Changes in proteinase 3 anti-neutrophil cytoplasm autoantibody levels in early systemic granulomatosis with polyangiitis (Wegener's) may reflect treatment rather than disease activity

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ABSTRACT

Objective. *To investigate the nature of the relationship between proteinase 3 anti-neutrophil cytoplasm autoantibody (PR3-ANCA) and relapse in patients with early systemic granulomatosis with polyangiitis (Wegener's) (GPA).*

Methods. *Clinical data from 16 relapsing and 12 non-relapsing patients with early systemic GPA from a randomised clinical trial were correlated to monthly PR3-ANCA values over 18 months. Each sample was examined using 9 different enzyme-linked immunosorbent assays (ELISAs) to ensure reliability of ANCA results. PR3-ANCA peaks were identified by the highest sum of logarithmic transformation values from all assays in samples after remission.*

Results. *A PR3-ANCA peak was identified in all relapsing and non-relapsing patients and coincided with relapse in all 14 evaluable relapsing patients. The monthly increment before the peak, however, was similar in relapsing and non-relapsing patients in all assays. Increments from remission to peak were higher in relapsing patients in 2/9 assays. PR3-ANCA values at entry and peak PR3-ANCA values were higher in relapsing patients in 3/9 and 2/9 assays, respectively. However, large overlaps of PR3-ANCA values prevented a distinction between relapsing and non-relapsing patients. The median time to reach peak values was 14 months in relapsing and 12 months in non-relapsing patients with scheduled termination of treatment at 12 months.*

Conclusion. *The predictive value for relapses of PR3-ANCA determinations confirm and extend previous reports. Although all relapses were related to PR3-ANCA increases, reduction or withdrawal of immunosuppression without relapse was also related to increases and may explain the lack of predictive value of sequential PR3-ANCA determinations.*

Introduction

Antineutrophil cytoplasm(ic) (auto) antibodies (ANCA) are directed against components of primary granules of neutrophils and monocytes. ANCAs with specificity for proteinase 3 (PR3) are the typical autoantibodies detected in granulomatosis with polyangiitis (Wegener's) (GPA) in Europe.

Traditional detection methods for ANCAs are indirect immunofluorescence (IF) for screening and immunochemical methods, such as enzyme-linked immunosorbent assays (ELISA) for target identification (1). In IF, PR3-ANCA appears as C-ANCA (classical or cytoplasmic ANCA).

Since the first reports on ANCA detection in GPA in 1985 it has been assumed (2, 3), that the C-ANCA titre would diminish when remission was attained and that titres would increase with relapses. However, since then studies of the value of serial PR3-ANCA determinations in predicting disease activity have yielded conflicting results. Recently data summarised by Tomasson *et al.* (4) concluded that measuring PR3-ANCA increments is not clinically useful for identifying relapses. The reasons for these conflicting results and the ensuing lack of clinical usefulness, however, are not clarified. As PR3-ANCA are increasingly suspected of playing a pathogenic role in GPA (5), we found it of interest to further explore the association between changes in PR3-ANCA values and relapses.

The present study is based on a protocol (RELANCA) associated to the ran-

domised clinical trials (RCTs) conducted by the European Vasculitis Study Group (EUVAS) in ANCA-associated systemic vasculitis (AASV) from 1996 to 2003 (6). This protocol aimed to assess the value of monthly measurements of PR3-ANCA in monitoring disease activity in newly diagnosed AASV patients. In one of the EUVAS RCTs, (NORAM) (7), which included only early systemic GPA patients, relapses were prominent. From this trial, all PR3-ANCA positive GPA patients with available sera were included to further explore the relationship between PR3-ANCA values and relapses, examining monthly samples over 18 months with 9 different ELISAs to ensure reliability of the PR3-ANCA determinations.

Materials and methods

Patients and serum sampling

The NORAM study was an unblinded, prospective RCT performed to determine whether methotrexate (MTX) could replace cyclosphosphamide (CYC) for the treatment of AASV in patients with serum creatinine below 150 μmol/l (7). The medication was given for 12 months and the patients followed for an additional 6 months. Serum samples were designed to be collected at entry, then monthly for 18 months and at any time of a clinically suspected relapse.

