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Association of Serum Calprotectin (S100A8/A9) Level With Disease Relapse in Proteinase 3–Antineutrophil Cytoplasmic Antibody–Associated Vasculitis

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Objective. **S100A8/A9 (calprotectin) has shown promise as a biomarker for predicting relapse in antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV). This study was undertaken to investigate serum S100A8/A9 level as a biomarker for predicting future relapse in a large cohort of patients with severe AAV.**

Methods. **Serum levels of S100A8/A9 were measured at baseline and months 1, 2, and 6 following treatment initiation in 144 patients in the Rituximab in ANCA-Associated Vasculitis trial (cyclophosphamide/azathioprine versus rituximab [RTX] for induction of remission) in whom complete remission was attained.**

Results. **Patients were divided into 4 groups: proteinase 3 (PR3)–ANCA with relapse (n = 37), PR3-ANCA without relapse (n = 56), myeloperoxidase (MPO)–ANCA with relapse (n = 6), and MPO-ANCA without relapse (n = 45). Serum S100A8/A9 level decreased in all groups during the first 6 months of treatment. The percentage reduction from baseline to month 2 was significantly different between patients who experienced a relapse and those who did not in the PR3-ANCA group ($P = 0.046$). A significantly higher risk of relapse was associated with an increase in S100A8/A9 level between baseline and month 2 ($P = 0.0043$) and baseline and month 6 ($P = 0.0029$). Subgroup analysis demonstrated that patients treated with RTX who had increased levels of S100A8/A9 were at greatest risk of future relapse ($P = 0.028$).**

Conclusion. **An increase in serum S100A8/A9 level by month 2 or 6 compared to baseline identifies a subgroup of PR3-ANCA patients treated with RTX who are at higher risk of relapse by 18 months. Since RTX is increasingly used for remission induction in PR3-ANCA–positive patients experiencing a relapse, S100A8/A9 level may assist in identifying those patients requiring more intensive or prolonged treatment.**

Granulomatosis with polyangiitis (Wegener's) (GPA) and microscopic polyangiitis (MPA) are classified as antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) and commonly affect the kidneys, lungs, and other organs (1). ANCA is detected in a significant proportion of patients with active GPA as well as those with MPA, while some patients may be ANCA negative (2,3). Although serial ANCA testing is often performed to help assess disease activity or to predict relapse, its usefulness is a subject of controversy (4). Other potential biomarkers of systemic inflammation have been identified to assist in distinguishing active vasculitis from inactive disease, but not

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for predicting relapse (5). More recently, investigators using a transcriptional profiling approach identified a subset of CD8+ T cell genes that was associated with a poor prognosis in various autoimmune diseases (6), and is therefore a potential biomarker in AAV, but it has yet to be validated.

Remission followed by relapse is common in patients with AAV, especially those who are positive for proteinase 3 (PR3)–ANCA, with reported relapse rates of up to 55% within the first 3 years of treatment (7). The risk of relapse continues in the long-term follow-up of these patients, and a proportion of patients progress to end-stage renal disease (8), which may represent progressive chronic kidney disease or ongoing relapsing vasculitis. However, since reliable biomarkers for relapse have not yet been found, therapy is not highly customized for individual patients. Therefore, similar regimens are used for many patients, potentially exposing some to unnecessary prolonged immunosuppression, while others who could benefit from more intensive treatment may not be easily identified.

S100A8 and S100A9 are members of the S100 family of proteins that form a heterodimer of S100A8/A9 (termed calprotectin), which is expressed in neutrophils, monocytes, and early-differentiated macrophages but not resident tissue macrophages (9,10). S100A8/A9 heterodimer is an endogenous ligand of Toll-like receptor 4 (11) as well as the receptor for advanced glycation end products (12,13). It is secreted locally by phagocytes at the site of inflammation, where it has a number of autocrine and paracrine proinflammatory effects on phagocytes (14–16) and the endothelium (17,18), both of which are implicated in the pathogenesis of vasculitis. Serum levels of the heterodimer S100A8/A9 are increased in several inflammatory and autoimmune conditions, including juvenile idiopathic arthritis (JIA), where levels have been shown to predict disease relapse (19,20), rheumatoid arthritis (RA) (16,21), systemic lupus erythematosus (SLE) (22), and Kawasaki disease (23). Measurement of fecal calprotectin is routinely performed to detect mucosal inflammation in inflammatory bowel disease (24).

