

Circulating Markers of Vascular Injury and Angiogenesis in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis

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Objective. To identify biomarkers that distinguish between active antineutrophil cytoplasmic antibody

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(ANCA)–associated vasculitis (AAV) and remission in a manner superior or complementary to established markers of systemic inflammation.

Methods. Markers of vascular injury and angiogenesis were measured before and after treatment in a large clinical trial in AAV: 163 subjects enrolled in the Rituximab in ANCA-Associated Vasculitis trial were screened for the present study. Serum levels of E-selectin, intercellular adhesion molecule 3 matrix metalloproteinase protein 1 (MMP-1), MMP-3, MMP-9, P-selectin, thrombomodulin, and vascular endothelial growth factor were measured at study screening (time of active disease) and at month 6. Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels had been measured at the time of the clinical visit. The primary outcome measure was the difference in marker level between screening and month 6 among patients whose disease was in remission (Birmingham Vasculitis Activity Score for Wegener's granulomatosis [BVAS/WG] score of 0) at month 6.

Results. All patients had severe active vasculitis at screening (mean \pm SD BVAS/WG score 8.6 ± 3.2). Among the 123 patients whose disease was clinically in remission at month 6, levels of all markers except E-selectin showed significant declines. MMP-3 levels were also higher among the 23 patients with active

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disease at month 6 than among the 123 patients whose disease was in remission. MMP-3 levels correlated weakly with ESR and CRP levels.

Conclusion. Many markers of vascular injury and angiogenesis are elevated in severe active AAV and decline with treatment, but MMP-3 appears to distinguish active AAV from remission better than the other markers studied. Further study of MMP-3 is warranted to determine its clinical utility in combination with conventional markers of inflammation and ANCA titers.

The disease group of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) includes granulomatosis with polyangiitis (Wegener's) (GPA) and microscopic polyangiitis (MPA), entities that share the features of necrotizing vasculitis of small blood vessels in multiple organ systems and positivity for ANCA. Before effective treatments were discovered, AAV was usually fatal after a monophasic illness. Aggressive immunosuppressive therapy has not led to cure, but instead has converted GPA and MPA into chronic diseases. Relapse is common but not universal, unpredictable in its timing, and highly variable in severity. Most patients require long-term immunosuppressive therapy to reduce the risk of severe relapse or to control musculoskeletal, constitutional, or upper airway symptoms.

Because of the highly variable course of disease, long-term management of AAV is challenging. Changes in ANCA titers correlate with changes in disease activity, but discordance between ANCA status and clinical status is high (1–5); in one large study, changes in proteinase 3 (PR3) ANCA titers explained only 8% of the observed changes in disease activity (5). Levels of the generic inflammation markers erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) are typically elevated in active AAV (6–8) but, in addition to being nonspecific with regard to other inflammatory conditions, these markers do not distinguish active AAV from remission as well as might be expected (6,8). Additional markers are needed to guide therapy and help distinguish highly active disease, mildly active disease, and remission.

Markers of vascular injury and the linked process of angiogenesis are of particular interest in vasculitis and have been investigated as biomarkers in AAV. For example, thrombomodulin is released by damaged endothelial cells, P-selectin is released by platelets activated by damaged microvessels, vascular endothelial growth factor (VEGF) is an inducible mediator of

vascular permeability and of angiogenesis following tissue damage, and multiple matrix metalloproteinases (MMPs) are induced during angiogenesis and tissue remodeling. Several of these markers have been reported to be elevated in patients with active AAV, in comparison to healthy controls, in comparison to patients whose disease is in remission, or both (9–14). Evaluation of these markers in a larger, independent cohort is needed.

Utilizing serum specimens collected during the conduct of a randomized, controlled, clinical trial of AAV, we performed a study to determine if markers of vascular injury and angiogenesis distinguish between active AAV and remission. We also assessed whether any of the newly identified markers distinguish important subsets of active AAV.

PATIENTS AND METHODS

Study design. Subjects for this study were enrolled in the Rituximab in ANCA-Associated Vasculitis (RAVE) trial, conducted by the RAVE-Immune Tolerance Network (RAVE-ITN) Research Group; members of the RAVE-ITN Research Group are listed in Appendix A. Samples from all subjects who completed 6 months in their original treatment groups and had adequate volumes of serum obtained at both the screening and month 6 visits ($n = 146$) were used in this study. Additional samples from screening visits were used from subjects who completed only 4 months in the trial ($n = 6$) and from subjects who were crossed over to the other treatment group during the first 6 months ($n = 11$).