The study was conducted according to the Declaration of Helsinki. Approval of ethics was obtained from each participating centre.

The NORAM study recruited 100 patients of which 84 completed the trial at 18 months. Sera from 45 patients were received at the EUVAS Serum Bank at the Statens Seruminstitut, Copenhagen, Denmark. Of these, 17 patient series were incomplete or PR3 ANCA negative and therefore excluded. Thus 28 PR3-ANCA-positive patients could be included (Fig. 1) in this study. The demographic parameters of the patients are given in Table I.

Relapses

Disease activity was recorded every month for the first 6 months, once every 3 months thereafter, and at relapse using the Birmingham Vasculitis Activity Score (BVAS) (8). Remission was defined as the absence of new or worse clinical activity whereas minor persistent activity in one affected organ scoring less than two points was allowed. Relapse was defined on the basis of clinical manifestations on an "intention to treat" basis and divided into categories of major and minor relapse (7). All but one of the 28 patients was in remission at month four. During the trial period 16 patients experienced 19 relapses, whereas 12 patients maintained remission. In the 3 patients with 2 relapses, the relapse with the highest BVAS was chosen. The median time from inclusion to relapse was 14 months.

The patients who suffered relapse during the study are called relapsing patients while those maintaining remission are called non-relapsing patients.

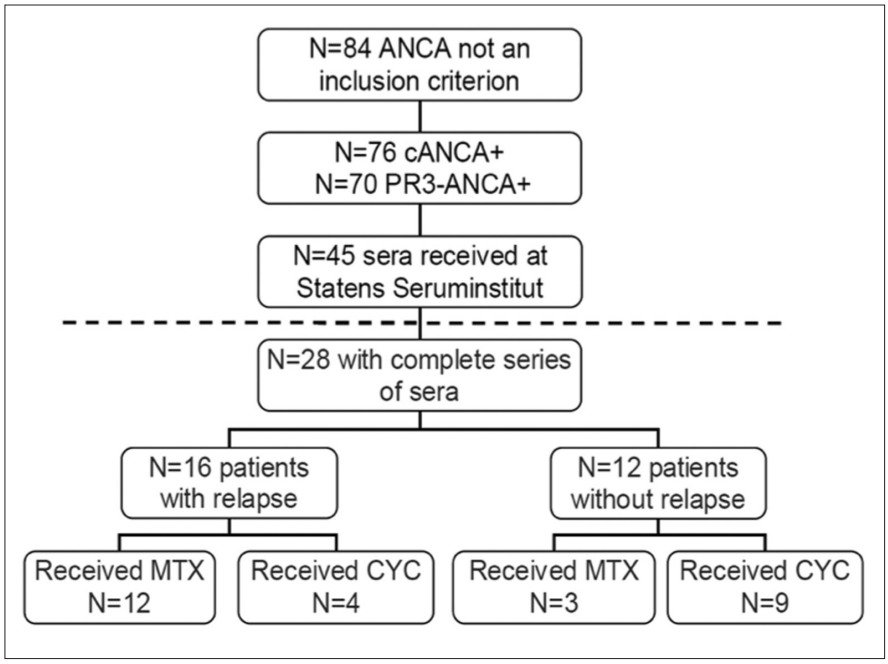

Fig. 1. Flow diagram of patients included from the original NORAM population.

Table I. Demographic data on study patients related to the entire NORAM population.

Patients	Relapsing	Non-relapsing	All	Original NORAM
Patients (n)	16	12	28	95
Age (years)	41.5 (29-61)	56.5 (26-78)	49.5 (26-78)	53.0(18-78)
Gender (female/male)	10/6	6/6	16/12	51/44
Treatment arm (MTX/CYC)	12/4	3/9	15/13	49/46
Creatinine (μmol/l)	72 (62-107)	93 (67-115)	80 (62-115)	84 (42-149)
BVAS at entry	18 (2-49)	16 (11-41)	17 (2-49)	15 (2-49)

Age, creatinine and BVAS shown as median (range); MTX: methotrexate; CYC: cyclosphosphamide; BVAS: Birmingham Vasculitis Activity Score; NORAM: study of CYC *versus* MTX in early systemic GPA patients.