We previously demonstrated that patients with active generalized AAV had elevated serum S100A8/A9 levels compared to patients in remission and healthy controls (25). Patients with focal and crescentic glomerulonephritis demonstrated glomerular infiltration of S100A8/A9-positive macrophages. Additionally, in a cohort of patients with early limited systemic disease, persistently elevated S100A8/A9 levels despite immunosuppressive treatment were found in patients who went on to experience a relapse (25). We did not have pretreatment samples available for analysis, so we concluded that failure to suppress S100A8/A9 levels with treatment was associated with relapse. To validate the findings of that study, we proceeded to measure serum S100A8/A9 levels in a large number of patients with severe AAV enrolled in the Rituximab in ANCA-Associated Vasculitis (RAVE) trial, to investigate whether a failure to suppress S100A8/A9 with conventional treatment was predictive of future disease flares.

PATIENTS AND METHODS

Trial details. The RAVE trial was a multicenter, randomized, double-blind trial comparing rituximab (RTX) with standard cytotoxic immunosuppression for the induction of remission by 6 months in patients with severe AAV. Patients were eligible if they had newly diagnosed or relapsing GPA or MPA, positive serum assays for PR3-ANCA or myeloperoxidase (MPO)–ANCA, and severe manifestations of disease. Standard immunosuppression for induction therapy consisted of oral cyclophosphamide (CYC) and glucocorticoids, compared to the investigational therapy of RTX and glucocorticoids. Patients in the CYC group were switched to maintenance therapy with azathioprine (AZA) if complete remission was achieved between months 4 and 6. Both groups received the same reducing glucocorticoid regimen as per trial protocol. The primary end point was defined as a Birmingham Vasculitis Activity Score for Wegener's Granulomatosis (BVAS/WG) (26) of 0 and the successful discontinuation of prednisone. Disease flare was defined as an increase in BVAS/WG of 1 point or more. The 18-month follow-up data were also published (27).

Study design. Serum samples from 188 patients were obtained for this study. Serum was analyzed at baseline (time 0), and months 1, 2, and 6 after the start of treatment. The primary goal was to determine the level of serum S100A8/A9 at these different time points and the relative changes in serum levels over time, and correlate these values with the duration of complete remission and time to relapse. For the purpose of this analysis, relapse episodes were defined as a disease flare from month 6 onward. Patients who were classified as not entering complete remission during the study were excluded from the analysis. Additional clinical and laboratory data were obtained from the trial database.

Measurement of serum S100A8/A9 levels. Samples were stored at −80°C until analyzed. S100A8/A9 was analyzed by sandwich enzyme-linked immunosorbent assay according to the recommendations of the manufacturer (BioLegend).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 6.0 and Stata v11. Variables were log-transformed where distributions were skewed. Nonparametric tests of significance were applied. For comparisons of 2 groups, the Mann-Whitney U test was used. To compare the absolute values at subsequent time points compared to baseline, a mixed-effects model was used. Correlations were assessed using nonparametric Spearman's rank correlation analysis. P values less than 0.05 were considered significant. For relapse-free survival analysis, Bonferroni correction was used for multiple comparisons, with significance defined as $P < 0.025$.

Risk of relapse was investigated using Kaplan-Meier survival analysis, and the influence of baseline renal involvement and change in ANCA titer on the relationship between changes in S100A8/A9 levels and relapse was examined using the Cox proportional hazards approach. This was an exploratory study; however, a post hoc power calculation using Friedman's method

Table 1. Baseline characteristics of the patients with ANCA-associated vasculitis*

	All patients (n = 144)	Anti-PR3 without relapse (n = 56)	Anti-PR3 with relapse (n = 37)	Anti-MPO without relapse (n = 45)	Anti-MPO with relapse (n = 6)
Age, median (range) years	52.5 (15–92)	51.5 (17–77)	51 (15–74)	55 (16–92)	75.5 (48–78)
Sex, no. female/male	70/74	22/34	16/21	29/16	3/3
Ethnicity, no.					
White	134	50	37	42	5
African American	6	3	0	3	0
Other	4	3	0	0	1
New diagnosis at presentation, no.	77 (53)†	28	12	35	2
Relapse at presentation, no.	67 (47)†	28	25	10	4
Renal failure at baseline, no.	76 (53)†	27	15	31	3
BVAS/WG at baseline, median (range)	8 (3–16)	10 (4–15)	7.5 (3–14)	8 (4–16)	10 (6–12)
Treatment arm, no. of patients					
Cyclophosphamide	68	26	17	23	2
Rituximab	76	30	20	22	4

* Baseline characteristics of the patients upon entry into the Rituximab in Antineutrophil Cytoplasmic Antibody (ANCA)–Associated Vasculitis trial. The patients were divided into 4 groups based on their relapse status at the end of the trial and the type of ANCA present. Anti-PR3 = anti–proteinase 3; anti-MPO = antimyeloperoxidase; BVAS/WG = Birmingham Vasculitis Activity Score for Wegener's Granulomatosis.
† Values are the number (%).

suggested there was 60% power to detect the effect size observed in subjects with PR3, assuming a probability of relapse of 0.31 in the reference group (those with a decrease in S100A8/A9 levels) (without adjustment for multiple comparisons).

