

**The association of serum calprotectin (S100A8/S100A9) levels with disease relapses in**

**PR3-ANCA-associated vasculitis**

Ruth J Pepper MBBS, MRCP, PhD<sup>1</sup>, Juliana B Draibe<sup>1</sup> MD, Ben Caplin MBBS, MRCP, PhD<sup>1</sup>, Fernando C Fervenza MD, MPH<sup>2</sup>, Gary S Hoffman MD<sup>3</sup>, Cees G.M Kallenberg MD, PhD<sup>4</sup>, Carol A Langford MD, MHS<sup>3</sup>, Paul A Monach MD, PhD<sup>5</sup>, Philip Seo MD<sup>6</sup>, Robert Spiera MD<sup>7</sup>, E. William St Clair MD<sup>8</sup>, Nadia K Tchao MD<sup>9</sup>, John H Stone MD, MPH<sup>10</sup>, Ulrich Specks MD<sup>2</sup>, Peter A Merkel MD, MPH<sup>11</sup>, AD Salama MBBS, PhD, FRCP<sup>1</sup> and RAVE-ITN Research Group

<sup>1</sup>UCL Centre for Nephrology, Royal Free Hospital, London, UK.

<sup>2</sup> Mayo Clinic, Rochester, MN, USA

<sup>3</sup> Cleveland Clinic Foundation, Cleveland, OH, USA

<sup>4</sup> University of Groningen, Groningen, The Netherlands

<sup>5</sup> Boston University, Boston, MA, USA

<sup>6</sup> Johns Hopkins University, Baltimore, MD, USA

<sup>7</sup> Hospital of Special Surgery, New York, NY, USA

<sup>8</sup> Duke University Medical Centre, Durham, NC, USA

<sup>9</sup> Immune Tolerance Network, San Francisco, CA, USA

<sup>10</sup> Harvard Medical School, Clinical Rheumatology, Boston, MA, USA

<sup>11</sup> University of Pennsylvania, Division of Rheumatology, Philadelphia, PA, USA

Correspondence to: Prof Alan D. Salama

UCL Centre for Nephrology

Royal Free Hospital

Rowland Hill Street

London NW3 2PF

Email: [a.salama@ucl.ac.uk](mailto:a.salama@ucl.ac.uk)

Tel: +442077940500 x36007

Fax: + 4402078302653

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## **ABSTRACT**

**Objectives:** S100A8/A9 (calprotectin) has shown promise as a biomarker for predicting relapse in AAV. This study investigated serum S100A8/A9 levels as a biomarker predicting future relapse in a large cohort of patients with severe ANCA-associated vasculitis (AAV).

**Methods:** Serum levels of S100A8/A9 were measured at baseline, months 2, and 6 following treatment initiation in 144 patients in the RAVE trial (cyclophosphamide/azathioprine vs. rituximab for induction of remission) who attained complete remission.

**Results:** Patients were divided into 4 groups: PR3-ANCA with (n=37), and without (n=56) relapse, and MPO-ANCA with (n=6) and without (n=45) relapse. Serum S100A8/A9 levels decreased in all groups during the first 6 months of treatment. The percentage reduction from baseline to month 2 was significantly different between relapsers and non-relapsers in the PR3-AAV group ( $p=0.046$ ). A significantly higher risk of relapse was associated with an increase in S100A8/A9 between baseline and month 2 ( $p=0.006$ ) and baseline and month 6 ( $p=0.0099$ ) for all patients. Subgroup analysis demonstrated it was patients treated with rituximab and who increased levels of S100A8/A9 who were at greatest risk of future relapse ( $p=0.028$ ).