The primary outcome measure in this study was the difference in the levels of various markers between active AAV (at screening) and remission (at month 6) in the same patients, as determined by analysis of the absolute changes in marker levels and analysis of receiver operating characteristic (ROC) curves. Secondary outcome measures included differences in marker levels between patients whose AAV was in remission and patients with persistent or recurrent active AAV at month 6, differences in marker levels among predefined clinical subsets at screening, and association of marker levels with the clinically apparent extent of disease (total Birmingham Vasculitis Activity Score for Wegener's granulomatosis [BVAS/WG] [15]) at screening.

Summary of clinical trial and clinical outcome measures. The RAVE trial was a randomized, double-blind, multicenter clinical trial that compared standard remission-induction therapy using oral cyclophosphamide (CYC) and glucocorticoids to experimental treatment using the B cell-depleting agent rituximab (RTX) and glucocorticoids, in 197 patients with new or recurrent, severe AAV (GPA or MPA) (16). All patients tested positive for antibodies to either PR3 or myeloperoxidase (MPO). Subjects randomized to receive CYC were switched to maintenance therapy with azathioprine (AZA) if their disease was clinically in remission between months 4 and 6. Subjects in the RTX arm were not prescribed a maintenance agent, but the great majority still had no

detectable B cells at month 6 and were therefore still considered to be “on treatment” at this time point. Ongoing or recurrent severe disease during the first 6 months led to blinded crossover to the other treatment arm or withdrawal from blinded treatment, and such patients were regarded as treatment failures in determining the clinical end points. Per protocol, glucocorticoid (prednisone) treatment was completely withdrawn before 6 months. Investigators had the option to reinstitute prednisone at no more than 10 mg/day to control recurrent symptoms of mild disease. The rates of achievement of remission were equivalent in the 2 treatment arms (16).

Activity of vasculitis, in the RAVE trial and in this ancillary study, was assessed using the BVAS/WG, in which each severe disease manifestation is assigned 3 points and each nonsevere (mild) manifestation is assigned 1 point. Remission is defined as a BVAS/WG score of 0; severe disease is defined as the presence of 1 or more severe manifestations. Every patient had at least 1 severe manifestation and a BVAS/WG score of at least 3 at screening. The primary clinical end point in the RAVE trial was the proportion of patients in each group whose disease was in remission at month 6 and were not taking prednisone. An important secondary end point was the proportion of patients whose disease was in remission at month 6 with or without prednisone at ≤ 10 mg/day.

All patients at all participating sites were enrolled using institutional review board (IRB)-approved protocols.

Clinical subgroups. The following subgroups were defined, based on data obtained at the screening visit: GPA versus MPA, PR3 ANCA versus MPO ANCA, active versus inactive renal disease, newly diagnosed versus relapsing disease, already taking versus not taking glucocorticoids, and already taking versus not taking any immunosuppressive drug (including glucocorticoids).

Selection and processing of serum samples. Serum was collected, processed, and stored at each study site at the time of study visits, subsequently shipped to a central repository, and then shipped to the study laboratory. All samples were kept frozen at -80°C until the day the assays were performed.

Biomarker assays. Levels of E-selectin, intercellular adhesion molecule 3 (ICAM-3), P-selectin, and thrombomodulin (as a 4-plex); MMP-1, MMP-3, and MMP-9 (as a 3-plex); and VEGF as a single assay were measured in serum using commercial electrochemiluminescence assays (17–19) according to the protocols of the manufacturer (Meso Scale Discovery). Standard curves were included on each plate. Laboratory personnel who obtained and processed the assay data were blinded with regard to the clinical data associated with the samples.

The precision of these assays has not been reported for serum. Therefore, in an independent set of 200 serum samples from patients with various vasculitides, levels of markers were measured in duplicate in order to estimate coefficients of variation (CVs). CVs were low for E-selectin (7.7%), P-selectin (9.4%), MMP-9 (6.1%), thrombomodulin (6.3%), and VEGF (7.0%), but were higher for ICAM-3 (12.7%), MMP-1 (18.3%), and MMP-3 (39.8%).

