risk of relapse. Changes in levels of ANCA are frequently but not always related to the disease course. Binding of ANCA to their respective surface antigens on primed leukocytes *in vitro* leads to activation and degranulation of these cells, and subsequent vascular damage. These data imply that qualitative differences between ANCA such as epitope specificity and immunoglobulin (sub)class characteristics, are also relevant. This might explain divergence between quantitative ANCA titers and clinical outcome. However, patients who remain positive for C-ANCA might also have an ongoing smouldering immune activation. The latter hypothesis requires further investigation and might be addressed by serially studying markers of immune activation, ANCA titers and clinical status.

As most relapses occur during tapering or shortly after stopping immunosuppression, extension of treatment in high-risk patients might prevent relapses [17]. Unfortunately, in some patients the time interval between becoming positive for C-ANCA and the occurrence of relapse was rather long, making it difficult to individualize treatment. Long-term immunosuppression is currently tested in an EUVAS study in which long-term (4 years) low-dose immunosuppressive therapy is compared with standard treatment with azathioprine maintenance for 18 months [18]. Although this study specifically aims at patients in whom an additional renal relapse would severely threaten renal survival and does not incorporate ANCA status, its outcome might indicate to what extent this strategy can be extrapolated to other subgroups. Obviously, the adverse effects of extended immunosuppressive therapy should outweigh the damage due to increased relapse rate.

As our study is retrospective and we studied relapse risks in patients on sequential treatment regimens, a prospective study is needed to confirm our data. Ideally, such a study should be combined with a clinical trial, stratifying treatment-groups according to C-ANCA status.

In conclusion, ANCA positivity at several time points during early follow-up identifies patients with PR3-ANCA-associated vasculitis at increased risk of experiencing relapse. ANCA-guided adjustment of immunosuppressive regimens may lead to a reduction in relapses and should be explored in prospective, randomized, controlled trials.

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