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

**Keywords:** ANCA vasculitis, disease activity, immunosuppression, relapse.

## INTRODUCTION

Granulomatosis with polyangiitis (GPA) and microscopic polyangiitis are classified as anti-neutrophil cytoplasm antibody (ANCA)- associated vasculitis (AAV), commonly affecting the kidneys, lungs, and other organs(1). ANCA is detected in a significant proportion of patients with active GPA as well as microscopic polyangiitis, 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 controversial(4). Other potential biomarkers of systemic inflammation have been identified to assist in distinguishing active vasculitis from inactive disease, but not for predicting relapse(5). More recently, a transcriptional profiling approach identified a subset of CD8+ T cell genes associated with a poor prognosis in various autoimmune diseases, and is therefore a potential biomarker in AAV, but has yet to be validated(6).

Remission followed by relapse is common in patients with AAV, especially those patients positive for 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 (ESRD)(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 customised 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 intense 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 (TLR4)(11) as well as the receptor for advanced glycation end-products (RAGE)(12, 13). S100A8/A9 is secreted locally at the site of inflammation by

phagocytes, where it has a number of autocrine and paracrine pro-inflammatory 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, where levels predicted disease relapse(19, 20), rheumatoid arthritis(16, 21), systemic lupus erythematosus (SLE)(22), and Kawasaki disease(23). Measurement of faecal calprotectin is routinely performed to detect mucosal inflammation in inflammatory bowel disease(24).

We previously demonstrated that patients with active generalised AAV had elevated serum S100A8/A9 levels compared to patients in remission and healthy controls. 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 relapse(25). In that analysis we did not have available pre-treatment samples for analysis, so we concluded that failure to suppress S100A8/A9 whilst on treatment was associated with relapse. To validate this study, we proceeded to measure levels of serum S100A8/A9 in a large number of patients with severe AAV enrolled in the Rituximab in ANCA-Associated Vasculitis (RAVE) trial, and investigate whether a failure to suppress S100A8/A9 with conventional treatment was predictive of future disease flares.

## **METHODS**

### **Trial details**

The RAVE trial was a multi-center, randomized, double-blind trial comparing rituximab with standard cytotoxic immunosuppression in patients with severe ANCA associated vasculitis for the induction of remission by 6 months. Patients were eligible if they had a new or relapsing diagnosis of GPA or MPA, positive serum assays for PR3-ANCA or 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 rituximab and glucocorticoids. Those patients in the CYC group were switched to maintenance therapy with azathioprine if complete remission was achieved between month 4 to 6. Both groups received the same reducing glucocorticoid regime. The primary end-point was defined as a Birmingham Vasculitis Activity Score for Wegener's Granulomatosis (BVAS/WG) 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 was also published(26).

### **Study design**

Serum from 188 patients was obtained for this study. The time-points included serum 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 onwards. Those patients who were classified as not entering complete remission during the study were excluded from analysis. Additional clinical and laboratory data was obtained from the trial database.

### **Measurement of serum S100A8/A9**

Samples were stored at -80C until analysis. S100A8/A9 was analysed by sandwich enzyme-linked immunosorbent assay (ELISA) as per manufacturer's instructions (BioLegend).

### **Statistical analysis**

All statistics were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego California, USA) and Stata v11 (StataCorp LP, Texas, US). Variables were log transformed where distributions

were skewed. Non-parametric tests of significance were applied. For comparing 2 groups, Mann-Whitney U test was used. To compare the absolute values at subsequent time-points compared to baseline, a mixed-effects model was utilised. Correlations were assessed using the non-parametric Spearman rank correlation analysis. Statistical significance was defined as  $p < 0.05$ . For relapse-free survival analysis, Bonferroni correction was used for multiple comparisons with significance defined at  $p < 0.025$ .

Risk of relapse was investigated using Kaplan-Meier survival analysis and the influence of baseline renal involvement and change in ANCA titre on the relationship between changes in S100A8/A9 and relapse examined using the Cox proportional hazards approach. This was an exploratory study however a post-hoc power calculation using Freedman method suggested there was a 60% power to detect the effect size observed in PR3 subjects assuming a probability of relapse of 0.31 in the reference group (S100A8/A9 decrease) (without adjustment for multiple comparisons).