PR3-ANCA assays

Nine different ELISAs were applied to follow levels of PR3-ANCA: 3 direct ELISAs (dE) and 4 capture ELISAs (cE) using human native (hn) PR3 only, and 2 direct ELISAs using human recombinant (hr) PR3 (human cDNA expressed in human cells, Euroimmun), one of these using a mixture of human native and human recombinant (hn-hr) PR3.

All assays were performed in accordance with the manufacturers' instructions and all values were recorded as exact values In cases with values exceeding the upper reading limit, dilutions were used to obtain the exact value. Company-defined borders between negative and positive values were thus not used. All participating laboratories were blinded as to the clinical status of the patients.

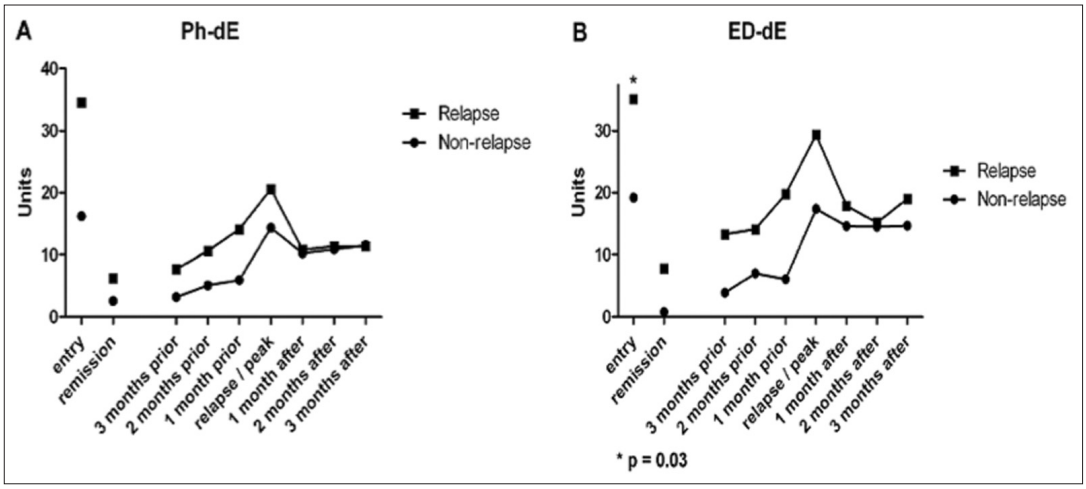

Fig. 2. Entry, remission and peak related monthly median PR3-ANCA values in relapsing and non-relapsing patients from two direct ELISAs (**A**: Ph-dE = Phadia's direct ELISA and **B**: ED-dE = EuroDiagnostica's direct ELISA) using native human PR3.