To explore the value of change in S100A8/A9 levels to predict relapse, we developed multivariable logistic regression models including clinical and laboratory parameters that might be used in clinical practice to assess the risk of relapse: age, sex, ethnicity, PR3-ANCA versus MPO-ANCA status, baseline renal involvement, RAVE treatment arm, C-reactive protein (CRP) level, and change in ANCA titer (over the same period as change in S100A8/A9 levels). The utility of the addition of change in S100A8/A9 levels to change in ANCA titer versus change in ANCA titer alone as a predictor of relapse was quantified with receiver operating curves (ROCs) generated from these logistic regression models.

RESULTS

Demographic characteristics and patient groups. Patients were divided into 4 groups depending on their ANCA subtype and whether they experienced a relapse during the 18 months of follow-up of the trial. For patients to be included in the analysis, they had to have entered complete remission, as defined by a BVAS/WG of 0 and the successful discontinuation of prednisone. Therefore, 44 patients (33 PR3-ANCA positive and 11 MPO-ANCA positive) in whom complete disease remission was not achieved were excluded from the analysis.

A total of 144 patients were included in the analysis (93 PR3-ANCA–positive patients [65%] and 51 MPO-ANCA–positive patients [35%]) (Table 1). The median age of the patients was 52.5 years (range 15–92), and 70 (49%) of the patients were women and 74 (51%) were men. Seventy-seven (53%) of the patients had a new diagnosis of AAV at the time of enrollment in the study, and 67 (47%) of the patients presented with relapsing disease. Seventy-six (53%) of the patients had renal involvement at baseline. The CYC/AZA arm included 68 patients (47%), and the RTX arm included 76 patients (53%).

Serum levels of S100A8/A9 at different time points. Serum levels of S100A8/A9 significantly decreased from baseline to months 1, 2, and 6 in the entire cohort, with a median level of 6,509 ng/ml (range 1,002–92,267) at baseline, 4,660 ng/ml (range 962–35,523) at month 1 ($P < 0.0001$ versus baseline), 4,004 ng/ml (range 1,020–25,814) at month 2 ($P < 0.0001$ versus baseline), and 3,141 ng/ml (range 346–19,383) at month 6 ($P < 0.0001$ versus baseline) (Figure 1A). The results for within-patient change using log S100A8/A9 multilevel model random intercept were highly significant at each time point. There was no significant difference in absolute S100A8/A9 level between MPO-ANCA–positive patients and PR3-ANCA–positive patients at any time point (Table 2) or between those patients who did and those who did not experience relapse at each time point, with the exception of a difference at month 2 between the PR3-ANCA–positive patients who experienced a relapse and the PR3-ANCA–positive patients who did not experience a relapse ($P = 0.05$) (Table 2). In addition, there was no difference in the absolute level of serum S100A8/A9 at any time point between those patients presenting with renal involvement (n = 76) and those presenting without renal involvement (n = 68).

Change in serum levels of S100A8/A9 between time points. We then investigated the percentage change in serum S100A8/A9 level between 2 time points in