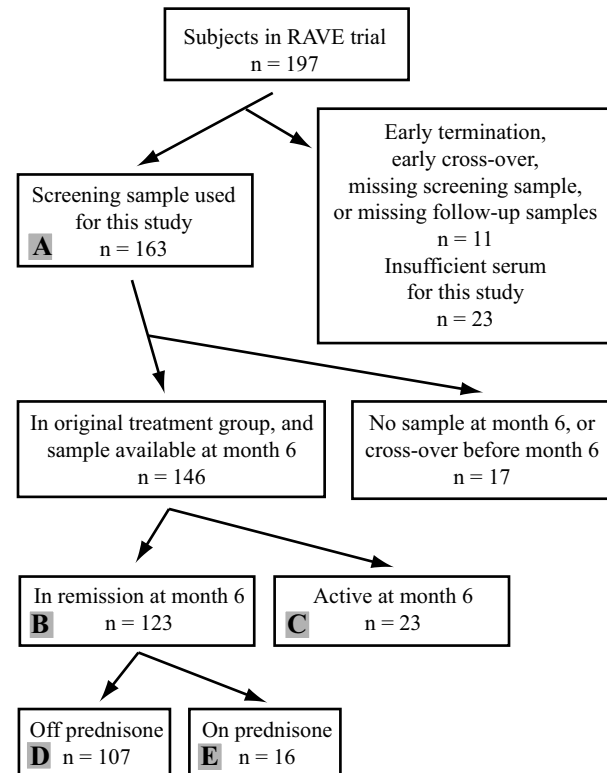


Figure 1. Disposition of the patients in the Rituximab in Antineutrophil Cytoplasmic Antibody (ANCA)-Associated Vasculitis (RAVE) trial in relation to selection and analysis of samples. B (versus subset of A) = primary analysis: comparison of marker levels in active ANCA-associated vasculitis at screening with marker levels in remission in the same 123 subjects. Group B was also used to evaluate for differential effects of treatment with cyclophosphamide/azathioprine or rituximab. B versus C = comparison of remission with relatively mild recurrent or persistent disease at month 6. A = all samples available at screening, for analysis of clinical subsets of ANCA-associated vasculitis and early effects of treatment. D versus E = comparison of the effects of low-dose prednisone among patients whose disease was in remission at month 6.

Healthy controls. Sera from 20 persons self-identified as being in good health (9 men and 11 women; median age 57 years) were collected at Boston University under an IRB-approved protocol and were assayed for all 8 experimental markers. This number of subjects was considered too low to enable strong conclusions in comparison to values obtained from patients with AAV. No reference values for healthy persons have been established for the experimental markers, and values often differ among different assays. More information was therefore gathered about reported “normal” values from the literature and from immunoassay manufacturers’ web sites. Data from several relatively large studies (≥ 50 subjects) and from manufacturers reporting values for at least 4 of the markers of interest are shown in Supplementary Table 1

(available on the *Arthritis & Rheumatism* web site at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)).

Additional laboratory data. Westergren ESR, CRP, and serum creatinine were assayed at the participating sites. Glomerular filtration rate (GFR, in ml/minute/1.73 m² body surface area) was estimated from serum creatinine (in mg/dl) using the Modification of Diet in Renal Disease study formula (20).

Statistical analysis. *Distributions, correlations, and adjustment for multiple testing.* Distributions of marker values were evaluated for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests, as well as visual inspection of histograms. Since none of the levels of any of the markers was normally distributed among patients with active vasculitis, data are reported as medians and interquartile ranges and were analyzed using nonparametric statistics. Correlations between pairs of markers were measured using Spearman's correlation coefficients. In all analyses, *P* values less than or equal to 0.05 after adjustment for multiple comparisons were considered significant; 10 markers were tested in each analysis by calculating the false discovery rate by the Benjamini and Hochberg method (21). All analyses were performed using SAS 9.1 (SAS Institute).

Distinguishing active AAV from remission. As described above, the primary outcome measure was the change in marker level from screening to month 6 among patients whose disease was in remission at month 6. The signed rank test was used to compare the distribution of the absolute changes in marker levels to the predicted null value. ROC curves were constructed using logistic regression, with marker level as the independent variable and AAV activity as the dichotomous (active or remission) dependent variable, and areas under these ROC curves (AUCs) were interpreted as indices of the markers' ability to distinguish active AAV from remission. An optimal cut point for distinguishing active AAV from remission was determined using the Youden index (22), which is the maximum sum of sensitivity and specificity.

Clinical subsets, renal function, and extent of active disease. Marker levels in clinical subsets were compared by Wilcoxon test. The effects of GFR on marker levels, performed on samples from patients whose disease was in remission at month 6 in order to be independent of the presence of active glomerulonephritis, were assessed by linear regression with GFR as the independent variable and marker level as the dependent variable. Association of marker levels with apparent extent of disease at screening was measured using Spearman's correlation coefficient between marker level and total BVAS/WG score. Because it is not clear that the BVAS/WG is a fully scalable linear measure, this analysis was considered supplemental to the primary analysis of active AAV versus remission.

