ARTHRITIS & RHEUMATOLOGY
Vol. 68, No. 7, July 2016, pp 1700–1710
DOI 10.1002/art.39637

Factors Determining the Clinical Utility of Serial Measurements of Antineutrophil Cytoplasmic Antibodies Targeting Proteinase 3

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Objective. **Relapse following remission is common in antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV), particularly with ANCAs directed at proteinase 3 (PR3). This study was undertaken to evaluate the association of an increase in PR3-ANCA level with subsequent relapse.**

Methods. **Data from the Rituximab versus Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) trial were used. Starting from the time of achieving complete remission, serial measurements by direct and capture enzyme-linked immunosorbent assays (ELISAs) were analyzed in 93 patients with PR3-ANCA, using Cox proportional hazards regression.**

Results. **An increase in PR3-ANCA level was identified in 58 of 93 subjects (62.4%) by direct ELISA and in 59 of 93 (63.4%) by capture ELISA. Relapses occurred in 55 of 93 subjects (59.1%), with 25 and 21 occurring within 1 year after an increase by direct ELISA and capture ELISA, respectively. An increase by direct ELISA was associated with subsequent severe relapses (hazard ratio [HR] 4.57; $P < 0.001$), particularly in patients presenting with renal**

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Supported by the NIH (National Center for Advancing Translational Sciences Clinical and Translational Science Award [CTSA] UL1-TR-000135). Enzyme-linked immunosorbent assay kits for antineutrophil cytoplasmic antibody (ANCA) testing were provided by Euroimmun (Lubeck, Germany). Data for the current study were obtained from the Rituximab versus Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) trial, which was performed with the support of the Immune Tolerance Network (NIH contract N01-AI-15416, protocol number ITN021AI), an international clinical research consortium headquartered at the University of California and supported by the National Institute of Allergy and Infectious Diseases, NIH, and the Juvenile Diabetes Research Foundation. Genentech and Biogen Idec provided the study medications and partial funding for the RAVE trial. At the Mayo Clinic and Foundation, Johns Hopkins University, Boston University, and the Cleveland Clinic, the RAVE trial was supported by the NIH (Mayo Clinic: National Center for Research Resources CTSA 1-UL1-RR-024150-01; Johns Hopkins University: National Center for Research Resources CTSA UL1-RR-025005 and career development awards K23-AR-052820 to Dr. Seo and K24-AR-049185 to Dr. Stone; Boston University: National Center for Research Resources CTSA UL1-RR-025771, grant M01-RR-00533, and career development award K24-AR-02224 to Dr. Merkel; Cleveland Clinic: National Center for Advancing Translational Sciences grant UL1-TR-000439). The RAVE trial at Boston University was also supported by the Arthritis Foundation (Investigator Award to Dr. Monach).

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Dr. Hoffman has received consulting fees, speaking fees, and/or honoraria from Roche, Regeneron, Sanofi, Prothena, AstraZeneca, MedImmune, and Celltrion (less than $10,000 each). Dr. Kallenberg has received consulting fees, speaking fees, and/or honoraria from Eli Lilly (less than $10,000). Drs. Stone and Specks have received consulting fees from Roche/Genentech (less than $10,000 each).

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Submitted for publication September 18, 2015; accepted in revised form February 9, 2016.

involvement (HR 7.94; $P < 0.001$) and alveolar hemorrhage (HR 24.19; $P < 0.001$). Both assays identified increased risk for severe relapse in the rituximab group (HR 5.80; $P = 0.002$ for direct ELISA and HR 4.54; $P = 0.007$ for capture ELISA) but not the cyclophosphamide/azathioprine group ($P = 0.103$ and $P = 0.197$, respectively).

Conclusion. **The association of an increase in PR3-ANCA level with the risk of subsequent relapse is partially affected by the PR3-ANCA detection methodology, disease phenotype, and remission induction treatment. An increase in PR3-ANCA level during complete remission conveys an increased risk of relapse, particularly severe relapse, among patients with renal involvement or alveolar hemorrhage and those treated with rituximab. Serial measurements of PR3-ANCA may be informative in this subset of patients, but the risk of relapse must be weighed carefully against the risks associated with therapy.**

Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) is a heterogeneous group of diseases including granulomatosis with polyangiitis (Wegener's) (GPA) and microscopic polyangiitis. AAV is defined by inflammation and necrosis predominantly of small blood vessels, along with necrotizing granulomatous tissue inflammation in GPA (1,2). Acute morbidity, mortality, and irreversible organ damage can be attributed to the underlying disease and to complications of immunosuppressive therapy (3–6). Remission is achieved with induction therapy in up to 90% of patients, but more than half of patients with severe disease experience relapses (5–7). In the absence of definitive indicators of oncoming relapses, balancing the risks of immunosuppression with the benefits of disease control remains challenging (8,9).

The diagnostic utility of ANCA testing has been widely accepted, whereas the clinical utility of ANCA as a biomarker of disease activity and predictor of relapses has remained a subject of controversy despite numerous investigations (10–32). A clear role for serial ANCA measurements in AAV as a whole has not been established, and it is not advised to make treatment decisions based on changes in ANCA titers alone (33,34). Methodologic issues, including the specific assays and the definition of an increase in the ANCA level, the ANCA type, the disease phenotype, and the treatment chosen to induce or maintain remission all deserve consideration when assessing the clinical utility of serial ANCA monitoring (9,33). It has been well documented that patients with ANCA targeting proteinase 3 (PR3-ANCA) have a higher risk of relapse than patients with ANCA against myeloperoxidase (MPO-ANCA) (7,31,35–38). Most recently, a single-center study of 166 AAV patients found that an increase in ANCA level (PR3-ANCA or MPO-ANCA) was predictive of relapses, particularly in patients who had presented with renal involvement, and in those with nonrenal severe disease (15).