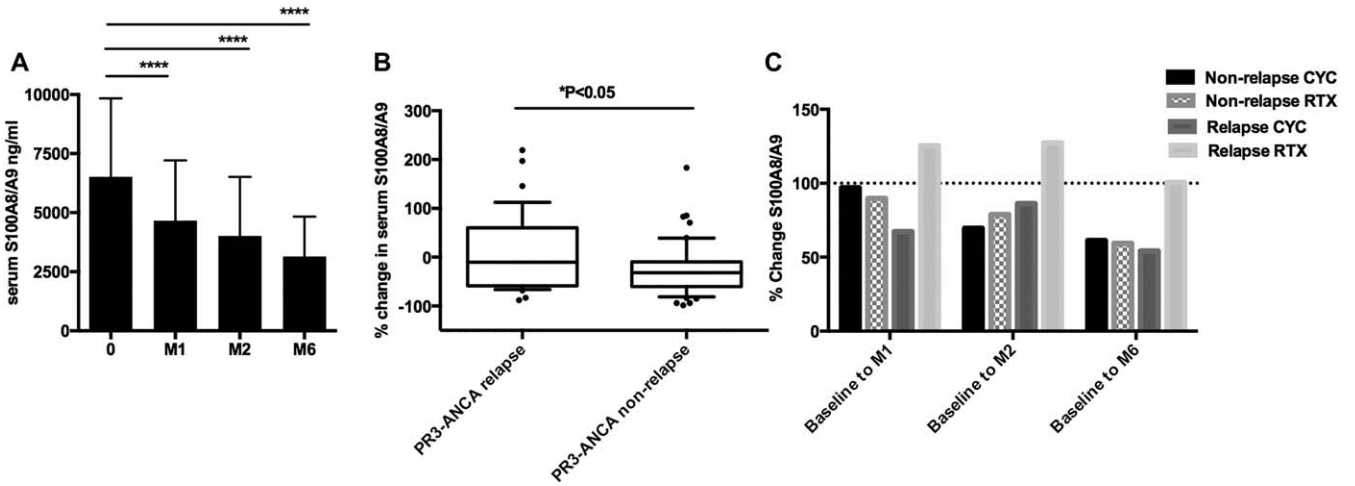


Figure 1. Percentage change in serum S100A8/A9 levels over time in patients with antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis. **A,** Absolute value of serum S100A8/A9 level in all patients (n = 144) at baseline, month 1, month 2, and month 6 after the initiation of treatment. Bars show the median and interquartile range. There were significant decreases in S100A8/A9 levels at each time point compared to baseline. **** = $P < 0.0001$. **B,** Percentage change in serum S100A8/A9 level from baseline to month 2 in proteinase 3 (PR3)–ANCA–positive patients who experienced a relapse and PR3-ANCA–positive patients who did not experience a relapse. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Circles indicate outliers. The median change in serum S100A8/A9 level from baseline to month 2 was a decrease of 10% (IQR −59%, 60%) in the group of patients who experienced a relapse and a decrease of 31% (IQR −60%, −9%) in the group of patients who did not experience a relapse ($P = 0.046$ by Mann-Whitney U paired *t*-test). **C,** Percentage change in S100A8/A9 level from baseline to months 1, 2, and 6 in PR3-ANCA–positive patients divided into 4 groups (those treated with cyclophosphamide [CYC] who did not experience a relapse, those treated with rituximab [RTX] who did not experience a relapse, those treated with CYC who did experience a relapse, and those treated with RTX who did experience a relapse).

individual patients according to relapse status. Since there were only 6 MPO-ANCA–positive patients who experienced a relapse, we focused our analysis on the PR3-ANCA–positive patient cohort.

There was a significant difference in the percentage change in serum S100A8/A9 level from baseline to month 2 between the PR3-ANCA–positive patients who experienced a relapse and the PR3-ANCA–positive patients who did not experience a relapse (Figure 1B), with patients who experienced a relapse demonstrating less suppression of serum S100A8/A9 levels. The median change in serum S100A8/A9 level at month 2 compared with baseline was a decrease of 10% in the group of patients who experienced a relapse (interquartile range [IQR] −59%, 60%), with a significantly greater degree of suppression in the group of patients who did not experience a relapse, of 31% (IQR −60%, −9%) ($P = 0.0457$). Therefore, a lower percentage reduction in serum S100A8/A9 level during the first 6 months of treatment identified a subset of patients who were at risk of disease relapse during the subsequent 12 months. The relative risk for relapse with an increase in S100A8/A9 level at month 2 compared to baseline was 1.81 (95% confidence interval [95% CI] 1.11–2.93), and the relative risk for relapse with an increase at month 6 compared to baseline was 1.76 (95% CI 1.1–2.83). Figure 1C shows the mean percentage change from baseline to month 1, baseline to month 2, and baseline to month 6. The patients are

Table 2. Serum S100A8/A9 levels in patients according to relapse status and ANCA subtype at different time points*