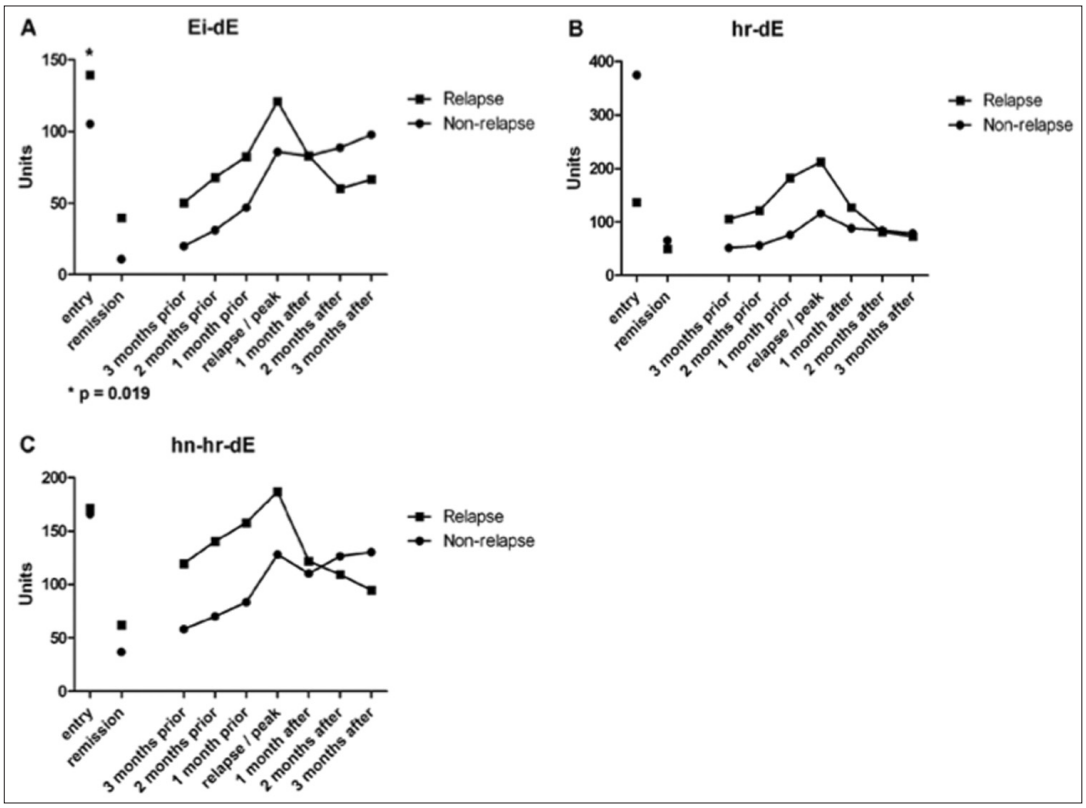

Fig. 3. Entry, remission and peak related monthly median PR3-ANCA values in relapsing and non-relapsing patients from three direct ELISAs from the same manufacturer (Euroimmun) using either native human PR3 (**A**: Ei-dE = Euroimmun's direct ELISA), recombinant human PR3 (**B**: hr-dE = human recombinant direct ELISA) or a mixture (**C**: hn-hr-dE = human native – human recombinant mixture direct ELISA).

– Detection of PR3-ANCA by Phadia
Phadia performed the aliquoting and labelling of all samples for the study. Phadia also performed their Varelisa PR3 ANCA (Ph-dE) and their Varelisa PR3 Capture (Ph-cE).

– Detection of PR3-ANCA by Statens Seruminstitut
All samples were examined using a third-generation anchor anti-PR3 assay (hs-cE) (Anti-PR3 HS ELISA, ORG 618, ORGENTEC Diagnostika GmbH, Germany).

– Detection of PR3-ANCA by EuroDiagnostica
EuroDiagnostica performed their direct ELISA (ED-dE) and their capture ELISA (ED-cE).

– Detection of PR3-ANCA by Euroimmun
Euroimmun performed four different ELISAs: Three direct ELISAs coated with hn PR3 (Ei-dE), hr PR3 (hr-dE) or a mixture of hn and hr PR3 (hn-hr-dE) and one capture ELISA using hn PR3 (Ei-cE).

Data handling
All PR3-ANCA values from each laboratory were merged into the original NORAM clinical and laboratory data files. The identity and exact time point of each serum sampling was carefully secured. When sera were missing, forward (or backward) imputation from the previous (or following) value was used.