RESULTS

Patient characteristics at screening and followup. As shown in Figure 1, 163 of the 197 patients in the RAVE trial (80 male and 83 female; median age 52 years [interquartile range 44–66]) were screened for the present study, based on the availability of sufficient serum samples and sufficient followup time. One hundred forty-six of these 163 patients had available samples from month 6 and had not undergone blinded crossover for reinduction therapy. Of these 146 patients, disease was in remission in 123 and active in 23 at month 6. Among the 123 patients whose disease was in remission, 16 were receiving prednisone ≤10 mg/day, and the remaining 107 were not receiving prednisone. The median BVAS/WG score at screening was 8 (interquartile range 6–10, range 3–16). Among the 23 patients with active disease at month 6, 4 had severe disease

Table 1. Changes in marker levels in patients with AAV transitioning from severe active disease (screening) to remission at month 6*

Marker	Screening (n = 123)	Remission (month 6) (n = 123)	Difference, screening minus remission	<i>P</i> †	Active disease (month 6) (n = 23)	<i>P</i> ‡
VEGF, ng/ml	0.666 (0.391, 1.14)	0.477 (0.283, 0.721)	0.139 (−0.007, 0.512)	<0.0001	0.612 (0.411, 1.120)	0.11
E-selectin, ng/ml	13.4 (10.1, 16.4)	12.7 (9.59, 17.3)	0.13 (−2.40, 2.80)	0.63	13.8 (10.4, 18.1)	0.45
ICAM-3, ng/ml	2.13 (1.61, 2.84)	1.70 (1.35, 2.29)	0.34 (−0.09, 0.95)	<0.0001	1.65 (1.11, 2.14)	0.24
P-selectin, ng/ml	120 (95.0, 150)	109 (83.7, 125)	16.2 (−4.84, 37.9)	<0.0001	108 (92.9, 121)	1.0
Thrombomodulin, ng/ml	5.27 (3.76, 6.95)	4.71 (3.80, 6.42)	0.43 (−0.67, 1.38)	0.0005	4.41 (3.32, 5.93)	0.47
MMP-1, ng/ml	27.5 (18.8, 50.4)	18.2 (11.6, 29.1)	9.20 (1.90, 21.9)	<0.0001	18.5 (12.3, 23)	0.64
MMP-3, ng/ml	71.6 (35.1, 127)	13.5 (9.69, 21.8)	54.1 (16.5, 103)	<0.0001	24.5 (13.6, 76.7)	0.005
MMP-9, ng/ml	332 (212, 554)	130 (87.5, 190)	196 (76.3, 383)	<0.0001	161 (122, 222)	0.046
ESR, mm/hour	39 (18, 61)	13 (7, 24)	20 (3, 39)	<0.0001	23 (10, 44)	0.038
CRP, mg/dl	1.2 (0.5, 3.9)	0.5 (0.3, 1.2)	0.7 (0, 3.1)	<0.0001	0.65 (0.3, 1.5)	0.60

* Values are the median (interquartile range). VEGF = vascular endothelial growth factor; ICAM-3 = intercellular adhesion molecule 3; MMP-1 = matrix metalloproteinase 1; ESR = erythrocyte sedimentation rate; CRP = C reactive protein.

† Significance of the difference between the value at screening and the value at month 6 when antineutrophil cytoplasmic antibody-associated vasculitis (AAV) was in remission, determined by signed rank test and adjusted for multiple comparisons.

‡ Significance of the difference between the value at month 6 among patients whose disease remained active and the value among those whose disease was in remission, determined by Wilcoxon test and adjusted for multiple comparisons.