To explore the value of change in S100A8/A9 to predict relapse, we developed multivariable logistical regression models including clinical and laboratory parameters that might be used in clinical practice to assess risk of relapse: age; sex; ethnicity; PR3 ANCA vs MPO ANCA status; baseline renal involvement; RAVE treatment arm; CRP; change in ANCA titre (over the same period as change in S100A8/A9). The utility of the addition of change in S100A8/A9 to change in ANCA titre versus change in ANCA titre alone as a predictor of relapse was quantified by Receiver operator curves (ROC) generated from these logistic regression models.

## **RESULTS**

### **Demographics and patient groups**

Patients were divided into 4 groups depending on their ANCA subtype and whether they relapsed during the 18 months of follow-up of the trial. For patients to be included in the analysis, they must

have entered complete remission, as defined by a BVAS/WG of 0 and the successful discontinuation of prednisone. Therefore, 44 patients (33 PR3-ANCA and 11 MPO-ANCA) were excluded from analysis as these patients did not achieve complete disease remission.

144 patients were included in the analysis (Table 1). 93 PR3-ANCA patients (65%) and 51 MPO-ANCA patients (35%). The median age of the patients included was 52.5 years (range 15-92) with 70 (49%) female and 74 (51%) male patients. 77 (53%) of the patients had a new diagnosis of AAV at the time of enrollment in the study and 67 (47%) patients presented with relapsing disease. 76 (53%) of patients had renal involvement at baseline. 68 patients (47%) were in the cyclophosphamide/azathioprine arm and 76 patients (53%) were in the rituximab arm.

#### **Serum levels of S100A8/A9 (calprotectin) at the difference time-points**

Serum S100A8/A9 levels significantly decreased from baseline to month 1, 2, and 6 in the whole cohort of patients, with median serum S100A8/A9 at baseline 6509 ng/ml (range 1002-92267), month 1 4660ng/ml (962-35523;  $p < 0.0001$  vs. baseline), months 2 4004 ng/ml (1020-25814;  $p < 0.0001$  vs. baseline), and month 6 3141 ng/ml (346-19383;  $p < 0.0001$  vs. baseline) (Figure 1(i)). The results for within patient change using log S100A8/A9 multilevel model random intercept is highly significant at each time point. There was no statistical difference in absolute S100A8/A9 levels between MPO-ANCA and PR3-ANCA patients at any time point (Table 2) or between those patients who did or did not relapse at each time point, with the exception of a difference between the relapsing and non-relapsing PR3-ANCA group at month 2 ( $p = 0.05$ )(Table 2). In addition, there was no difference in the absolute levels of serum S100A8/A9 at any time point between those patients presenting with ( $n = 76$ ) or without ( $n = 68$ ) renal involvement.

#### **Change in serum levels of S100A8/A9 at the different time points.**

We then investigated the percentage change of serum S100A8/A9 for individual patients between two time-points according to relapse status. As there were only six MPO-ANCA patients who relapsed, we focused our analysis on the PR3-ANCA patient cohort.

There was a significant difference in the percentage change in serum S100A8/A9 between the PR3-ANCA relapsers and non-relapsers at baseline compared to month 2 levels (Figure 1), with relapsing patients demonstrating less serum S100A8/A9 suppression. The median change in serum S100A8/A9 at month 2 compared with baseline was a decrease of 10% in the relapsing groups (IQR -59% to +60%), with a significantly greater degree of suppression in the non-relapsing group of 31% (IQR -60 to -9%)( $p < 0.0457$ ). Therefore, an increase in serum S100A8/A9 identifies a subset of patients during the first 6 months who are at risk of disease relapse during the subsequent 12 months. The relative risk for relapse with an increase in S100A8/A9 at month 2 compared to baseline was 1.81 (CI 1.11-2.93), and at month 6 compared to baseline was 1.76 (CI 1.1-2.83). Figure 1 (iii) demonstrates the mean % change from (i) baseline to month 1, (ii) baseline to month 2 and (iii) baseline to month 6. The patients are classified into 4 groups according to relapse status and treatment arm. This data demonstrates that those patients treated with rituximab who went onto relapse, increased serum S100A8/A9 between the time-points.

