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

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Objective. We performed a retrospective evaluation of whether c-ANCA titres (indirect immunofluorescence) and anti-proteinase 3 (PR3)-ANCA levels (ELISA) at diagnosis and following immunosuppressive treatment are predictive of relapse of ANCA-associated vasculitis.

Methods. Patients diagnosed with PR3-ANCA-associated vasculitis between 1991 and 2002, with at least 2 yr of follow-up, and treated with cyclophosphamide and corticosteroids only (1991–1996) or switched to azathioprine after induction of remission with cyclophosphamide and corticosteroids (1997–2002) were included. ANCA were assessed by immunofluorescence and direct PR3-specific ELISA at diagnosis and 3, 6, 12, 18 and 24 months after diagnosis. Actuarial relapse-free survival was analysed with the log rank test.

Results. We studied 87 patients positive for PR3-ANCA: 46 were on cyclophosphamide maintenance therapy and 41 switched to azathioprine. Overall actuarial relapse-free survival was 72% at 2 yr and 34% at 5 yr. Relapse-free 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 (immunofluorescence) or PR3-ANCA (ELISA) until 24 months after diagnosis had a lower risk of relapse ($P = 0.01$ and $P = 0.02$, respectively). Positive c-ANCA (immunofluorescence) titres at 3 [relative risk (RR) 2.0; 95% confidence interval (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 yr after diagnosis. 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 of relapse within 5 yr. In the azathioprine group, a positive c-ANCA titre at the time of switching to azathioprine (RR 2.2; 95% CI 1.0–5.4) was associated with relapse.

Conclusion. Positive c-ANCA (immunofluorescence) and PR3-ANCA (ELISA) titres during early follow-up identify patients at increased risk of relapse.

KEY WORDS: ANCA-associated vasculitis, Relapse, Treatment switch, Follow-up, PR3-ANCA.

Small vessel vasculitides predominantly affecting the respiratory tract and kidneys, and associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCA) can nowadays be treated successfully, resulting in a 10-yr 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 experiencing 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 titre in patients with PR3-ANCA-associated vasculitis at the time of switching to azathioprine, after induction of stable remission for at least 3 months by

cyclophosphamide, is associated with an increased risk of relapse [6]. As many relapses occur during tapering or shortly after stopping therapy, we hypothesize that persistent or reappearing ANCA titres following induction of remission and during tapering of immunosuppressive therapy may identify a subgroup of patients at increased risk of relapse. In order to test this hypothesis, we retrospectively analysed serial c-ANCA titres (IIF) and PR3-ANCA levels [enzyme-linked immunosorbent assay (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 of relapse.

Patients and methods

Patients and treatment

Eighty-seven patients diagnosed with PR3-ANCA-associated vasculitis who received induction treatment with cyclophosphamide and corticosteroids and were followed for at least 2 yr 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 centre. Thirty-five patients could not be included: 18 patients with less than 2 yr of follow-up (10 died, eight were transferred to another centre), 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 yr 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 4–6 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 2–4 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 out-patient clinic at least once a month during the first year and once every 3 months thereafter. From 1997 on, patients were increasingly switched to azathioprine (1.5–2 mg/kg daily), following 3 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 3, 6 and 12 months after diagnosis were calculated.

At diagnosis, at 3, 6, 12, 18 and 24 months and at relapse, serum ANCA titres 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 r.p.m. for 10 min 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 titres during follow-up were collected from the patient records. c-ANCA had been detected by IIF as described previously [9]. ANCA titres had been analysed by serial dilution of sera starting at 1:20. When a cytoplasmic 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 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 microtitre plates were coated at 37°C for 1.5 h with PR3 (1 μg/ml) in coating buffer (0.1 M sodium carbonate, pH 9.6), which was inactivated by 5 mM phenylmethyl-sulphonyl fluoride. Blocking buffer [0.1 M Tris–HCl, 0.3 M NaCl, 1% bovine serum albumin (BSA)] was subsequently added and left for 1 h. 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 dilutions 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 s.D. 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, 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 the log rank test with relapse-free survival as the dependent variable. The end-point for survival analysis was the occurrence of relapse. Patients without relapse were censored 5 yr after diagnosis, in 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, San Diego, CA, USA). 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 yr vs 47 yr 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

TABLE 1. Clinical characteristics at diagnosis

	All patients ($n = 87$)
At diagnosis	
Male/female: n (%)	55 (63%)/32 (37%)
Age (yr)[a]	55 (14–80)
c-ANCA titre (IIF)[a]	1:320
CRP (mg/l)[b]	126.3 ± 96.7
Creatinine (μmol/l)[a]	117 (40–918)
BVAS[a]	25 (7–48)
Organ involvement	
Ear, nose and throat: n (%)	77 (89%)
Lung: n (%)	46 (53%)
Kidney: n (%)	67 (77%)

[a]Median (range); [b]mean ± s.D.