Consequently, we hypothesized that the methodology used to measure ANCA levels, the disease phenotype, and the remission induction regimen all affect the clinical utility of serial measurements of ANCA titers. To test this hypothesis, we evaluated the relationship between PR3-ANCA titers and the risk of relapse within 1 year of an increase in a well-defined cohort of patients with severe AAV.

PATIENTS AND METHODS

Patients. This study used serum samples and clinical data collected during the Rituximab versus Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) trial. Details of the RAVE trial protocol have been described elsewhere (4,7). Briefly, patients were randomized to receive rituximab or cyclophosphamide, along with a prespecified prednisone taper, for induction therapy and were followed up prospectively according to protocol at baseline; at weeks 1, 2, 3, and 4; at months 2, 4, and 6; and every 3 months until month 18. Thereafter, patients were seen every 6 months until common closeout of the trial (which was the month 18 time point for the last patient enrolled). Additional visits occurred at the discretion of patients and providers to evaluate disease activity or medication toxicity. At each visit, the Birmingham Vasculitis Activity Score for Wegener's Granulomatosis (BVAS/WG) was used in conjunction with clinical assessment to evaluate disease activity, and serum samples were obtained and stored at −80°C (4,7).

Each of the 197 trial participants had either new or relapsing disease at the time of enrollment. A positive serum assay for PR3-ANCA or MPO-ANCA was required for enrollment. The present study focused on the 131 patients with PR3-ANCA because of the higher number of patients and relapses in this group. In contrast, of the 66 MPO-ANCA–positive patients, complete remission was achieved in 52, and only 15 experienced a relapse (4,7).

Disease activity. *Assessments.* The BVAS/WG instrument was used to document disease activity at each study visit. A score of ≥1 reflects active disease within the 28 days prior to assessment, while 0 indicates remission (39). Complete remission was defined as a BVAS/WG of 0, along with a prednisone dose of 0 mg. Disease relapse was defined as any new disease activity, with an increase in BVAS/WG of ≥1 point after achievement of complete remission. A relapse was considered severe if the BVAS/WG was >3, if a new major item was present, or if, in the judgment of the clinician, reinitiation of induction therapy was warranted (40).

Disease manifestations and disease phenotype categories. The organ manifestations present at enrollment and at each study visit were recorded by expert clinicians (study investigators) using the BVAS/WG instrument. The disease phenotype categories used for this analysis are based on the BVAS/WG items recorded at the time of enrollment. BVAS/WG items considered to reflect underlying necrotizing granulomatous inflammation included mouth ulcers, retroorbital mass/proptosis, bloody nasal discharge, sinus involvement, salivary gland enlargement,