	All patients (n = 144)	Anti-PR3 without relapse (n = 56)	Anti-PR3 with relapse (n = 37)	Anti-MPO without relapse (n = 45)	Anti-MPO with relapse (n = 6)
Baseline	6,509 (1,002–92,267)	6,304 (1,894–92,267)	5,522 (1,341–29,920)	6,869 (1,002–29,653)	8,385 (3,174–13,639)
Month 1	4,660 (962–35,523)	4,914 (1,463–18,248)	4,649 (1,317–26,071)	4,109 (1,077–35,523)	4,632 (962.2–18,290)
Month 2	4,004 (1,020–25,814)	3,900 (1,112–9,413)	4,898 (1,798–19,152)†	3,481 (1,020–19,037)	2,651 (1,364–25,814)
Month 6	3,141 (346–19,383)	2,941 (711–19,383)	3,976 (751–10,517)	3,070 (346–12,818)	4,202 (944–18,839)
Month 12	3,428 (408–24,235)	3,283 (408–15,033)	3,304 (513–24,235)	3,428 (1,021–13,070)	3,707 (2,784–4,694)

* Values are the median (range) ng/ml. ANCA = antineutrophil cytoplasmic antibody; anti-PR3 = anti–proteinase 3; anti-MPO = antimyeloperoxidase.
† $P = 0.05$ versus anti-PR3 without relapse, by Mann-Whitney U paired *t*-test.

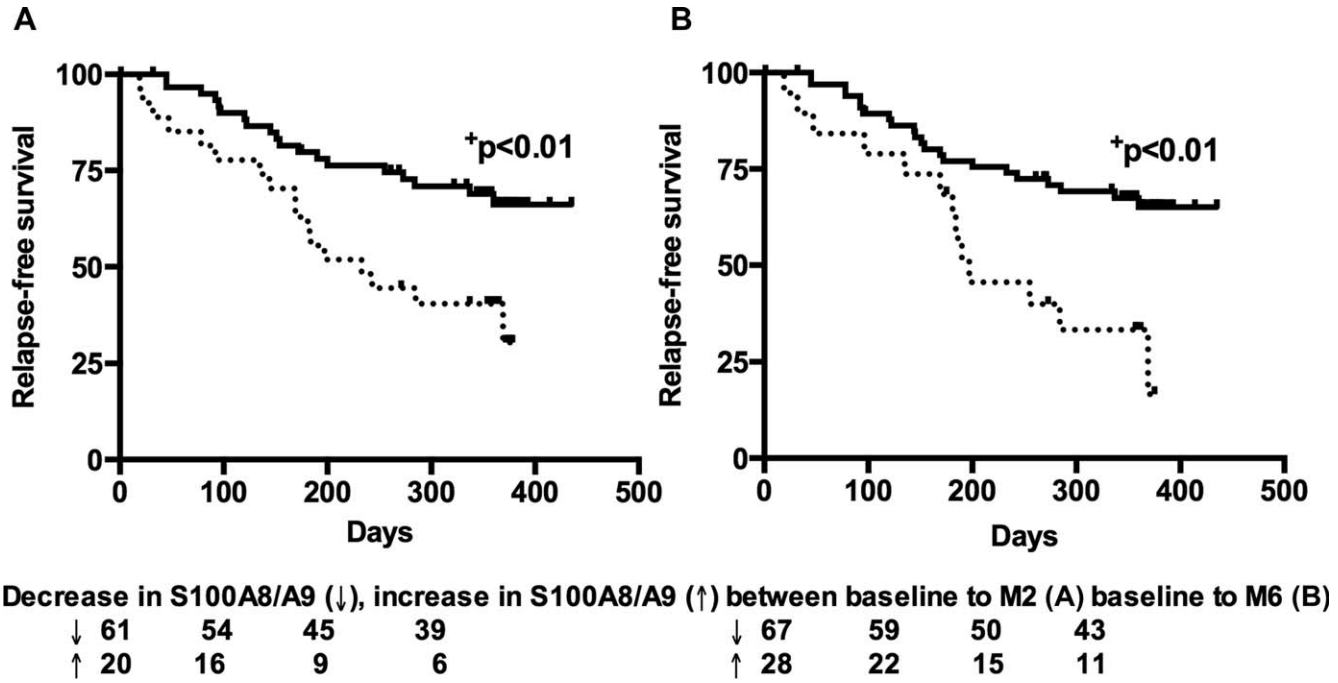


Figure 2. Kaplan-Meier curves showing relapse-free survival among patients with proteinase 3 (PR3)–antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis with a decrease in serum S100A8/A9 levels (solid lines) and those with an increase in serum S100A8/A9 levels (broken lines) from baseline to month 2 (**A**) and from baseline to month 6 (**B**). The x-axis shows days of follow-up beginning 6 months after the start of treatment. Data were adjusted for multiple comparisons, and *P* values less than 0.025 were considered significant. Values are the number of patients at risk (patients who remained relapse free). There was a significant difference between groups in relapse-free survival, with those patients with increased serum S100A8/A9 levels having a significantly lower rate of relapse-free survival ($P = 0.0043$ for baseline to month 2 and $P = 0.0029$ for baseline to month 6).