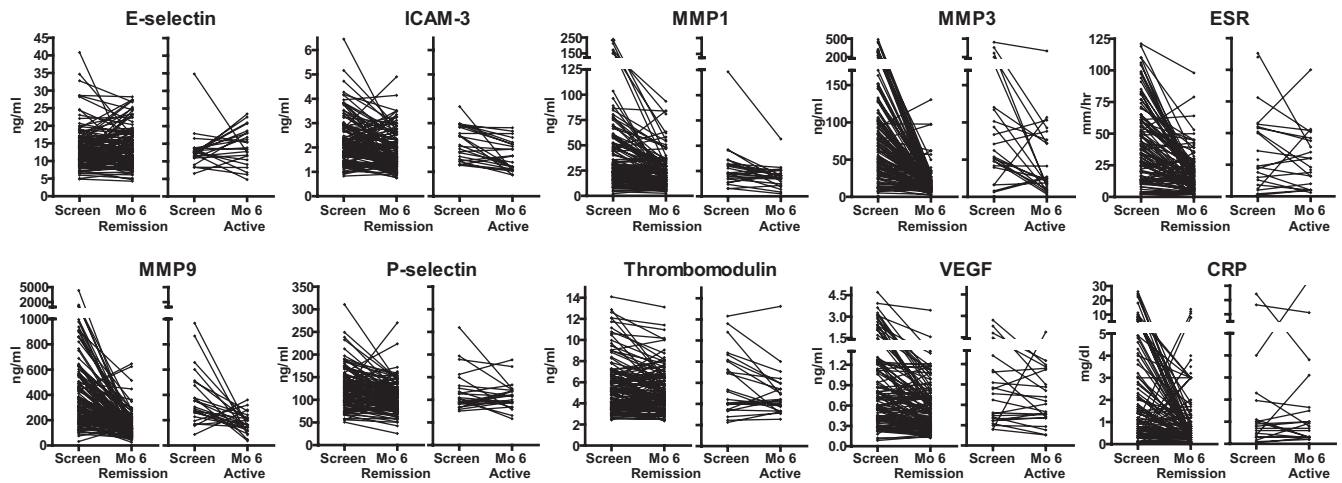


Figure 2. Marker levels in patients with antineutrophil cytoplasmic antibody-associated vasculitis at screening and at month 6, stratified by remission ($n = 123$) or recurrent disease ($n = 23$) at month 6. Each line connects the data obtained on 1 patient. ICAM-3 = intercellular adhesion molecule 3; MMP-1 = matrix metalloproteinase 1; ESR = erythrocyte sedimentation rate; VEGF = vascular endothelial growth factor; CRP = C-reactive protein.

(BVAS/WG scores 4–8) and 19 had nonsevere disease (BVAS/WG scores 1–2).

Of the 163 patients evaluated at screening, 127 had been diagnosed as having GPA and 36 as having MPA, 113 were positive for anti-PR3 and 50 for anti-MPO, and 80 had active glomerulonephritis. Seventy-seven of the patients had a new diagnosis of AAV and 86 had established diagnoses with a severe relapse. At screening, 79 patients were receiving glucocorticoids, and 90 were receiving some immunosuppressive drug

(glucocorticoids, other drugs, or both); per protocol, initiation or escalation of immunosuppressive drugs to treat the current episode of AAV occurred <14 days before initiation of randomized treatment with CYC or RTX.

Marker levels during severe AAV and remission.

Table 1 and Figure 2 show the results of marker measurements at screening (when all patients had severe active disease) and month 6. The ESR and serum levels of CRP, ESR, ICAM-3, MMP-1, MMP-3, MMP-9,

Table 2. Discrimination between active vasculitis and remission by different markers in patients who had active AAV at screening and remission at month 6*

Marker	AUC of ROC curve	Optimal cut point†	Sensitivity for active AAV, %‡	Specificity for active AAV, %§
VEGF	0.64	0.747	44	78
ICAM-3	0.63	1.64	74	49
P-selectin	0.62	129	42	80
Thrombomodulin	0.56	5.07	55	59
MMP-1	0.68	18.7	76	52
MMP-3	0.89	33.2	79	90
MMP-9	0.86	234	73	87
ESR	0.77	31	60	86
CRP	0.68	0.85	60	69

* AUC = area under the curve; ROC = receiver operating characteristic (see Table 1 for other definitions).

† Youden index, i.e., the maximum sum of sensitivity and specificity. Values are ng/ml except for ESR (mm/hour) and CRP (mg/dl).

‡ Percentage of patients in whom the marker level at screening was higher than the optimal cut point ($n = 119$ for ESR and CRP; $n = 123$ for all other markers).

§ Percentage of patients whose disease was in remission at month 6 in whom the marker level was lower than the optimal cut point ($n = 119$ for ESR and CRP; $n = 123$ for all other markers).