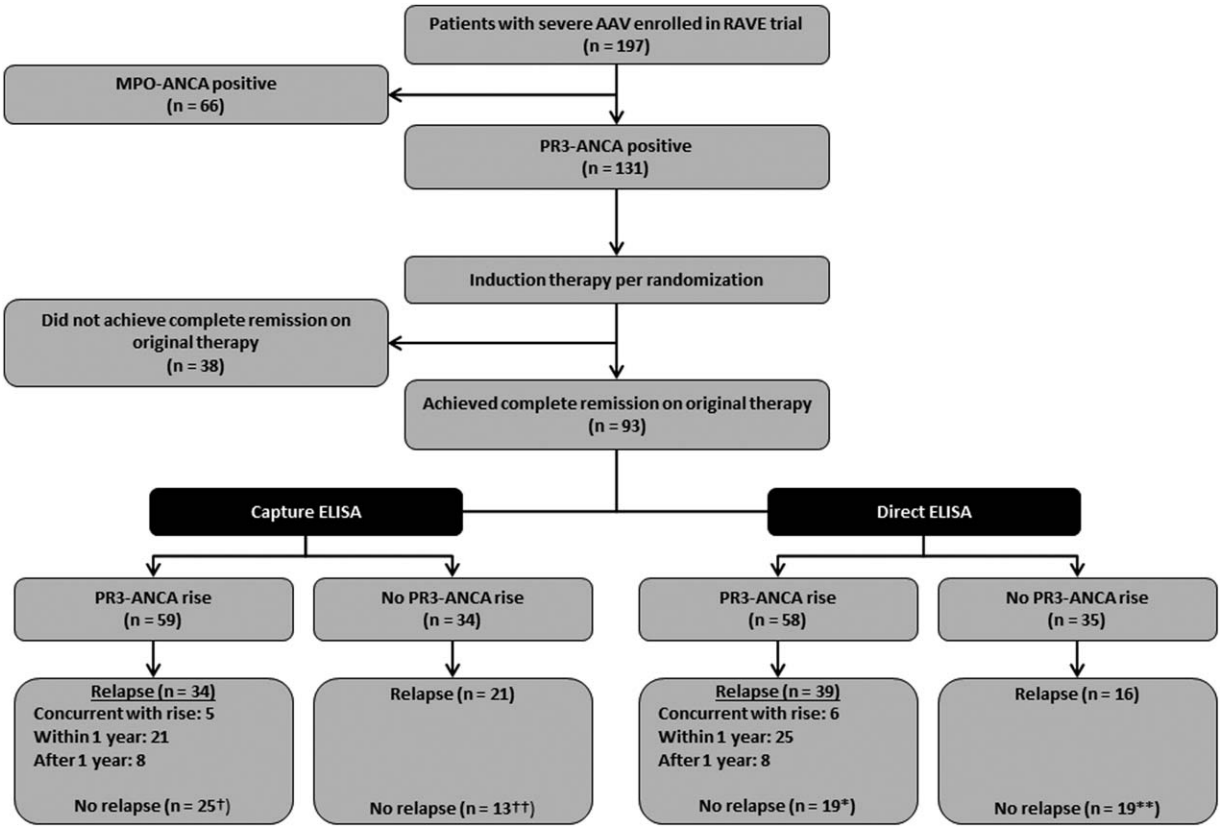

Figure 1. Patient selection and outcomes. † = Six of the 25 patients had <1 year of follow-up after the detection of an increase in antineutrophil cytoplasmic antibody (ANCA) levels. †† = One of the 13 patients had <1 year of follow-up after achieving complete remission. * = Four of the 19 patients had <1 year of follow-up after the detection of an increase in ANCA level. ** = Two of the 19 patients had <1 year of follow-up after achieving complete remission. AAV = ANCA-associated vasculitis; RAVE = Rituximab versus Cyclophosphamide for ANCA-Associated Vasculitis; MPO = myeloperoxidase; PR3 = proteinase 3; ELISA = enzyme-linked immunosorbent assay.

subglottic inflammation, conductive deafness, other major or minor ear/nose/throat involvement, pulmonary nodule/cavity, endobronchial involvement, meningitis, and cord lesion. In contrast, capillaritis was defined as the presence of one or more of the following BVAS/WG items: cutaneous purpura, scleritis, retinal hemorrhage or exudate, sensorineural deafness, mesenteric ischemia, alveolar hemorrhage, hematuria, red blood cell casts on urinalysis or glomerulonephritis, increase in creatinine level, sensory peripheral neuropathy, or motor mononeuritis multiplex.

Patients were considered to have renal disease if any renal item on the BVAS/WG (hematuria, red blood cell casts or glomerulonephritis, increase in creatinine level, or "other") was scored. A patient was categorized as having alveolar hemorrhage only if that item was scored on the BVAS/WG. All other BVAS/WG items cannot be clearly attributed to either necrotizing granulomatous inflammation or capillaritis and were, therefore, not considered to categorize the patient one way or another. Using this approach, all patients could be clearly assigned to 1 or more of 5 groups subjected to analysis: granulomatous disease only, any granulomatous disease, any capillaritis, renal involvement, and alveolar hemorrhage at enrollment.

ANCA testing. *Assays.* Standardized direct enzyme-linked immunosorbent assays (ELISAs) for PR3-ANCA and MPO-ANCA (supplied by Euroimmun) were performed on baseline serum samples from all 197 patients (28). For patients found to have PR3-ANCA, serial samples were tested with the direct ELISA, as well as with a capture ELISA developed in our laboratory and described previously, using a monoclonal antibody to PR3 (MCPR3-2) (41). For each ELISA described here, stored serum samples from serial visits for each individual patient were run on the same assay plate at a single laboratory from the second thaw cycle of each sample. The definitions used for an increase by each assay were selected to be outside the intraassay and interassay coefficients of variation, which have been published elsewhere (Euroimmun assay test instruction sheet [14]), and these definitions are consistent with those used in prior studies involving these assays (7,14). The value at each visit was compared to the lowest value within the preceding 6 months.

Definitions. For the direct ELISA, a PR3-ANCA titer of ≥20 units was considered positive. An increase in PR3-ANCA level was defined as a doubling of the result, or an increase to at least 40 units if the assay had previously become negative, within the preceding 6 months (7).

For the capture ELISA, a level, expressed as net absorbance, of ≥0.10 was considered positive. An increase in PR3-ANCA level was defined as a doubling of the result, with an absolute increase of at least 0.40, within the preceding 6 months (14).

Statistical analysis. All analyses were performed using SAS, version 9.3 (SAS Institute). Descriptive data are presented as the mean ± SD, median (interquartile range), or percentage. Cox proportional hazards models were used to assess whether an increase in PR3-ANCA level was associated with subsequent relapse. Separate analyses were performed with the event of interest being "any" relapse or "severe" relapse. For these analyses, the date of complete remission was time 0. All patients who experienced an increase in PR3-ANCA level without previously experiencing the specified type of relapse event were identified. An increase in PR3-ANCA level was modeled using a binary time-varying covariate. For a given patient, this variable has a value of "0" from time 0 to the date that an increase in PR3-ANCA level is detected and a value of "1" following this date. Using this approach, increases detected concurrent with a relapse event are treated as if no increase occurred. Since the primary question of interest was whether patients who experience an increase in PR3-ANCA level are at increased risk of relapse during the first 12 months following the increase, the primary analyses were performed with data censored at last follow-up for patients who did not experience an increase in PR3-ANCA level, and at 12 months following the increase or at last follow-up (whichever was shorter) for patients who did have an increase in PR3-ANCA level.

Findings from the proportional hazards regression are summarized using the hazard ratio (HR) with corresponding 95% confidence interval. Since the prospective, time-dependent survival analysis precludes the calculation of sensitivity and specificity of a PR3-ANCA titer increase for relapse in this data set, we calculated the concordance index (c-index), which is an extension of the concept of the receiver operating characteristic (ROC) curve, providing a measure of predictive discrimination (42). Similar to the interpretation of the area under the ROC curve, a c-index of 0.5 indicates no discrimination, and a c-index between 0.7 and 0.8 is considered to indicate adequate discrimination (43).

To further characterize the cumulative percentage of patients who experienced a relapse following an increase in PR3-ANCA level, a Kaplan-Meier analysis that included only patients who experienced an increase in PR3-ANCA level was performed. For this analysis, the date of the increase was used as time 0.

All analyses were performed for the entire cohort and for patient subsets defined according to disease phenotype categories, new diagnosis versus relapsing disease, and treatment groups. In all cases, *P* values less than 0.05 (2-tailed) were considered significant.