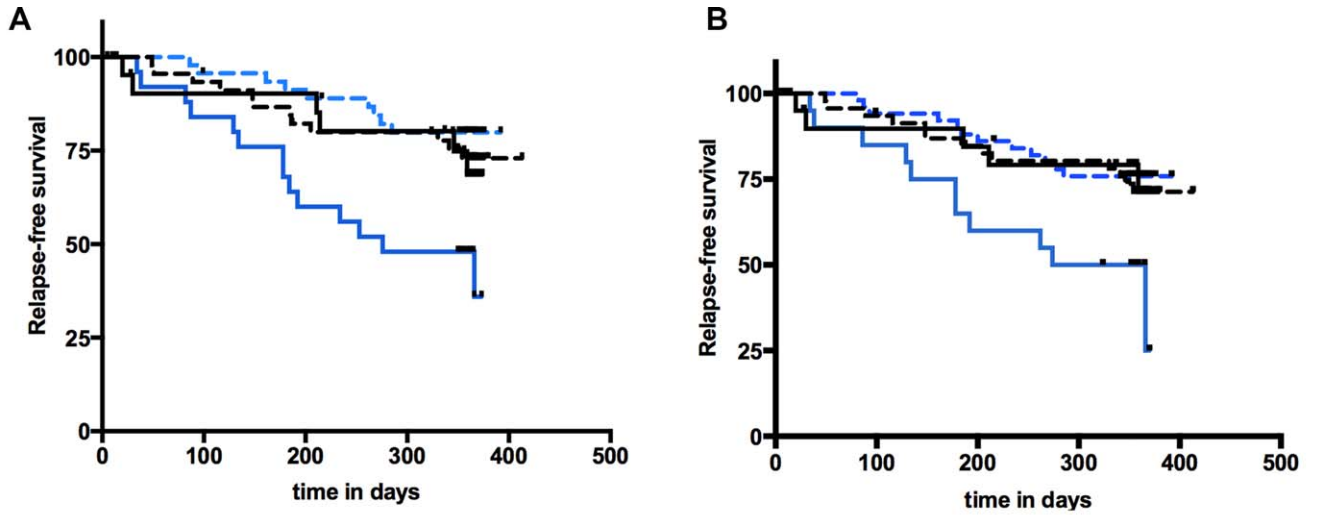
classified into 4 groups according to relapse status and treatment arm. These data demonstrate that patients treated with RTX who later experienced a relapse showed increases in serum S100A8/A9 levels between time points.

Increase in serum levels of S100A8/A9 predicts earlier relapse. When patients were categorized according to whether they had an increase or decrease in serum S100A8/A9 level between 2 time points, patients who demonstrated an increase in serum S100A8/A9 level from baseline to month 2 and from baseline to month 6 had significantly earlier and more frequent relapses than patients who showed a decrease in serum S100A8/A9 level ($P = 0.004$ for baseline to month 2 and $P = 0.003$ for baseline to month 6) (Figure 2). When these groups were divided according to the different treatment arms, the patients in the RTX treatment arm with increased levels of serum S100A8/A9 between time points demonstrated significantly earlier relapses (Figure 3).

Cox regression analysis was used to determine the risk of a future relapse for the PR3-ANCA–positive patients. An increase in ANCA titer from baseline to month 2 was associated with a hazard ratio (HR) of 0.85 (95% CI 0.55–1.13), while an increase in serum S100A8/A9 level had a significantly higher HR of 2.2 (95% CI 1.17–4.26) ($P = 0.016$). This model was unaffected by whether patients had renal involvement at trial entry, but model fit was improved by the addition of change in ANCA titer.

To further investigate the potential of serum S100A8/A9 level as a biomarker of relapse risk in patients treated with RTX early in the induction period, multivariable logistic regression models were developed. Only the addition of change in ANCA titer (over the same time period, e.g., from baseline to month 2 or month 6) improved model fit. ROC for change from baseline to month 2 demonstrated an area under the curve (AUC) of 0.55 for an increase in ANCA titer and an AUC of 0.77 for the combination of an increase in ANCA titer and an increase in serum S100A8/A9 level ($P = 0.028$ for the difference between curves). At 6 months, the AUC for an increase in ANCA titer was 0.69, while the combination of increases in both ANCA titer and serum S100A8/A9 level generated a higher AUC of 0.75 (*P* not significant).