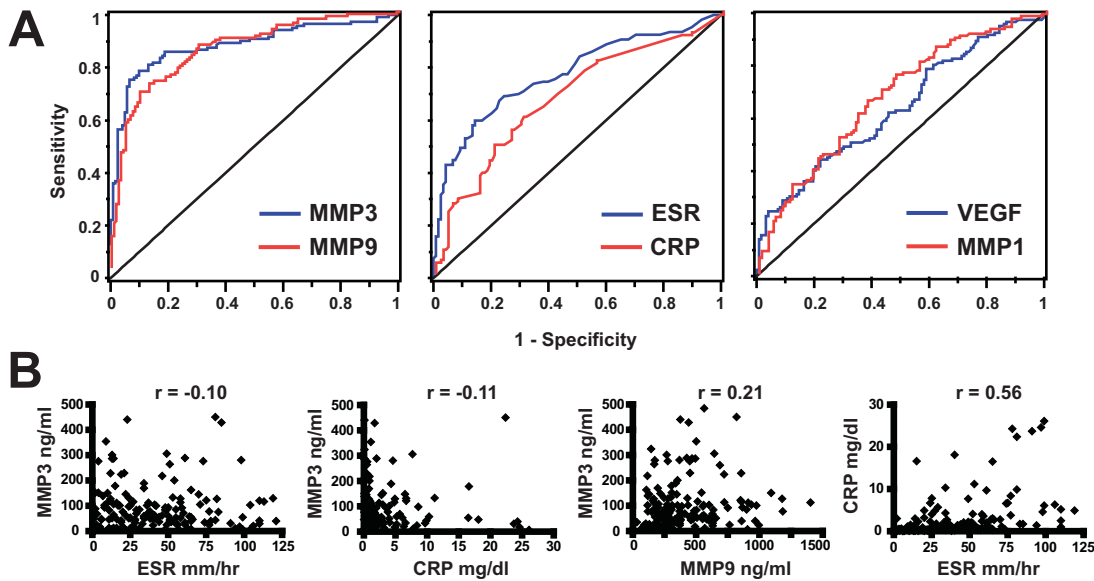


Figure 3. Comparison of the ability of various markers to distinguish active antineutrophil cytoplasmic antibody-associated vasculitis (AAV) from AAV in remission. **A**, Receiver operating characteristic curves of selected markers, constructed using the values obtained during active disease and remission in 123 patients. **B**, Comparison of the values of selected pairs of markers in patients with active AAV (at screening); r values are the Spearman correlation coefficients. See Figure 2 for other definitions.

P-selectin, thrombomodulin, and VEGF were all significantly higher ($P < 0.05$ after adjustment for 10 simultaneous comparisons) at screening than at month 6 in the 123 patients whose disease was in remission at 6 months (Table 1). Only E-selectin showed no significant change.

ROC analysis was used to better define how well the different markers were able to distinguish active AAV from remission in these 123 patients. As shown in Table 2 and Figure 3A, MMP-3 and MMP-9 distinguished disease states well ($AUC > 0.8$), whereas performance of the other experimental markers was modest ($AUC < 0.7$). Using a cutoff value of 33.2 ng/ml (as calculated using the Youden index), MMP-3 had a sensitivity of 0.79 for active AAV and a specificity (referring to AAV in remission, not to persons in good health or with other diseases) of 0.9. For MMP-9, sensitivity was 0.73 and specificity was 0.87 at a cutoff value of 234 ng/ml.

Review of data on marker levels in sera from healthy persons, using either the same assay platform (our own 20 controls, and data from the manufacturer) or different assays, was informative for further comparison of MMP-3 and MMP-9 as biomarkers (see Supplementary Table 1, available on the *Arthritis & Rheuma-*

tism web site at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)). Concentrations of MMP-3 are similar in different assay systems, and the majority of values seen in patients with active AAV in this study appeared to be above the normal range. In contrast, levels of MMP-9 vary widely with different assays, and it appears likely that most of the values we obtained in patients with active AAV were within the normal range.

Marker levels in AAV at different levels of severity. At screening, at which time each patient had at least 1 severe manifestation of AAV but total BVAS/WG scores ranged from 3 to 16, levels of several markers correlated with total BVAS/WG scores, but the correlations were modest, with coefficients never exceeding 0.3 (Supplementary Figure 1, [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)). MMP-3, which showed the strongest association with active AAV relative to remission, showed no correlation with BVAS/WG scores.

Twenty-three patients had active disease at month 6. After adjustment for multiple comparisons, only MMP-3 showed significantly higher levels in patients with recurrent or persistent, usually mild disease, compared with patients whose disease was in remission at month 6 (Table 1). Levels of ICAM-3, MMP-1,

Table 3. Markers whose levels differed significantly among patients stratified by clinical subtype or treatment*