RESULTS

Baseline characteristics of the patients. Of the 131 patients with PR3-ANCA at the time of enrollment in the RAVE trial, complete remission was achieved in 93 (71.0%) receiving the therapy to which they were originally randomized. The remaining 38 patients were excluded from this analysis (Figure 1).

The mean ± SD age of the 93 patients was 50.1 ± 14.2 years. The majority of the patients were male (60.2%; n = 56) and white (93.5%; n = 87). More than half of the patients had relapsing disease rather than a new diagnosis of AAV at enrollment (55.9%; n = 52 versus 44.1%; n = 41). The median duration of follow-up from the time of complete remission was 35.6 months, with 87 of 93 patients (93.5%) having follow-up of at least 1 year after achievement of complete remission. Additional baseline characteristics of this cohort are presented in Table 1.

Table 1. Baseline demographic and clinical characteristics*

Age at enrollment, mean ± SD years	50.1 ± 14.2
Sex, no. (%) female/male	37 (39.8)/56 (60.2)
Race, no. (%)	
White	87 (93.5)
Black	3 (3.2)
Other	3 (3.2)
Ethnicity, no. (%)	
Not Hispanic or Latino	90 (96.7)
Hispanic or Latino	2 (2.2)
Unknown	1 (1.1)
AAV type, no. (%)	
GPA	90 (96.7)
MPA	3 (3.2)
Newly diagnosed at enrollment, no. (%)	41 (44.1)
Organ involvement at enrollment according to BVAS/WG, no. (%)	
Ear, nose, and throat	70 (75.3)
Constitutional	65 (69.9)
Renal	60 (64.5)
Pulmonary	50 (53.7)
Mucous membranes and eyes	31 (33.3)
Cutaneous	20 (21.5)
Nervous system	19 (20.4)
Cardiovascular	1 (1.1)
Gastrointestinal	1 (1.1)
Other	7 (7.5)
BVAS/WG at enrollment, mean ± SD	8.1 ± 3.1
ESR at enrollment, mean ± SD mm/hour	36.0 ± 28.6
CRP at enrollment, mean ± SD mg/dl	5.0 ± 12.4
Creatinine at enrollment, mean ± SD mg/dl	1.0 ± 0.65
Treatment group, no. (%)	
Rituximab	50 (53.7)
Cyclophosphamide	43 (46.2)
Time to complete remission, median (IQR) months	5.9 (5.8–6.2)
Duration of follow-up after complete remission, median (IQR) months	35.6 (24.6–42.6)

* AAV = antineutrophil cytoplasmic antibody–associated vasculitis; GPA = granulomatosis with polyangiitis (Wegener's); MPA = microscopic polyangiitis; BVAS/WG = Birmingham Vasculitis Activity Score for Wegener's Granulomatosis; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IQR = interquartile range.

Relationship of PR3-ANCA titer increases to subsequent relapses. An increase in PR3-ANCA level was identified in 58 of 93 subjects (62.4%) by direct ELISA and in 59 of 93 subjects (63.4%) by capture ELISA (Figure 1); 46 patients were found to have an increase using both assays, although not necessarily at the same visit. To estimate the relative increase in risk of a disease relapse within 1 year conveyed by a PR3-ANCA titer increase, and to assess how well the model discriminates those patients at increased risk of relapse, we calculated HRs and c-indices, respectively (Table 2). The number of patients with an increase in PR3-ANCA level followed by a relapse and the timing of the relapses are

Table 2. Increase in PR3-ANCA level and relapse within 1 year*

		Capture ELISA			Direct ELISA		
	No.†	HR (95% CI)‡	*P*	c-index	HR (95% CI)‡	*P*	c-index
All subjects	93						
Any relapse	55	1.15 (0.62–2.13)	0.648	0.50	2.24 (1.24–4.08)	0.008	0.59
Severe relapse	42	1.71 (0.80–3.71)	0.169	0.55	4.57 (2.16–10.37)	<0.001	0.67
According to disease phenotype at enrollment							
Granulomatous only	15						
Any relapse	13	0.36 (0.09–1.38)	0.136	0.38	0.65 (0.17–2.54)	0.532	0.49
Severe relapse	11	0.48 (0.11–2.16)	0.340	0.41	1.01 (0.23–4.48)	0.989	0.53
Any granulomatous disease	76						
Any relapse	47	1.18 (0.61–2.30)	0.625	0.50	2.28 (1.18–4.42)	0.014	0.59
Severe relapse	38	1.38 (0.62–3.09)	0.428	0.53	4.35 (1.82–10.44)	0.001	0.65
Any capillaritis manifestation	77						
Any relapse	42	1.42 (0.70–2.86)	0.331	0.52	2.63 (1.35–5.19)	0.004	0.60
Severe relapse	31	2.42 (0.98–6.14)	0.056	0.59	7.78 (3.10–22.61)	<0.001	0.71
Renal involvement	60						
Any relapse	28	1.02 (0.44–2.33)	0.954	0.48	2.16 (1.00–4.64)	0.049	0.59
Severe relapse	21	2.33 (0.82–6.93)	0.116	0.58	7.94 (2.72–29.18)	<0.001	0.71
Alveolar hemorrhage	24						
Any relapse	14	1.69 (0.48–6.24)	0.414	0.54	9.45 (2.58–34.63)	<0.001	0.76
Severe relapse	11	3.18 (0.78–14.94)	0.118	0.61	24.19 (3.05–447.20)	<0.001	0.81
According to treatment group							
Cyclophosphamide	43						
Any relapse	24	0.63 (0.22–1.70)	0.370	0.42	1.51 (0.59–4.17)	0.400	0.55
Severe relapse	19	0.40 (0.09–1.47)	0.197	0.42	2.84 (0.87–11.40)	0.103	0.62
Rituximab	50						
Any relapse	31	1.90 (0.85–4.32)	0.117	0.57	3.09 (1.37–7.06)	0.006	0.60
Severe relapse	23	4.54 (1.61–15.05)	0.007	0.68	5.80 (2.06–19.77)	0.002	0.68
According to disease status at enrollment							
Relapsing disease	52						
Any relapse	35	0.53 (0.23–1.20)	0.126	0.42	2.35 (1.13–4.96)	0.022	0.59
Severe relapse	27	0.76 (0.27–2.08)	0.603	0.48	4.31 (1.67–12.51)	0.004	0.67
New diagnosis	41						
Any relapse	20	3.00 (1.12–8.67)	0.032	0.60	1.99 (0.72–5.80)	0.187	0.57
Severe relapse	15	5.04 (1.49–20.33)	0.013	0.67	4.99 (1.41–23.37)	0.020	0.66