Identification of peak values
As the PR3-ANCA values from the different assays were based upon different units and different reference sera,

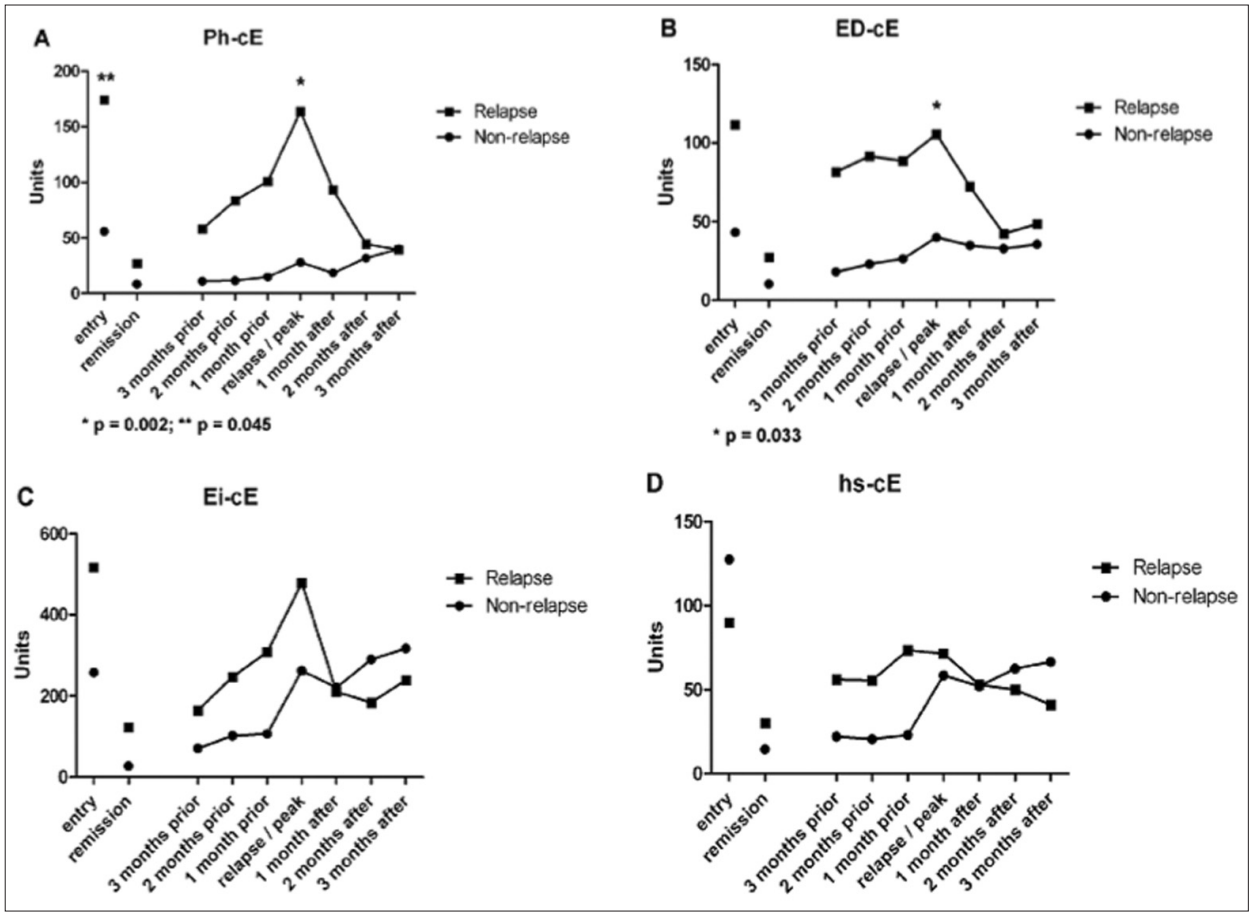

Fig. 4. Entry, remission and peak related monthly median PR3-ANCA values in relapsing and non-relapsing patients from four capture ELISAs (**A**: Ph-cE = Phadia's capture ELISA; **B**: ED-cE = EuroDiagnostica's capture ELISA; **C**: Ei-cE = Euroimmun's capture ELISA and **D**: hs-cE = high sensitivity capture ELISA) using native human PR3.