#### **Increase in serum levels of S100A8/A9 predicts earlier relapse**

When patients were categorized into whether they had an increase or decrease in serum S100A8/A9 between 2 time-points, those patients who demonstrated an increase in serum S100A8/A9 from baseline to month 2 ( $p = 0.004$ ) and baseline to month 6 ( $p = 0.003$ ), demonstrated significantly earlier and more frequent relapses than those patients who suppressed serum S100A8/A9 (Figure 2).



When these groups were divided according to the different treatment arms, it was those patients within the rituximab treatment arm with increased levels of serum S100A8/A9 between the time points who demonstrated significantly earlier relapses (Figure 3).

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

To further investigate the potential of serum S100A8/A9 as a biomarker to stratify relapse risk in patients treated with rituximab early in the induction period multivariable logistic regression models were developed. Only addition of change in ANCA titre (over the same time period e.g. 2- or 6-months compared to baseline) improved model fit. Receiver operator curves (ROC) for change from baseline to month 2 demonstrated an AUC of 0.55 for an increase in ANCA and an AUC of 0.77 for the combination of an increase in ANCA and serum S100A8/A9 ( $p=0.028$  for a difference between curves). At 6 months, the AUC for an increase in ANCA is 0.69, while the combination of increase in both ANCA and serum S100A8/A9 generated a higher AUC of 0.75 ( $p=NS$ ).

### **New versus relapsing disease**

77 patients at time of inclusion into the trial presented with new disease while 67 presented with relapsing disease; these sub-cohorts were then analysed separately. At baseline, those patients (MPO-ANCA and PR3-ANCA combined) presenting with new disease had a statistically significant higher level of serum S100A8/A9 than those patients presented with relapsing disease, with a median level of 7439 ng/ml (1002-92267) compared to 4933 ng/ml (1341-14653)( $p=0.0008$ ). There was no difference comparing new disease versus relapsing disease at the later time-points. Analysing

the patients with PR3-ANCA only, there was a significantly greater absolute value of serum S100A8/A9 between those patients presenting with new disease compared to relapsing disease at baseline ( $p=0.009$ ), and at month 6 ( $p=0.029$ ).

### **Correlations**

Combining the patients with PR3-ANCA and MPO-ANCA, correlations of serum S100A8/A9 with clinical parameters were analysed. At baseline, there was a weak correlation between the serum levels of S100A8/A9 and C-reactive protein (CRP) [ $r=0.22$  ( $p=0.016$ )], total white cell count (WCC) [ $r=0.23$  ( $p=0.01$ )], and BVAS/WG score [ $r=0.27$  ( $p=0.002$ )]. This correlation persisted at month 1, with CRP [ $r=0.24$  ( $p=0.005$ )] and WCC [ $r=0.34$  ( $p<0.0001$ )]. However, by month 2 there remained only a weak correlation with WCC [ $r=0.32$  ( $p=0.0002$ )]. There was no correlation between serum S100A8/A9 levels and BVAS/WG at months 1, 2, or 6.

Comparing the subset of patients with relapsing PR3-ANCA vasculitis, this subset again had a weak correlation between serum S100A8/A9 and CRP at baseline ( $r=0.4$ )( $p=0.017$ ). However at months 1, 2 and 6 this correlation was not significant. This demonstrates that the traditional markers of inflammation and activity such as CRP remain suppressed during remission prior to a future relapse, unlike S100A8/A9.

Patients treated with cyclophosphamide/azathioprine had a lower overall WCC at month 1 and month 2 compared to those patients treated with rituximab, however, there was no difference in the neutrophil and monocyte counts, cells known to express S100A8/A9, at these time points (data not shown).