	VEGF, ng/ml	ICAM-3, ng/ml	Thrombomodulin, ng/ml	MMP-1, ng/ml	MMP-3, ng/ml	MMP-9, ng/ml	ESR, mm/hour
AAV type							
GPA (n = 127)		1.98 (1.47, 2.71)	4.96 (3.54, 6.53)			368 (235, 569)	
MPA (n = 36)		2.79 (2.17, 3.35)	6.97 (4.16, 8.69)			280 (180, 370)	
P		0.0001	0.003			0.014	
ANCA specificity							
PR3 (n = 113)		1.98 (1.47, 2.72)	4.78 (3.49, 5.97)				
MPO (n = 50)		2.36 (1.89, 2.96)	6.94 (4.18, 8.83)				
P		0.003	<0.0001				
New or established (relapsing) AAV							
New (n = 77)	0.914 (0.592, 1.33)	2.49 (2.04, 2.98)	5.81 (4.44, 7.86)				49 (26, 79.5)
Relapsing (n = 86)	0.430 (0.316, 0.720)	1.78 (1.39, 2.36)	4.41 (3.43, 6.15)				34 (13v52)
P	<0.0001	<0.0001	<0.0001				0.001
Active renal disease							
Yes (n = 80)			6.94 (5.36, 8.79)		95.4 (52.2, 163)		
No (n = 83)			3.96 (3.32, 4.78)		50.9 (29.3, 93.7)		
P			<0.0001		<0.0001		
Treatment at screening							
Yes (n = 90)	0.461 (0.367, 1.46)	1.84 (1.43, 2.42)	4.44 (3.43, 6.18)	23.0 (17.4, 31.5)			31 (13, 54)
No (n = 73)	0.812 (0.539, 1.28)	2.43 (1.83, 2.97)	5.87 (4.51, 7.78)	35.8 (22.6, 60.4)			50 (25, 75)
P	0.001	0.001	0.0003	0.0008			0.006
Glucocorticoids at screening							
Yes (n = 79)		1.83 (1.43, 2.42)	4.41 (3.42, 5.53)	22.9 (18.1, 30.4)			29 (13, 52)
No (n = 84)		2.37 (1.79, 2.96)	5.73 (4.18, 7.27)	32.3 (21.8, 55.7)			49 (25, 70)
P		0.0003	0.001	0.002			0.008

* For each clinical subtype or treatment category, data on the markers whose levels were significantly different between the 2 groups ($P < 0.05$ by Wilcoxon test after adjustment for multiple comparisons) are shown. (Data on all markers are shown in Supplementary Tables 2–4, on the *Arthritis & Rheumatism* web site at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).) Values are the median (interquartile range). GPA = granulomatosis with polyangiitis (Wegener's); MPA = microscopic polyangiitis; ANCA = antineutrophil cytoplasmic antibody; PR3 = proteinase 3; MPO = myeloperoxidase (see Table 1 for other definitions).

MMP-3, and MMP-9 declined significantly compared to the levels at screening among the 23 patients who had active disease at month 6 (analysis not shown, but see Figure 2). Neither P-selectin nor thrombomodulin showed a significant decline. However, these latter findings can probably be attributed to the smaller numbers of patients in this analysis, since the median values of P-selectin and thrombomodulin were similar among the 23 patients with active disease and the 123 whose disease was in remission (see Table 1).

Marker levels in clinical subgroups. Among patients with active AAV at screening, those with GPA had higher levels of MMP-9 and lower levels of ICAM-3 and thrombomodulin than did those with MPA. Not surprisingly, these trends were similar in groups defined by PR3 or MPO ANCA specificity (Table 3 and Supplementary Table 2, [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)).

Levels of MMP-3 and thrombomodulin were higher in patients with active renal disease than in those without (Table 3 and Supplementary Table 3, [http://](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)

[onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)). Since the effects of active renal disease are not easily distinguishable from effects of GFR with these data, the effect of GFR on levels of these markers among patients whose disease was in remission at month 6 was also measured. GFR affected levels of both thrombomodulin (0.54 ng/ml increase per 10 ml/minute decrease in GFR) and MMP-3 (2.1 ng/ml increase per 10 ml/minute decrease in GFR) among patients with AAV in remission. However, when these values were compared to the ranges of values seen in patients with active AAV (see Table 1), GFR appeared to be a minor contributor to levels of MMP-3.

Effects of treatment. Patients already being treated with immunosuppressive drugs at screening had lower ESRs, and lower levels of ICAM-3, MMP-1, thrombomodulin, and VEGF than did untreated patients (Table 3 and Supplementary Table 4, [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)). Levels of 4 of these markers (all except MMP-1) were also higher in patients with newly diagnosed AAV

than in those with relapsing disease (Table 3 and Supplementary Table 3). Effects of treatment during the first 6 months were also evaluated. Among patients whose disease was in remission at month 6, only levels of MMP-1 differed between those who had received CYC/AZA and those who had received RTX (Supplementary Table 5, [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)). Patients who had resumed treatment with prednisone (≤ 10 mg/day) had somewhat higher levels of MMP-3 and MMP-9 than those who were not receiving glucocorticoids (Supplementary Table 5). The most important conclusion to be drawn from these analyses is that the findings regarding MMP-3 cannot be explained by direct effects of particular medications.

Correlations between marker levels. The correlation coefficients between levels of the marker that best distinguished between severe active disease and remission based on ROC analysis (MMP-3) and levels of established markers of inflammation were quite low (with ESR, $r = -0.10$ at screening; with CRP, $r = -0.11$ at screening) (Figure 3B). In contrast, ESR and CRP correlated relatively well with each other ($r = 0.56$ at screening). No other correlations between pairs of markers exceeded 0.4 (data not shown). Overall, these results suggest that MMP-3 provides additional information about active AAV beyond what is obtained with ESR or CRP.