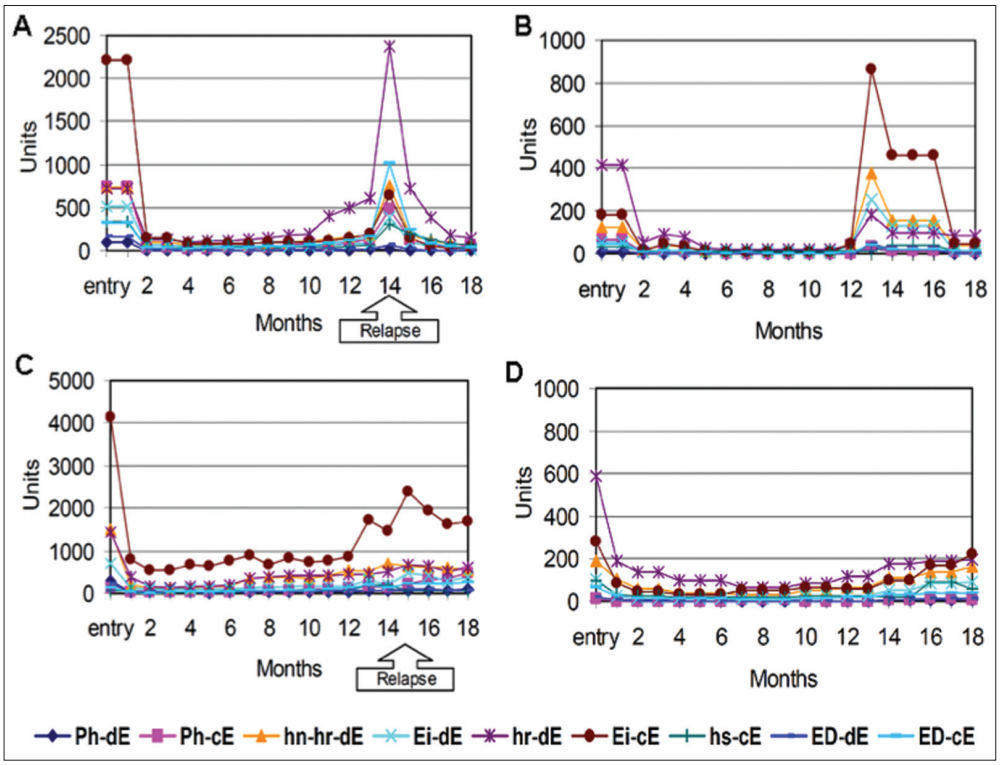

Fig. 5. Monthly PR3-ANCA values for each of the 9 tests for a relapsing patient (**A**) and a non-relapsing patient (**B**) with very pronounced rise in PR3-ANCA titre. Similarly, PR-ANCA values of two patients representing the general PR3-ANCA profile for relapsing patients (**C**) and non-relapsing patients (**D**) are shown.

all PR3-ANCA values from all 9 assays were transformed to logarithmic values. The sums of the logarithmic values at each month for each patient were then calculated. The highest sum value then identified the "PR3-ANCA peak value". This peak value could be identified in relapsing as well as in non-relapsing patients, making it possible to compare increments related to peak values in both groups.

Increments

For each assay the increments in absolute PR3-ANCA values were calculated A) from time of remission (month 4) till time of the peak value defined above, B) from the previous month till time of the peak value or C) from the previous month for all time points after month 4. The two increments before the peak values (A and B) were compared between the relapsing and the non-relapsing group. The largest monthly increment after month 4 (C) was recorded in order to determine whether it coincided with the time of relapse in each of the 9 assays for each of the relapsing patients.

Statistics

All comparisons of data were performed using the Wilcoxon rank sum test one-sided. P-values <0.05 were considered statistically significant. The S-PLUS programme was used for all calculations.