### **DISCUSSION**

This study demonstrates that S100A8/A9 can help identify those patients with AAV at risk of a future disease relapse. Laboratory parameters such as CRP and ANCA titers are often used to assist with

determining disease activity, however, these measurements have a limited use in helping predict a future flare. Data regarding monitoring change in ANCA titre as a marker of future disease activity are somewhat inconsistent. Measurement of MPO-ANCA in patients has been reported to be more reliable an indicator of relapse or disease activity than those patients positive for PR3-ANCA (4), while longitudinal ANCA measurements may have significant benefit in predicting relapse, but mostly in those patients with renal involvement(27). However, a previous meta-analysis of numerous reports related to ANCA monitoring was unable to draw firm conclusions due to the heterogeneity in the methods of differing studies(28), with a more recent meta-analysis demonstrating the limited use of serial ANCA measurements(4). It has been clearly demonstrated from numerous cohorts that patient with PR3-ANCA are at greater risk of relapse than patients with MPO-ANCA (29-31) and these data were confirmed in the RAVE study(32). 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, and inflammatory bowel disease) at risk of relapse(6), but this requires further validation.

The identification of a biomarker to predict relapse may permit individualization of treatment, such that patients who may be at a lower risk of a future disease relapse are exposed to lower levels of immunosuppression, with a 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. Using S100A8/A9 to predict relapse has also be demonstrated in juvenile idiopathic arthritis, with an absolute serum level measured during disease remission accurately predicting future disease flares(20).

Our current data 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 with MPO-ANCA relapsed, therefore it was not possible to draw conclusions regarding the MPO-ANCA positive

cohort, however, up to 50% of patients with PR3-ANCA had relapsed within 400 days following complete remission. Analyzing the PR3-ANCA patient sub-cohort demonstrated that those patients who did not relapse during the trial had significantly greater decreases in the level of serum S100A8/A9 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 demonstrated 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 population.

Interestingly, there was a correlation of serum S100A8/9 levels with BVAS/WG at baseline but this correlation was lost at later time points, suggesting that the elevated levels of serum S100A8/A9 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 appreciated from clinical assessment or more conventional markers. Further analysis demonstrated that it was the patients treated with rituximab with failure to suppress serum S100A8/A9 who were at most 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 this study) in rheumatoid arthritis treated with biological agents demonstrated the significant decline in S100A8/A9 in patients treated with adalimumab, infliximab and rituximab who responded to therapy. Interestingly, in those patients treated with rituximab, the non-responders significantly increased serum S100A8/A9 at 16 weeks unlike the non-responders treated with infliximab and adalimumab (33). Similar to these data, in juvenile idiopathic arthritis, after the withdrawal of methotrexate, levels of S100A8/A9 during remission were significantly higher in those patients who went onto relapse (19).

Our results suggest that changes in serum S100A8/A9 may help predict those patients treated with rituximab at risk of relapse, with an increase in S100A8/A9 following rituximab therapy putting the patients at risk of disease relapse. It does, however, remain unclear why some patients treated with cyclophosphamide/azathioprine, with a similar relapse rates as the rituximab group, relapsed despite decreases in the serum level of S100A8/A9, and these differences in the biological processes surrounding S100A8/9 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 rituximab treated group than the group treated with cyclophosphamide/azathioprine. Additionally, in this study, patients with renal disease or alveolar haemorrhage, the rise in ANCA titer had greater predictive value for subsequent relapse (34).

In our study, cyclophosphamide resulted in suppression of S100A8/A9 in patients who did proceed to relapse suggesting there is a difference in disease relapse mechanisms between those patients treated with more selective B-cell depletion therapy compared to those patients treated with cyclophosphamide.