DISCUSSION

This investigation of a series of biomarkers related to microvascular damage and/or angiogenesis in a large group of patients with AAV demonstrated that many of the markers were elevated in patients with severe active AAV and declined significantly with treatment. Levels of MMP-3 distinguished active AAV from remission better than did ESR and CRP, and MMP-3 levels correlated poorly with levels of these 2 markers. MMP-3 was also the only marker that appeared to be present in higher levels among the small number of patients with milder active disease at month 6 than among those patients whose disease was in remission. These results indicate that serum MMP-3 is particularly worthy of further study as an additional laboratory test to assist in the evaluation of patients with AAV and management of the disease.

Two markers, MMP-3 and thrombomodulin, differed significantly in patients stratified by the presence or absence of active renal disease. However, since MMP-3 was also highly associated with active AAV in

general, and since thrombomodulin levels remained elevated in many patients who achieved remission, prospects for using these markers to help screen for active renal disease in AAV are limited. Since markers of damage to microvascular beds rather than markers of granulomatous disease were chosen for study, it was not surprising that levels of most markers were similar in patients with GPA or MPA. Among those that did differ, caution in interpretation may still be indicated (e.g., for thrombomodulin), since the prevalence of renal disease is higher in MPA than in GPA.

The main strengths of this study derive from the use of a large cohort of patients with clinical data collected in a standardized manner at defined times. The study was large enough to allow comparison of the performance of experimental markers to each other and to that of established markers of inflammation.

A limitation of the study is the absence of detailed information about treatment, particularly the dosing of glucocorticoids at and shortly before the screening visit. However, it was encouraging that treatment (yes or no, either limited to glucocorticoids or including all immunosuppressive drugs) at the time of screening was not associated with higher levels of any marker. Association of treatment with lower levels of several markers indicated that if early treatment had any influence on the results, it was to bias the study against showing significant differences between active AAV and remission. Strictly speaking, this study (like most others) cannot distinguish between effects of successful treatment and direct effects of treatment independent of their success; only longitudinal studies, with repeated measurements in patients who either experience flares or whose disease remains in remission with various levels of treatment (as will be possible using future samples from the RAVE trial), would allow for analysis of such distinctions. Finally, the study had limited power to assess marker levels during mild relapses and was not designed to assess prediction of future relapses or to distinguish AAV from other disease states that might elevate marker levels.

Two technical details merit discussion. First, in a separate study, the CV of measurement error for the MMP-3 assay was 39.8%. However, large measurement errors bias against finding significant differences between groups or significant changes within a subject over time (23). More importantly, MMP-3 (using reagents developed for a commercial capture enzyme-linked immunosorbent assay [R&D Systems]) was included in a multiplex assay platform that was applied to the same set

of samples (Monach PA, Warner R, Tomasson G, et al: unpublished observations), and MMP-3 levels obtained with these 2 different assays correlated highly with one another ($R^2 = 0.91$). Second, serum levels of MMP-1, P-selectin, and VEGF are reported to be higher than plasma levels (24), probably because these proteins can be released from platelets; it is not clear whether serum or plasma levels of these markers will serve as better biomarkers of inflammatory microvascular diseases.

The source of circulating MMP-3 in AAV is not apparent from review of the literature. MMP-3 can be produced by a wide range of cell types, including microvascular endothelial cells stimulated in vitro (25,26). Study of MMP-3 in the vasculature has focused on diseases of large arteries. Genetic polymorphisms in the MMP-3 gene have been associated with multiple cardiovascular outcomes related to atherosclerosis (27), and with risk of Kawasaki disease or with aneurysm formation in that disease (28–30). Circulating MMP-3 levels were found to be elevated in a cohort of patients with Takayasu arteritis (31). Indeed, in one of the two studies in which MMP-3 levels were measured and found to be elevated in patients with GPA, the marker was chosen to address the hypothesis that GPA leads to accelerated atherosclerosis (13,14). Study of MMP-3 in other autoimmune diseases has focused on its stronger association with inflammatory arthritis than with involvement of other organ systems (32). The latter study also highlighted the concern that glucocorticoid treatment may elevate levels of MMP-3 (32). However, our data provide evidence against the notion that glucocorticoids are the major driver of MMP-3 levels in AAV.

Studies of patients with severe active disease are an appropriate initial screen for markers of interest in pursuing clinically relevant goals. Investigation of MMP-3 and other promising biomarkers in samples collected during long-term followup of patients from the RAVE trial and other cohorts will allow for additional evaluation of the clinical utility of these markers in combination with other clinical and laboratory data.

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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. Merkel 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.

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