Serum levels of S100A8/A9 in newly diagnosed versus relapsing disease. At the time of inclusion into the trial, 77 patients presented with new disease while 67 presented with relapsing disease; these subcohorts were



Decrease in S100A8/A9 (↓), increase in S100A8/A9 (↑) between baseline to M2 (A) baseline to M6 (B)
CYC = cyclophosphamide arm, RTX = rituximab arm

CYC↑	21	19	19	17	20	18	17	16
CYC↓	45	43	37	36	46	44	39	37
RTX↑	26	22	16	13	21	18	13	11
RTX↓	48	44	42	36	53	48	44	38

Figure 3. Relapse-free survival according to trial treatment group. The x-axis shows days of follow-up beginning 6 months after the start of treatment. Patients were divided into 4 groups depending on treatment arm and relapse status: patients treated with rituximab (RTX) who had an increase in S100A8/A9 level (solid blue lines), patients treated with RTX who had no increase in S100A8/A9 level (broken blue lines), patients treated with cyclophosphamide (CYC) who had an increase in S100A8/A9 level (solid black lines), and patients treated with CYC who had no increase in S100A8/A9 level (broken black lines) from baseline to month 2 (**A**) and from baseline to month 6 (**B**). Values are the number of patients free of relapses according to treatment arm and whether there was an increase or decrease in S100A8/A9 level. Patients treated with RTX who had an increase in S100A8/A9 level had significantly more relapses than patients treated with RTX with a decrease in S100A8/A9 level ($P = 0.006$ for baseline to month 2 and $P = 0.028$ for baseline to month 6).

analyzed separately. At baseline, patients (both MPO-ANCA positive and PR3-ANCA positive combined) presenting with new disease had significantly higher serum levels of S100A8/A9 than patients presenting with relapsing disease, with a median level of 7,439 ng/ml (range 1,002–92,267) compared to 4,933 ng/ml (range 1,341–14,653) ($P = 0.0008$). There was no difference between new disease and relapsing disease at the later time points. When only the PR3-ANCA–positive patients were analyzed, there was a significantly greater absolute value of serum S100A8/A9 levels in patients presenting with new disease compared to those presenting with relapsing disease at baseline ($P = 0.009$) and at month 6 ($P = 0.029$).

Correlations of serum S100A8/A9 level with clinical parameters. Correlations of serum S100A8/A9 level with clinical parameters were analyzed in the combined group of patients with PR3-ANCA and patients with MPO-ANCA. At baseline, there was a weak correlation between serum S100A8/A9 level and CRP level ($r = 0.22$, $P = 0.016$), total white blood cell count ($r = 0.23$, $P = 0.01$), and BVAS/WG ($r = 0.27$, $P = 0.002$). The correlations persisted at month 1 for CRP level ($r = 0.24$, $P = 0.005$) and white blood cell count ($r = 0.34$, $P < 0.0001$). However, by month 2 there remained only a weak correlation with white blood cell count ($r = 0.32$, $P = 0.0002$). There was no correlation between serum S100A8/A9 level and BVAS/WG at months 1, 2, or 6. When the subset of patients with relapsing PR3-ANCA vasculitis was analyzed, a weak correlation between serum S100A8/A9 level and CRP level at baseline was observed ($r = 0.4$, $P = 0.017$). However, at months 1, 2, and 6 this correlation was not significant. This demonstrates that traditional markers of inflammation and activity, such as CRP level, remain suppressed during remission prior to a future relapse, unlike S100A8/A9 level.

Patients treated with CYC/AZA had a lower overall white blood cell count at month 1 and month 2 compared to patients treated with RTX; however, there was no difference in the numbers of neutrophils and monocytes, cells known to express S100A8/A9, at these time points (data not shown).