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

**Table 1: Baseline patient characteristics**

	<b>All patients</b>	<b>Anti-PR3 non-relapse</b>	<b>Anti-PR3 relapse</b>	<b>Anti-MPO non-relapse</b>	<b>Anti-MPO- relapse</b>
<b>Total number of patients</b>	144	56	37	45	6
<b>Age years (median/range)</b>	52.5 (15-92)	51.5 (17-77)	51 (15-74)	55 (16-92)	75.5 (48-78)
<b>Female/Male</b>	70/74	22/34	16/21	29/16	3/3
<b>Ethnicity</b>	W134, B6,O4	W50, B3, O3	W37	W42, B3	W5, O1
<b>New diagnosis at presentation</b>	77 (53%)	28	12	35	2
<b>Relapse at presentation</b>	67 (47%)	28	25	10	4
<b>Renal failure at Baseline</b>	76 (53%)	27	15	31	3
<b>BVAS at baseline (median/range)</b>	8 (3-16)	10 (4-15)	7.5 (3-14)	8 (4-16)	10 (6-12)
<b>Cyclophosphamide</b>	68	26	17	23	2
<b>Rituximab</b>	76	30	20	22	4

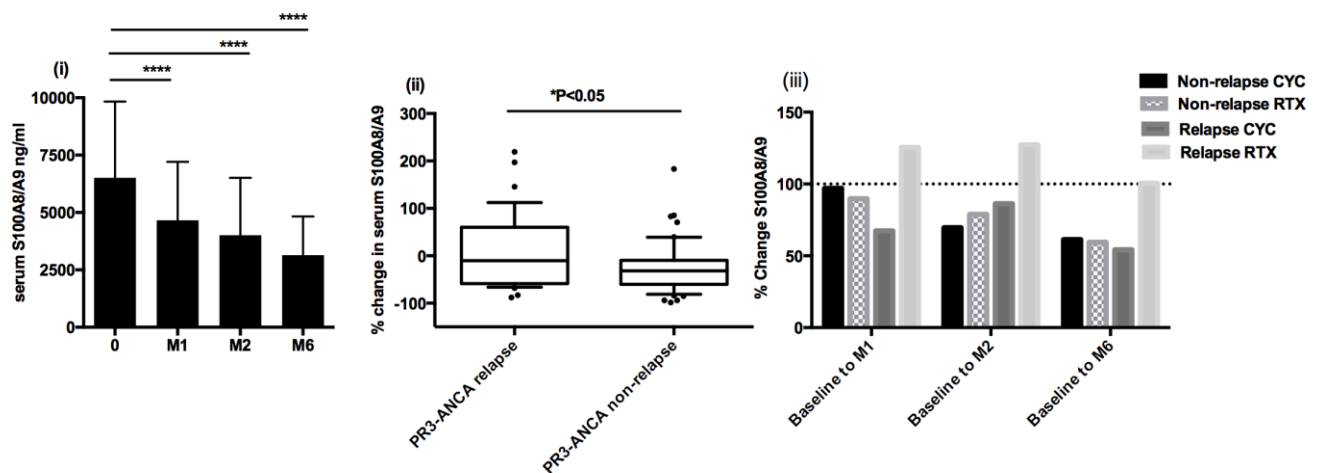
Baseline characteristics of the patients at baseline upon entry into the RAVE trial [(W) White, (B) Black, (O) Other]. The patients were divided into 4 groups based on their relapse status at the end of the trial and the type of ANCA present.

**Table 2: Serum S100A8/A9 levels in patients according to relapse status and ANCA subtype at different timepoints.** Serum levels are reported with the median and range. Values analysed using Mann-Whitney U-paired t-test.