* PR3-ANCA = antineutrophil cytoplasmic antibodies directed at proteinase 3; ELISA = enzyme-linked immunosorbent assay; HR = hazard ratio; 95% CI = 95% confidence interval; c-index = concordance index.
† Total number of first relapse events of the specified type after complete remission for each subcategory during follow-up.
‡ Determined by Cox proportional hazards regression.

listed for the entire cohort (Figure 1) and for each subgroup (Figure 2). The median time to relapse and the proportion of patients experiencing a relapse by specific time points following an increase in PR3-ANCA level are presented in Table 3.

Disease relapse occurred in 55 of 93 subjects (59.1%). An increase in PR3-ANCA titer as measured by direct ELISA was associated with an increased risk of any relapse within 1 year ($P = 0.008$) and an increased risk of a severe relapse within 1 year ($P < 0.001$). However, an increase detected by capture ELISA was not associated with an increased risk of either type of relapse ($P = 0.648$ and $P = 0.169$, respectively) (Table 2). Moreover, the c-indices of 0.59 for any relapse and 0.67 for severe relapse detected by direct ELISA indicate that the model approached an adequate level of discrimination only for severe relapse (Table 2). Thirty-nine of the 58 patients (67.2%) who had an increase in PR3-ANCA level detected by direct ELISA had a subsequent relapse during the entire observation period, and only 25 experienced a relapse within 1 year (Figure 1). The median time to any relapse following an increase detected by direct ELISA was 11.8 months (Table 3). Sixteen of the 35 patients (45.7%) who did not have an increase in PR3-ANCA level detected by direct ELISA also experienced a relapse (Figure 1). Subsequent declines in PR3-ANCA value of ≥50% occurred in 7 of the 58 patients with an increase detected by direct ELISA and in 3 of the 59 patients with an increase detected by capture ELISA; none of these patients experienced a relapse within 1 year of the increase.

Factors affecting the risk of relapse following a PR3-ANCA titer increase. We further investigated the relative increase in risk of relapse conveyed by a PR3-ANCA titer increase in patient subsets defined according to various factors of clinical relevance.

Granulomatous Only (N = 15)

Capture ELISA						Direct ELISA					
Rise	10	CYC, RTX (3, 7)	No rise	5	CYC, RTX (3, 2)	Rise	11	CYC, RTX (5, 6)	No rise	4	CYC, RTX (1, 3)
Relapse	8	(3, 5)	Relapse	5	(3, 2)	Relapse	9	(5, 4)	Relapse	4	(1, 3)
Concurrent	1	(0, 1)				Concurrent	1	(1, 0)			
≤ 1 year	4	(1, 3)				≤ 1 year	5	(2, 3)			
> 1 year	3	(2, 1)				> 1 year	3	(2, 1)			
No relapse	2	(0, 2)	No relapse	0	(0, 0)	No relapse	2	(0, 2)	No relapse	0	(0, 0)

Renal Involvement (N = 60)

Rise	33	CYC, RTX (15, 18)	No rise	27	CYC, RTX (14, 13)	Rise	36	CYC, RTX (16, 20)	No rise	24	CYC, RTX (13, 11)
Relapse	14	(4, 10)	Relapse	14	(7, 7)	Relapse	19	(6, 13)	Relapse	9	(5, 4)
Concurrent	2	(1, 1)				Concurrent	5	(0, 5)			
≤ 1 year	10	(3, 7)				≤ 1 year	13	(6, 7)			
> 1 year	2	(0, 2)				> 1 year	1	(0, 1)			
No relapse	19†	(11, 8)	No relapse	13††	(7, 6)	No relapse	17*	(10, 7)	No relapse	15**	(8, 7)

† 5 (2,3) of the 19 had < 1 year of follow-up after PR3-ANCA rise
†† 1 (0,1) of the 13 had < 1 year of follow-up after complete remission
* 4 (1, 3) of the 17 had < 1 year of follow-up after PR3-ANCA rise
** 2 (1, 1) of the 15 had < 1 year of follow-up after complete remission

Alveolar Hemorrhage (N = 24)

Rise	14	CYC, RTX (2, 12)	No rise	10	CYC, RTX (6, 4)	Rise	18	CYC, RTX (5, 13)	No rise	6	CYC, RTX (3, 3)
Relapse	8	(0, 8)	Relapse	6	(4, 2)	Relapse	14	(4, 10)	Relapse	0	(0, 0)
Concurrent	1	(0, 1)				Concurrent	3	(0, 3)			
≤ 1 year	6	(0, 6)				≤ 1 year	10	(4, 6)			
> 1 year	1	(0, 1)				> 1 year	1	(0, 1)			
No relapse	6†	(2, 4)	No relapse	4	(2, 2)	No relapse	4*	(1, 3)	No relapse	6	(3, 3)