Results

PR3-ANCA profiles

The median PR3-ANCA values in both groups for each of the 9 assays are shown in Figures 2-4. In both groups and in all assays the median PR3-ANCA values increased with time from remission as medication was tapered. In the relapse group, PR3-ANCA values decreased again after relapse as treatment was re-induced or increased, while PR3-ANCA values after the peak value in non-relapsing patients were unchanged or continued rising. The peak values were reached after a median of 14 months (range 6–17) in relapsing patients and 12 months (range 6–17) in non-relapsing patients. There was no difference in PR3-ANCA profiles between treatment arms. However, when splitting up data after termination of treatment from each treatment arm in relapsing and non-relapsing patients, CYC treated relapsing patients had higher PR3-ANCA values than MTX treated patients, while the opposite was true for non-relapsing patients. These differences did not reach statistical significance due to the small numbers of patients in the sub-groups.

PR3-ANCA peak values related to relapses

In 14 of 16 relapsing patients, the peak value corresponded exactly to the relapse. In the two divergent patients, imputed values made it difficult to know, which of the two neighbouring time points actually represented the peak value.

PR3-ANCA value increments related to relapse status of the patients

In order to evaluate, whether a positive increment (rise in PR3-ANCA value) would identify a relapse, three types of increments were evaluated.

A. The increments of PR3-ANCA from remission to relapse (in relapsing patients) or peak value (in non-relapsing patients) were significantly higher in relapsing patients using the Ph-cE assay (p=0.034) (Fig. 4A) and the hr-dE assay (p=0.002) (Fig. 3B), whereas the differences in the other assays were insignificant.

B. The increments from the month before the relapse (in relapsing patients) or peak value (in non-relapsing patients) were almost identically distributed (*i.e.* similar PR3-ANCA value increases in both groups) with no statistical differences at all.

C. The largest monthly increment after remission did not coincide with the time of relapse in the majority of the 9 assays (median 7.5 of the assays in the 16 relapsing patients). None of the assays came out with a better performance than other assays.

Figures 5 A-B show PR3-ANCA values in two patients with very pronounced PR3-ANCA rises, one from each group, illustrating the lack of sensitivity of an ANCA titer rise to identify a relapse. Figures 5 C-D show the PR3-ANCA values in two patients with low PR3-ANCA peaks (also one from each group), reflecting the PR3-ANCA profiles generally found in this material.

PR3-ANCA values at relapse/peak related to the relapse status of the patients

In all 9 assays median values in relapsing patients at time of relapse were higher than in non-relapsing patients at time of the peak value, but this was only significant in two assays (Ph-cE: p=0.002; ED-cE: p=0.033). However, for both of these assays, there was a large overlap in values (Ph-cE: relapsing patients: 16.0-1530.0, non-relapsing patients: 1.2-483.5 units/ml; ED-cE: relapsing patients: 16.0-824.8, non-relapsing patients: 1.7–210.0 units/ml).

PR3-ANCA values at entry related to the relapse status of the patients

Entry values were higher in relapsing patients as tested with the following assays: Ph-dE, Ph-cE, Ei-dE, Ei-cE, ED-dE and ED-cE, although the differences were significant only with Ph-cE (p=0.045), Ei-dE (p=0.019) and ED-dE (p=0.030). Also in these 3 assays, there was a large overlap in values (Ph-cE: relapsing patients: 17.0–1486.0, non-relapsing patients: 5.5–467.0 units/ml; Ei-dE: relapsing patients: 58.90–702.66, non-relapsing patients 11.89–233.54 units/ml; ED-dE: relapsing patients: 8.5–311.7, non-relapsing patients: 3.4–83.7 units/ml).

The results of the 2 direct assays from Euroimmun (Fig. 3) using human recombinant antigen are deviant as the assay using only recombinant antigen has a lower median entry value for relapsing patients while the assay using a combination of the natural and recombinant antigen yields the same median value for the two groups. The hs-cE behaves like the hr-dE, also with a lower median entry value in the relapse group.

Discussion

This prospectively collected cohort of PR3-ANCA positive patients experiencing frequent relapses during standardised treatment was followed monthly as to their clinical data and ANCA status. This permitted assessment of the value of PR3-ANCA for monitoring disease activity and prediction of

relapses in 28 patients for whom complete sera sets were available. Although rises in PR3-ANCA occurred in relapsing patients, similar rises were also seen in those without relapse and reduction or withdrawal in immunosuppressive medication appeared to have the strongest influence on PR3-ANCA level.