<b>Median/ Range</b>	<b>All</b>	<b>Anti-PR3 non- relapse</b>	<b>Anti-PR3 relapse</b>	<b>P Value</b>	<b>Anti-MPO non-relapse</b>	<b>Anti-MPO- relapse</b>	<b>P value</b>
<b>Baseline ng/ml</b>	6509 (1002-92267)	6304 (1894-92267)	5522 (1341-29920)	p=0.66	6869 (1002-29653)	8385 (3174-13639)	p=0.56
<b>month 1 ng/ml</b>	4660 (962-35523)	4914 (1463-18248)	4649 (1317-26071)	p=0.65	4109 (1077-35523)	4632 (962.2-18290)	p=0.8
<b>month 2 ng/ml</b>	4004 (1020-25814)	3900 (1112-9413)	4898 (1798-19152)	p=0.05	3481 (1020-19037)	2651 (1364-25814)	p=0.4
<b>month 6 ng/ml</b>	3141 (346-19383)	2941 (711-19383)	3976 (751-10517)	p=0.47	3070 (346-12818)	4202 (944-18839)	p=0.17
<b>month 12 ng/ml</b>	3428 (408-24235)	3283 (408-15033)	3304 (513-24235)	p=0.80	3428 (1021-13070)	3707 (2784-4694)	p=0.85

## Figures

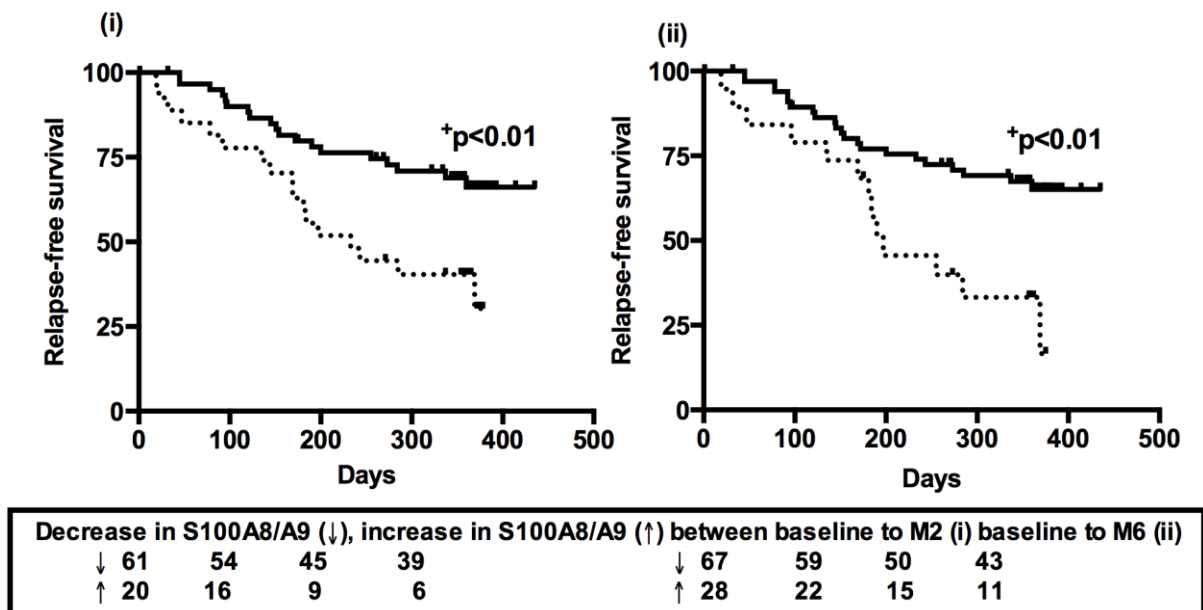
Figure 1



Graphs to demonstrate the % change in serum S100A8/A9. (i) Demonstrates the absolute values of serum S100A8/A9 at each time point in all patients (n=144): baseline, month 1, month 2 and month 6 with significant decreases in the value compared to baseline [all  $p < 0.0001$ ] (ii) shows the results comparing the % change in the PR3-ANCA patients serum levels at month 2 compared to baseline. The median change in serum S100A8/A9 at month 2 compared to baseline was a decrease of 10% in the relapsing groups (IQR -59% to +60%), and a decrease of 31% in the non-relapsing group (IQR -60 to -9%) ( $p = 0.046$ ) [Mann-Whitney U-paired t-test]. (iii) Graph demonstrates the % change in PR3-ANCA patients divided into 4 groups (RTX relapse and non-relapse, CYC relapse and non-relapse). The % change in S100A8/A9 is baseline compared to month 1 (M1), month 2 (M2) and month 6 (M6).