† 1 (0, 1) of the 6 had < 1 year of follow-up after PR3-ANCA rise
* 1 (0, 1) of the 4 had < 1 year of follow-up after PR3-ANCA rise

Rituximab (N = 50)

Rise	35	No rise	15	Rise	32	No rise	18
Relapse	22	Relapse	9	Relapse	23	Relapse	8
Concurrent	3			Concurrent	5		
≤ 1 year	14			≤ 1 year	14		
> 1 year	5			> 1 year	4		
No relapse	13†	No relapse	6††	No relapse	9*	No relapse	10**

† 4 of the 13 had < 1 year of follow-up after PR3-ANCA rise
†† 1 of the 6 had < 1 year of follow-up after complete remission
* 3 of the 9 had < 1 year of follow-up after PR3-ANCA rise
** 1 of the 10 had < 1 year of follow-up after complete remission

Cyclophosphamide (N = 43)

Rise	24	No rise	19	Rise	26	No rise	17
Relapse	12	Relapse	12	Relapse	16	Relapse	8
Concurrent	2			Concurrent	1		
≤ 1 year	7			≤ 1 year	11		
> 1 year	3			> 1 year	4		
No relapse	12†	No relapse	7	No relapse	10*	No relapse	9**

† 2 of the 12 had < 1 year of follow-up after PR3-ANCA rise
* 1 of the 10 had < 1 year of follow-up after PR3-ANCA rise
** 1 of the 9 had < 1 year of follow-up after complete remission

Figure 2. Increases in the levels of antineutrophil cytoplasmic antibodies (ANCAs) against proteinase 3 (PR3) and relapses by subgroup. ELISA = enzyme-linked immunosorbent assay; CYC = cyclophosphamide; RTX = rituximab.

PR3-ANCA detection methodology. The method of measurement used to quantify PR3-ANCA titer changes over time was a crucial determinant of risk associated with a titer increase. An increase in PR3-ANCA level detected by direct ELISA was consistently associated with an increased risk of severe relapse within 1 year throughout all analyzed disease subsets, except for patients who had only disease manifestations attributable to necrotizing granulomatous inflammation at enrollment. In contrast, such an association was detected by capture ELISA only in patients randomized to receive rituximab (Table 2).

Type of relapse. The type of relapse screened for also seems to be a relevant factor affecting the relationship. The increase in risk of severe relapse within 1 year

Table 3. Kaplan-Meier estimates for time to relapse following an increase in PR3-ANCA level*

	Median time to relapse, months	Cumulative relapse, percent of patients (95% confidence interval)		
		6 months	12 months	18 months
All subjects with an increase in PR3-ANCA level				
Any relapse (n = 52)	11.8	33 (19–45)	50 (33–63)	60 (43–72)
Severe relapse (n = 60)	19.1	27 (15–38)	43 (29–55)	49 (34–61)
Renal involvement at enrollment				
Any relapse (n = 31)	19.1	30 (12–45)	46 (24–61)	46 (24–61)
Severe relapse (n = 37)	22.5	25 (9–38)	40 (21–55)	44 (24–59)
Alveolar hemorrhage at enrollment				
Any relapse (n = 15)	5.2	53 (20–73)	69 (32–86)	77 (39–91)
Severe relapse (n = 17)	8.5	41 (12–60)	61 (28–79)	67 (34–84)
Rituximab treatment				
Any relapse (n = 27)	11.5	35 (14–51)	57 (32–63)	65 (40–80)
Severe relapse (n = 34)	11.8	30 (13–45)	50 (29–65)	57 (36–71)
Cyclophosphamide treatment				
Any relapse (n = 25)	17.2	32 (11–48)	44 (21–61)	54 (29–71)
Severe relapse (n =26)	22.5	23 (5–38)	35 (14–51)	40 (17–56)

* Analyses included only individuals who experienced an increase in the level of antineutrophil cytoplasmic antibodies (ANCAs) against proteinase 3 (PR3), as detected by direct enzyme-linked immunosorbent assay, during follow-up while at risk for the specified type of relapse. Increases detected concurrent with the specified type of relapse were treated as if no increase had occurred. Time zero corresponds to the date of the increase in PR3-ANCA level. The number of patients with an increase before "severe relapse" is higher than that before "any relapse" because for each grouping, 1 or more patients experienced an increase in PR3-ANCA level concurrent with or after a non-severe relapse, while still "at risk" for (before the occurrence of) a severe relapse.

following an increase in PR3-ANCA level detected by direct ELISA was consistently higher than that for any relapse throughout all subgroups (Table 2). Of the 42 patients experiencing a severe relapse in this cohort, 32 had a preceding increase in PR3-ANCA level detected by direct ELISA, with 23 experiencing a relapse within 1 year and 9 experiencing a relapse later. Four patients had an increase in PR3-ANCA level detected at the time of relapse, while only 6 of the 42 patients (14.3%) had a severe relapse without a previous or concurrent increase in PR3-ANCA level. This compares to 16 of the 55 patients (29.1%) with any flare not having experienced a preceding or concurrent increase in PR3-ANCA level (Figure 1).