TABLE 2. Clinical characteristics at relapse

	Patients experiencing relapse ($n = 52$)
At relapse	
c-ANCA titre (IIF)[a]	1:320
CRP (mg/l)[b]	50.8 ± 53.0
Creatinine (μmol/l)[a]	118 (71–1243)
BVAS[a]	12.5 (3–24)
Organ involvement	
Ear, nose and throat: n (%)	34 (65%)
Lung: n (%)	5 (10%)
Kidney: n (%)	33 (63%)

[a]Median (range); [b]mean $\pm$ S.D.

cyclophosphamide maintenance received a mean dosage of 0.46 ± 0.21 g/kg cyclophosphamide during 16 ± 6 months; patients in the azathioprine group first received a mean dosage of 0.23 ± 0.13 g/kg cyclophosphamide during 6 ± 4 months, and subsequently 0.51 ± 0.32 g/kg azathioprine during 14 ± 9 months.

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 yr 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, $P = 0.34$]. 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 five patients were positive for c-ANCA. These five 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 titres 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 negative for c-ANCA at least once 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%) 6 months after diagnosis (Fig. 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 again became positive for c-ANCA (Fig. 1), and c-ANCA titres at these time points were significantly higher than at 3 and 6 months after diagnosis (c-ANCA titres at 12 months *vs* 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 3, 6 and 12 months after diagnosis on the one hand and ANCA titres, changes in titres or occurrence of relapse at the other hand.
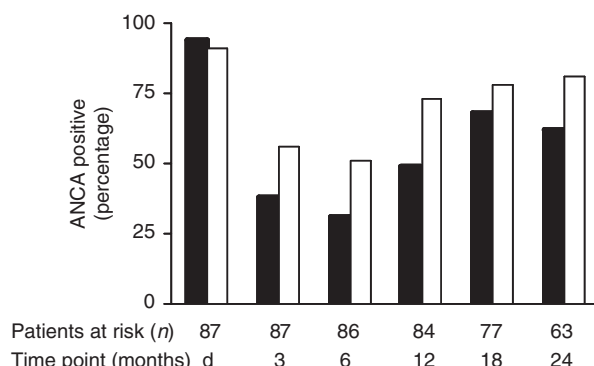

FIG. 1. Percentage of patients who were positive for c-ANCA by IIF ($\geq 1:40$) (solid squares) or positive for PR3-ANCA by direct ELISA (≥ 10 U/ml) (open squares) at diagnosis (d) and during follow-up 3, 6, 12, 18 and 24 months after diagnosis. At diagnosis all patients ($n = 87$) were positive by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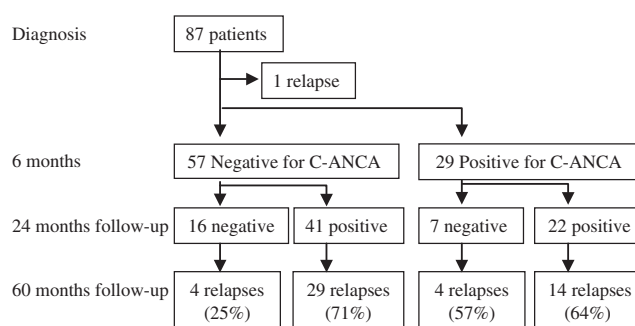

FIG. 2. Relapse rate within 60 months according to c-ANCA status at 6 months after diagnosis and during 24 months of follow-up.
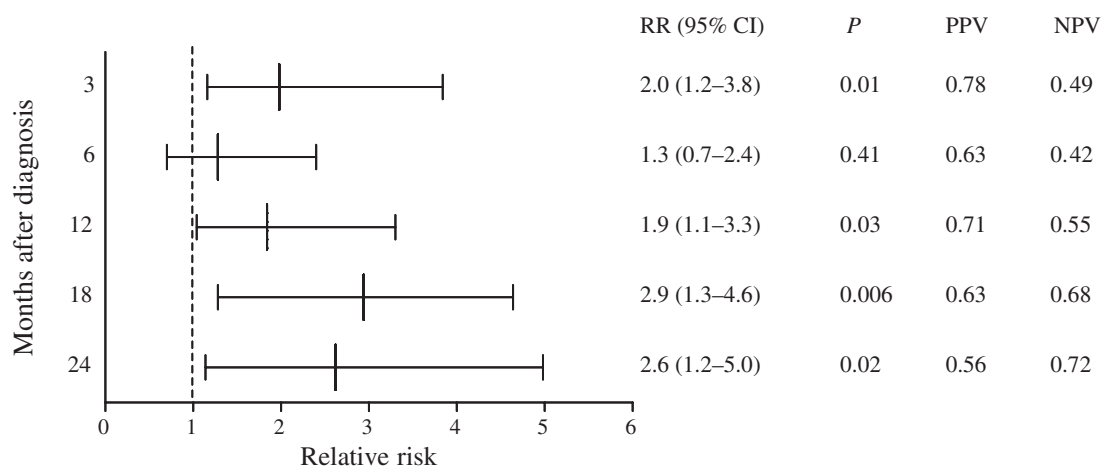
c-ANCA titres and risk of relapse

Compared with 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 of relapse ($P = 0.012$) (Fig. 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 the relapse rate.