DISCUSSION

This study demonstrates that S100A8/A9 level can help identify those patients with AAV who are at risk of a future disease relapse. Laboratory parameters such as CRP level and ANCA titer are often used to assist with determining disease activity; however, these measurements have limited use in helping predict a future flare. Data regarding monitoring change in ANCA titer as a marker of future disease activity are somewhat inconsistent. The level of MPO-ANCA has been reported to be a more reliable indicator of relapse or disease activity than PR3-ANCA (4), while longitudinal ANCA measurements may have significant benefit in predicting relapse, but mainly in those patients with renal involvement (28). However, a previous meta-analysis of numerous studies related to ANCA monitoring was unable to yield firm conclusions due to the heterogeneity of study methods (29), and a more recent meta-analysis demonstrated the limited use of serial ANCA measurements (4). It has been clearly demonstrated from numerous cohorts that patients with PR3-ANCA are at greater risk of relapse than patients with MPO-ANCA (30–32), and these data were confirmed in the RAVE study (33). However, to date no alternative biomarker for relapse has been validated for clinical use. A promising gene expression profiling approach has identified a novel CD8+ signature that defines a subgroup of patients (with SLE, AAV, or inflammatory bowel disease) at risk of relapse (6), but this requires further validation.

The identification of a biomarker to predict relapse may permit tailoring of treatment to individuals, such that patients who may be at lower risk of a future disease relapse are exposed to lower levels of immunosuppression, with potentially reduced treatment-related morbidity and mortality, while those patients who are at increased risk of subsequent flares may be treated with augmented therapy, which may minimize disease-related morbidity and mortality. S100A8/A9 levels have also been used to predict relapse in JIA, with absolute serum level measured during disease remission accurately predicting future disease flares (20).

The findings of the present study suggest that it is the change in an individual patient's level of S100A8/A9 from the time of diagnosis that stratifies the patient's relapse risk. Only a small number of patients who were positive for MPO-ANCA experienced a relapse; therefore, it was not possible to draw conclusions regarding the MPO-ANCA–positive cohort. However, up to 50% of patients who were positive for PR3-ANCA had experienced a relapse within 400 days following complete remission. Analysis of the PR3-ANCA–positive patient subcohort demonstrated that those patients who did not experience a relapse during the trial had significantly greater decreases in serum S100A8/A9 level early during their immunosuppressive therapy. Moreover, those patients who had increased levels of serum S100A8/A9 between selected early time points were at a significantly greater risk of relapse than those patients who had decreased levels between these time points. This is the first time that we have been able to stratify the risk of relapse within the PR3-ANCA–positive population.

Interestingly, there was a correlation of serum S100A8/A9 level with BVAS/WG at baseline, but this correlation was lost at later time points, suggesting that the elevated levels of serum S100A8/A9 observed during apparent clinical remission (with a BVAS/WG of 0) may imply a degree of subclinical inflammation and a risk of subsequent disease relapse not easily apparent from clinical assessment or more conventional markers. Further analysis demonstrated that patients who were treated with RTX and did not have a decrease in serum S100A8/A9 levels were most at risk of a future relapse, implying that mechanisms of achieving relapse may not all be equivalent, from the point of view of disease pathophysiology. A recent study investigating S100A8/A9 (defined as MRP8/14 in that study) in RA patients treated with biologic agents demonstrated a significant decline in S100A8/A9 levels in patients treated with adalimumab, infliximab, and RTX who responded to therapy. Interestingly, in those patients treated with RTX, the nonresponders had significantly increased serum S100A8/A9 levels at 16 weeks, unlike the nonresponders treated with infliximab and adalimumab (34). Similar to these data, in JIA, after discontinuation of methotrexate, levels of S100A8/A9 during remission were significantly higher in those patients who went on to experience a relapse (19).

Our results suggest that changes in serum S100A8/A9 levels may help identify those patients treated with RTX who are at risk of relapse, with an increase in S100A8/A9 levels following RTX treatment indicating a risk of disease relapse. It does, however, remain unclear why some patients treated with CYC/AZA, with a similar relapse rate as the RTX group, experienced a relapse despite decreases in serum S100A8/A9 levels, and these differences in the biologic processes surrounding S100A8/A9 require further investigation. This is similar to a recent study using the same cohort of patients in which the risk of relapse following an increase in PR3-ANCA was higher in the group treated with RTX than in the group treated with CYC/AZA. Additionally, in that study, in patients with renal disease or alveolar hemorrhage, the increase in ANCA titer had greater predictive value for subsequent relapse (35). In our study, CYC treatment resulted in the suppression of

S100A8/A9 level in patients who did experience a relapse, suggesting that there is a difference in disease relapse mechanisms between patients treated with more selective B cell depletion therapy compared to those treated with CYC.

Finally, in those patients at risk of relapse, as determined by consecutive measurement of serum S100A8/A9 level and calculation of percentage change in S100A8/A9 level, it may be tempting to suggest augmentation of immunosuppressive therapy, or at least, that they not be subjected to treatment minimization or withdrawal. This approach should be tested in a prospective manner to confirm the utility of our data.

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. Pepper 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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