Clinical phenotype. To determine whether the risk of relapse conveyed by an increase in PR3-ANCA level differs by clinical disease phenotype at presentation, we categorized patients into 5 clinically distinguishable groups: those with granulomatous disease only (n = 15), those with any granulomatous disease manifestations with or without additional manifestations attributable to capillaritis (n = 76), those with any capillaritis with or without additional granulomatous disease manifestations (n = 77), those with renal involvement (n = 60), and those with alveolar hemorrhage (n = 24) at enrollment. As expected, there was significant overlap between the 3 middle groups, but mutual exclusivity between the patients with granulomatous disease only and the alveolar hemorrhage group. We observed a gradient of c-indices for the associations of PR3-ANCA level, as detected by direct ELISA, with subsequent severe relapses ranging from 0.53 for those with granulomatous disease only, to 0.65 for those with any granulomatous disease, to 0.71 for both any capillaritis and renal involvement, to 0.81 for the alveolar hemorrhage group. The c-indices for the association with any relapse were similar, ranging from 0.49 for granulomatous disease only to 0.76 for alveolar hemorrhage. Data are presented in Table 2 and Figure 2.

In patients with renal involvement or alveolar hemorrhage at presentation, the increased risk of a severe flare within 1 year following an increase in PR3-ANCA level detected by direct ELISA was higher than for the entire cohort and higher than for any relapse (Table 2). This relationship was most pronounced for patients with alveolar hemorrhage at the time of enrollment (n = 24). Of these patients, 14 (58.3%) experienced disease relapse. The median time to any relapse following an increase detected by direct ELISA was 5.2 months in this subgroup, and the time to severe relapse was 8.5 months. As shown in Figure 2, no relapses occurred in this group without a preceding or concurrently detected increase in PR3-ANCA level using direct ELISA, while 4 patients had an increase without relapse during follow-up. In contrast, for patients who had only granulomatous disease manifestations at presentation, the risk of relapse was very high, but completely unrelated to PR3-ANCA levels.

Treatment regimen. The association of an increase in PR3-ANCA level with a subsequent relapse within 1 year differed between patients randomized to receive cyclophosphamide followed by azathioprine (conventional immunosuppression) and those who received only rituximab (Table 2). In contrast to the patients randomized to receive rituximab, no increase in risk of relapse within 1 year following an increase in PR3-ANCA level (using either assay methodology) was detected in patients treated with conventional immunosuppression. Of the 50 patients randomized to receive rituximab, 31 (62.0%) experienced disease relapse. Figure 2 shows the increases in PR3-ANCA levels and relapses for patients receiving rituximab and those receiving cyclophosphamide/azathioprine. Among those receiving rituximab, 23 experienced a severe relapse, with 18 of these being preceded by an increase in PR3-ANCA level determined by direct ELISA (14 occurring within 1 year of the increase and 4 more than 1 year after the increase). Three patients had an increase detected at the time of the severe relapse, and only 2 patients had a severe relapse without a preceding or concurrent increase in PR3-ANCA level detected by direct ELISA.

DISCUSSION

The clinical utility of serial ANCA measurements in AAV has remained a subject of controversy for almost three decades. Our analysis of serial PR3-ANCA measurements obtained in the context of a large randomized, double-blind, double-dummy controlled treatment trial and their relationship to the risk of relapse within 1 year provides novel insights with bearing on clinical practice. The information that can be derived from serial PR3-ANCA measurements is not only influenced by the assay methodology used, but more importantly by the patient-specific clinical context. Specifically, the risk of relapse conveyed by a PR3-ANCA titer increase depends on the type of relapse to be predicted, the clinical presentation of the patient, and the treatment chosen for the patient. Serial PR3-ANCA testing may be useful for the anticipation of severe relapses in patients presenting with disease manifestations caused by capillaritis, such as renal involvement or alveolar hemorrhage, or those treated with rituximab.

The methodology used for ANCA detection affects the clinical utility of serial PR3-ANCA testing. Methods with high analytical sensitivity are very useful for initial diagnostic screening, whereas analytical specificity, which often comes at the expense of analytical sensitivity, is the basis for diagnostic accuracy. While accurate determination of PR3-ANCA positivity is important for diagnostic purposes, titers are relevant as a patient-specific baseline. Assays with the highest sensitivity may not provide as much of an amplitude change over baseline compared to less sensitive assays. Our data obtained in the RAVE cohort revealed no difference in sensitivity at baseline between direct ELISA and capture ELISA (data not shown), but far fewer patients turned negative for PR3-ANCA with complete remission by capture ELISA (12 of 93) than by direct ELISA (36 of 93). This is consistent with the recognized specific performance characteristics of the two methodologies (28,44,45). Although the total number of increases detected by each assay was similar, the proportion of patients who did not go on to have a disease relapse after an increase was consistently higher using the capture ELISA, as was the number of patients who experienced disease relapse without an increase. Our findings obtained with capture ELISA in the RAVE trial cohort are consistent with our previous observations in the Etanercept Plus Standard Therapy for Wegener's Granulomatosis (WGET) cohort (14). Compared to capture ELISA, the direct ELISA was better at gauging the risk of relapses following a PR3-ANCA titer increase throughout most of the clinical subsets.

The PR3-ANCA response and the associated likelihood of relapses appear to be related to the disease phenotype. We observed no association between increase in PR3-ANCA level and risk of relapse in patients who only had disease manifestations of necrotizing granulomatous inflammation at enrollment. In contrast, the highest c-indices for any relapse and severe relapses were found in patients with alveolar hemorrhage at baseline and for severe relapses in patients with renal disease at baseline, both disease manifestations that are most clearly linked to capillaritis, rather than larger vessel or granulomatous involvement. Our findings are consistent with the recent findings of Kemna et al (15) in patients with active renal involvement at baseline. Taken together, these data imply a better relationship between PR3-ANCA levels and capillaritis than between PR3-ANCA levels and necrotizing granulomatous inflammation. This is consistent with experimental investigations of the pathogenic role of ANCA. In vitro studies have shown that ANCA can induce neutrophil adhesion to endothelial cells, as well as neutrophil degranulation (46,47), both hallmarks of capillaritis. Moreover, some animal models of PR3-ANCA disease develop capillaritis involving the kidneys and lungs but not granulomatous inflammation (48,49).