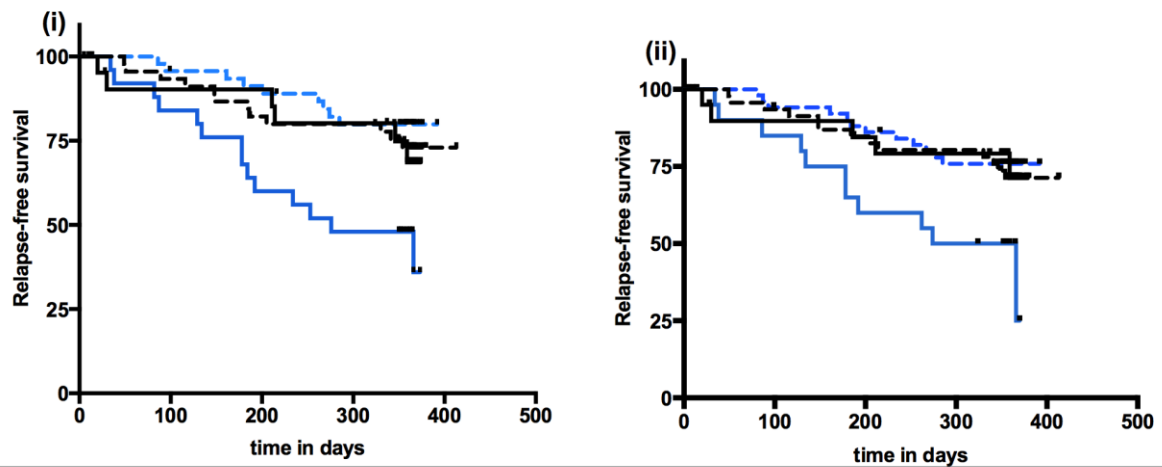


Figure 2



Graphs (i) and (ii) are Kaplan-Meier survival curves of the PR3-ANCA patients remaining relapse-free. Times in days represents the duration of complete remission. The data has been adjusted for multiple comparisons and significance defined as  $p < 0.025$ . The patients are divided into 2 groups: those with a decrease (solid line) and those who had an increase (dotted line) in serum S100A8/A9 between the following time-points: (i) Baseline to month 2 data: (ii) Baseline to month 6. The numbers represent those patients remaining relapse free during follow-up. There is a significant difference in relapse-free survival, with those patients increasing serum S100A8/A9 having a significantly lower relapse-free survival. (i) baseline to month 2 ( $p = 0.0043$ ), (ii) baseline to month 6 ( $p = 0.0029$ ). Numbers at risk included.

Figure 3



Decrease in S100A8/A9 (↓), increase in S100A8/A9 (↑) between baseline to M2 (i) baseline to M6 (ii)								
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

Relapse-free survival divided into the trial treatment groups of all the patients. The x-axis demonstrates days follow-up from 6 months. The patients are divided into 4 groups depending on treatment arm and relapse status. [Blue solid line= patients treated with rituximab with an increase in S100A8/A9][blue dotted line= patients treated with rituximab with no increase in S100A8/A9][black solid line= patients treated with cyclophosphamide with an increase in S100A8/A9][black dotted line= patients treated with cyclophosphamide with no increase in S100A8/A9]. The numbers on the graph represent the number of patients free of relapses categorized into treatment arm and whether there was an increase or decrease in S100A8/A9. Patients treated with rituximab who increased S100A8/A9 demonstrate significantly more relapses than patients with a decrease in S100A8/A9.(i) baseline to month 2 (p=0.006) (ii) baseline to month 6 (p=0.028).

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