Positive c-ANCA titres 3, 12, 18 and 24 months after diagnosis were associated with a significantly increased risk of relapse (Fig. 3a). A positive c-ANCA titre at the switch to azathioprine was significantly associated with increased risk of relapse (RR 2.2; 95% CI 1.0–5.6).

PR3-ANCA

At diagnosis, PR3-ANCA levels, as measured by direct ELISA, were less than 10 units/ml in nine patients. One of these patients

a. C-ANCA positivity and relapse risk

Months after diagnosis	RR (95% CI)	P	PPV	NPV
3	2.0 (1.2–3.8)	0.01	0.78	0.49
6	1.3 (0.7–2.4)	0.41	0.63	0.42
12	1.9 (1.1–3.3)	0.03	0.71	0.55
18	2.9 (1.3–4.6)	0.006	0.63	0.68
24	2.6 (1.2–5.0)	0.02	0.56	0.72

b. PR3-ANCA positivity and relapse risk

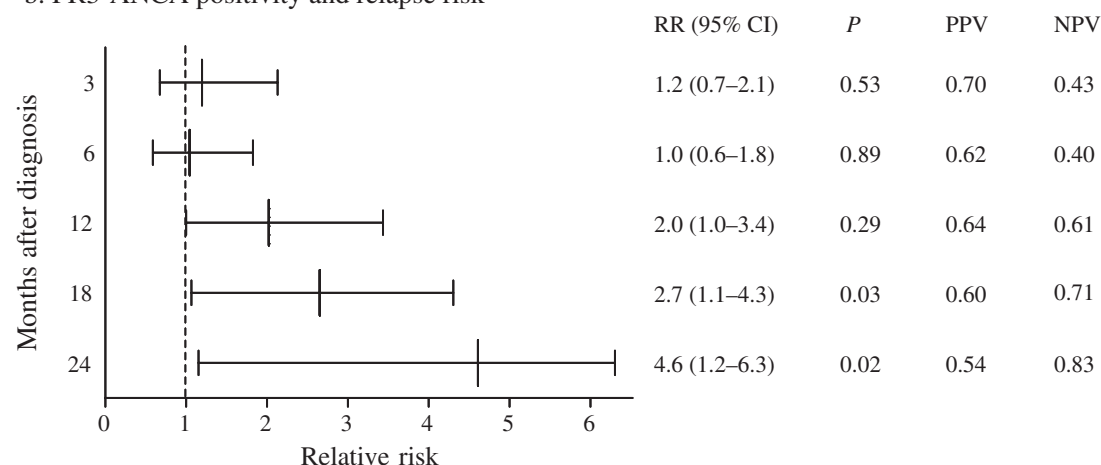| Months after diagnosis	RR (95% CI)	P	PPV	NPV
3	1.2 (0.7–2.1)	0.53	0.70	0.43
6	1.0 (0.6–1.8)	0.89	0.62	0.40
12	2.0 (1.0–3.4)	0.29	0.64	0.61
18	2.7 (1.1–4.3)	0.03	0.60	0.71
24	4.6 (1.2–6.3)	0.02	0.54	0.83

Fig. 3. (a) Relative risk of relapse in patients positive for c-ANCA 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 3, 6, 12, 18 and 24 months compared with patients negative for PR3-ANCA at these time points. Lines indicate relative risk and 95% confidence interval. P, P-value; PPV, positive predictive value; NPV, negative predictive value.

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 titres, 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. Three months after diagnosis, 38 patients were negative for PR3-ANCA and 6 months after diagnosis 42 patients were negative for PR3-ANCA (Fig. 1). An increasing number of patients were positive for PR3-ANCA 12, 18 and 24 months after diagnosis (Fig. 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 titres and changes in titres.

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 with 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 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 of experiencing a relapse (Fig. 3b). However, patients positive for PR3-ANCA 18 months and 24 months after diagnosis had a significantly increased risk of experiencing relapse (Fig. 3b).

Discussion

Most patients with ANCA-associated 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 yr, 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 titres during follow-up contributes to the identification of patients at increased risk of 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 the start of treatment. In particular, the limited number of patients who were c-ANCA/PR3-ANCA negative 18 and 24 months after diagnosis had a strongly reduced risk of experiencing 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 the 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 last 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 leucocytes *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 titres and the clinical outcome. However, patients who remain positive for c-ANCA might also have ongoing smouldering immune activation. This last hypothesis requires further investigation and might be addressed by serially studying markers of immune activation, ANCA titres 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 a EUVAS study in which long-term (4 yr) low-dose immunosuppressive therapy is compared with standard treatment with azathioprine maintenance for 18 months [18]. Although this study specifically aims to include 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 was 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.

Rheumatology	Key messages
	• A major proportion of patients with PR3-ANCA-associated vasculitis experience relapses of disease during long-term follow-up.
	• Positive c- and PR3-ANCA titres during early follow-up identify patients at increased risk of relapse.
	• Patients with PR3-ANCA-associated vasculitis who remain persistently negative for c- or PR3-ANCA after induction of remission have a strongly reduced risk of relapse.

Acknowledgements

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