In this study, the risk of relapse following an increase in PR3-ANCA level was higher in the rituximab group than in the cyclophosphamide/azathioprine group regardless of disease phenotype. Since most increases in PR3-ANCA levels in the rituximab group occurred following B cell reconstitution, this finding might also suggest that an increase in PR3-ANCA level occurring in a patient not actively receiv-

ing immunosuppressive therapy is of different clinical significance than an increase occurring in a patient receiving maintenance immunosuppression. For patients treated with cyclophosphamide/azathioprine, our study confirms the findings of previous studies that have led to the widely accepted conclusion that when the disease is in complete remission, intensification of conventional immunosuppression is not justified in response to an increase in PR3-ANCA titer (14,16,19,22,24,25). However, in the subset of patients presenting with renal disease or alveolar hemorrhage, an increase in PR3-ANCA levels may convey an increased risk of subsequent flare regardless of therapy choice.

This is the first study to formally evaluate the clinical utility of serial PR3-ANCA testing following remission induction with rituximab in a prospective blinded treatment trial. Even though the risk of relapse following an increase in PR3-ANCA titer was higher in rituximab-treated patients than in the conventional treatment group, a sizable proportion of PR3-ANCA titer increases were not followed by a relapse during the observation period. The risk of retreatment thus needs to be carefully weighed against the risk associated with continued observation in this treatment group, too.

The role of rituximab in remission maintenance remains under investigation. The scheduled application of 500 mg of rituximab every 6 months after remission induction with cyclophosphamide has been shown to be superior to remission maintenance with azathioprine (50). The scheduled dosing of 1,000 mg of rituximab every 4 months regardless of B cell counts and ANCA level, versus azathioprine for maintenance of remission following induction with rituximab, in patients experiencing a relapse is the subject of an ongoing remission maintenance trial (ClinicalTrials.gov identifier: NCT01697267). Our findings in the rituximab-treated patients of the RAVE trial suggest that another approach, consisting of individually timed retreatment with rituximab prompted by increases in PR3-ANCA level in the context of B lymphocyte monitoring, might be an alternative that minimizes the cumulative dose of rituximab with the potential to improve the balance between disease control and immunosuppression. This approach has been used successfully at one center for patients with chronically relapsing PR3-ANCA (51), and an ongoing randomized controlled trial compares individually timed application of rituximab to fixed-interval dosing (ClinicalTrials.gov identifier: NCT01731561).

Our study has several limitations. First, the noted distinctions among various disease phenotypes are based on BVAS/WG forms completed during the RAVE trial, per the judgment of expert clinicians. We were not able to verify these further for the present analysis with additional clinical data, such as bronchoalveolar lavage findings in patients noted to have alveolar hemorrhage. Second, our study was not designed to address the effect of rare events, such as a substantial disease phenotype change between baseline and relapse.

Third, the strategy of running all serum samples from a specific patient on a single assay plate for each ELISA decreases the coefficient of variation and allows the most accurate assessment of the relative change over time. However, this is not typical for serial testing performed in routine clinical practice, where samples are rarely tested in parallel with preceding samples. For the appropriate interpretation of clinical test results, it is therefore important to be aware of the published interassay and intraassay variation of each assay used. The specific assays evaluated here, the MCPR3-2 capture ELISA and the Euroimmun direct ELISA, allow for comparison of our findings with previous results obtained using these well characterized assays (14,28,52) but should not simply be extrapolated to other assays.

Fourth, the intervals between PR3-ANCA measurements in our patients were 3 months up to month 18, and 6 months thereafter. Since the goal was to determine the utility of PR3-ANCA levels to predict relapses, concurrently detected increases were treated as if no increase had occurred for all analyses. It is possible that more frequent measurements of PR3-ANCA could have identified an increase earlier in these patients, as suggested by the 2012 meta-analysis of the role of serial ANCA measurements, which found that a higher frequency of testing was associated with better prediction of relapses (34). This might be particularly useful for rituximab-treated patients, since all but one of the relapses with concurrently detected PR3-ANCA increases occurred in rituximab-treated patients. Additional studies are needed to determine the impact of more frequent measurements or alternative means of comparing a change in levels over time.

Finally, in the present study, we examined only patients with PR3-ANCA since the greater number of patients and relapse events in this group better allowed us to evaluate the prediction of relapses. Further study is needed to determine the relationship between MPO-ANCA levels and disease relapse.

In conclusion, the risk of relapse following a PR3-ANCA titer increase after complete remission is partially dependent on the methodology used to monitor PR3-ANCA, the phenotype of disease presentation, and the treatment chosen to induce remission. Within the overall study population, an increase in ANCA titer was poorly predictive of a subsequent disease flare. However, among the subset of patients with renal disease or alveolar hemorrhage, and among patients treated with rituximab, an increase in

ANCA titer had greater predictive value for subsequent relapse. These findings should be confirmed in an independent cohort. Furthermore, while serial measurements of PR3-ANCA may portend an increased risk of relapse in this subset of patients with AAV, the risk of relapse needs to be weighed carefully against any risks associated with therapy.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Specks had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Fussner, Schroeder, Spiera, Stone, Specks.

Acquisition of data. Fussner, Hummel, Schroeder, Silva, Cartin-Ceba, Snyder, Hoffman, Kallenberg, Langford, Merkel, Monach, Seo, Spiera, St.Clair, Tchao, Stone, Specks.

Analysis and interpretation of data. Fussner, Schroeder, Cartin-Ceba, Merkel, Tchao, Stone, Specks.

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