We have used a novel approach to evaluate the predictive value of rising PR3-ANCA values by applying a mathematical method to identify a maximal PR3-ANCA peak value in relapsing as well as non-relapsing patients based on the results of all 9 assays.

The lack of predictive potential of the increments from remission to the peak value is further supported by our findings when comparing increments over the last month before the peak value. Moreover, the majority of maximal monthly increments measured throughout the trial period in the relapsing patients were not related to a relapse.

Although our results confirm the original and later observations of recurrence of PR3-ANCA in relation to relapses (2, 3, 9-11), our results clearly demonstrate that tapering off immunosuppressive treatment alone causes similar increases in PR3-ANCA values in relapsing as well as non-relapsing patients, which makes it impossible to differentiate relapsing patients from non-relapsing patients based merely on the increase in PR3-ANCA values. Indeed, it appears that the influence of treatment on PR3-ANCA levels is stronger than the effect of disease activity (Fig. 5 A-D). This effect is the same in the MTX as in the CYC treated patients and therefore may mainly reflect the effects of the corticosteroid treatment given to each patient. Using the results of changes of PR3-ANCA values to predict a relapse in patients with early systemic GPA can therefore only be meaningful if possible changes in treatment is considered as well. This has not been done in previous studies and merits further investigation.

In 6 of the 9 assays we found the median value at entry to be higher in relapsing patients. This finding is new and bears resemblance to the association described between high values of PR3-ANCA at entry and decreased patient survival (12), especially as this phenomenon is observed when using simple monoclonal antibody capture ELISAs.

More striking is our finding that all assays consistently identified the median peak value at relapse in the relapsing patients as being higher than in the non-relapsing patients. This finding is also new and consistent with findings of an association between positive PR3-ANCA measured by capture ELISA and a later relapse (13-16). However, in contrast to Sanders *et al*. (16), we did not find higher PR3-ANCA levels after month 12 in the relapsing patients, which may be due to differences in treatment regimens. Due to the large overlap between values at entry as well as at relapse/peak in relapsing and non-relapsing patients, the individual PR3-ANCA values could not be used to predict relapse.

In the report from the WGET trial (17), using time of remission as reference point, they found a lack of consistent changes in PR3-ANCA values over time. This is in contrast to the present findings using either time from start of treatment or time of relapse/peak as the reference point. This is not due to differences in the assays used, as all our assays showed the same pattern, but may instead reflect that we have used monthly sampling as compared to 3 monthly sampling in the WGET trial, the homogeneity of our GPA patients, the early stage of the disease, and their particular treatment in the NORAM trial.

The large variability in the performance of the different PR3-ANCA assays has been described previously (18, 19). We also confirm that the ratio between values of the 9 assays for each patient may change during the course of the trial (20). Capture ELISA for detecting a PR3-ANCA rise has been reported to be superior for detecting a relapse compared with standard ELISA (13-15, 21), but this was not confirmed in our study.

The present results are based only on a small group of patients with PR3-ANCA positive early systemic GPA. This subgroup of GPA patients have a lower risk of developing renal disease and a high frequency of relapses (22, 23, 24) mainly in the ear, nose and throat area. Our conclusions may be less valid for PR3-ANCA positive GPA patients with more widespread vasculitic disease. Our observations support a pathogenic role for PR3-ANCA because changes in PR3-ANCA values were closely related to changes in treatment, all relapses were related to increases in PR3-ANCA and as PR3-ANCA values at entry and relapse/peak were higher in relapsing patients. The recently described animal model (5) suggested but did not confirm a causative role for PR3-ANCA in vasculitis. The practical clinical and pathogenic implications must therefore await the results of further investigations.

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Phadia, Euroimmun and EuroDiagnostica performed their own PR3-ANCA-tests.

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