

# Immunoglobulin (Ig)M antibodies to proteinase 3 in granulomatosis with polyangiitis and microscopic polyangiitis

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## Introduction

The anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are defined in most patients by characteristic clinical and histopathological features and the presence of ANCA [1]. However, patients with AAV present heterogeneously, to the extent that three separate clinicopathological variants of AAV have been defined: granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg–Strauss syndrome). Even within these syndromes the inflammatory activity can affect different organ

## Summary

Anti-neutrophil cytoplasmic antibodies (ANCA) appear to play an important role in the pathogenesis of ANCA-associated vasculitis (AAV). However, ANCA alone are not sufficient to generate disease, and some evidence suggests that infectious triggers may serve as inciting events for AAV disease activity. Antibodies of the immunoglobulin (Ig)M isotype often serve as markers of recent infection, and IgM ANCA have been identified previously in patients with AAV, although the frequency and clinical relevance of IgM ANCA is not well established. We sought to characterize IgM ANCA more clearly by creating a novel enzyme-linked immunosorbent assay (ELISA) for IgM antibodies to proteinase 3 [IgM proteinase 3 (PR3)–ANCA], which we applied to two large, clinically well-characterized trial cohorts of patients with granulomatosis with polyangiitis and microscopic polyangiitis. In the first cohort, IgM PR3–ANCA occurred with a frequency of 15.0%, and were associated with a higher degree of disease severity and a trend towards a higher rate of alveolar haemorrhage (29.6 *versus* 15.7%,  $P = 0.10$ ). Analysis of follow-up samples in this cohort showed that the presence of IgM PR3–ANCA was transient, but could recur. In the second cohort, IgM PR3–ANCA occurred with a frequency of 41.1%, and were also associated with a higher degree of disease severity. A higher rate of alveolar haemorrhage was observed among those with IgM PR3–ANCA (45.3 *versus* 15.8%;  $P < 0.001$ ). The association of transient IgM PR3–ANCA with an acute respiratory manifestation of AAV suggests a possible link between an infectious trigger and AAV disease activity.

**Keywords:** alveolar haemorrhage, ANCA-associated vasculitis, anti-neutrophil cytoplasmic antibodies, granulomatosis with polyangiitis, immunoglobulin M, microscopic polyangiitis

systems in different patients, and in any single patient the organs affected and the severity of disease activity can vary over time [2].

Our understanding of the pathogenesis of AAV has improved significantly over the last couple of decades, allowing a more targeted approach to therapy. However, many important questions remain unanswered. In predisposed patients abnormal T and B lymphocyte regulation leads to the production of ANCA, and their role in neutrophil and monocyte activation seem to be crucial for the development of disease manifestations such as glomerulonephritis or pulmonary capillaritis, although ANCA alone

are not sufficient for the disease process to play out fully in humans [3–5]. Moreover, the specific triggers of a primary ANCA immune response, such as infections, and the mechanisms leading subsequently to the loss of tolerance and persistence of ANCA in predisposed patients remain unclear.

The most prevalent and best-studied ANCA with accepted diagnostic utility are of the immunoglobulin (Ig)G isotype. IgM ANCA and IgA ANCA have also been identified [6–9]. Early studies investigating the clinical significance of the various ANCA isotypes suggested an association of circulating IgM ANCA with certain disease manifestations, particularly alveolar haemorrhage [6,10]. However, these associations and their clinical relevance were questioned by others [7,11,12]. Because these early reports on IgM ANCA were derived from somewhat small cohorts, and the theory of infections as triggering co-factors of disease development and activity has persisted for decades, we conducted the present study to revisit the prevalence and clinical associations of IgM PR3-ANCA in two large clinically well-characterized trial cohorts.

## Material and methods

### Patient populations

The study population consisted of two cohorts. The participants of the Wegener's Granulomatosis Etanercept Trial (WGET) comprised the first cohort, and the subset of 129 PR3-ANCA-positive subjects enrolled into in the Rituximab *versus* Cyclophosphamide for AAV trial (RAVE) made up the second cohort. We chose to study the two cohorts separately for two principle reasons: first, the entrance criteria of the two studies differed significantly, so there is substantial heterogeneity between the two groups; and secondly, the separation of the two cohorts provided the opportunity to test and validate associations identified in our study of the first cohort. All patients enrolled into the trials provided written informed consent that included consent for the use of biospecimens in ancillary studies, and both trials were approved by the institutional review boards at each participating site.

The design of WGET, the baseline clinical characteristics of study participants and the primary trial results have been described in detail elsewhere [13,14]. Briefly, WGET was a multi-centre, randomized, placebo-controlled trial that evaluated the use of etanercept for remission maintenance in GPA. The WGET study population consisted of 180 patients who were enrolled at eight centres across the United States. Each patient who enrolled in WGET met the following criteria: (1) the diagnosis of GPA was supported by fulfilling at least two of the five modified American College of Rheumatology criteria for the classification of Wegener granulomatosis, and (2) there was evidence of disease activity within 28 days of enrolment, with a

Birmingham Vasculitis Activity Score for Wegener Granulomatosis (BVAS/WG) of at least 3 [15].

The details of the design of RAVE, along with the participants' baseline clinical data and the trial's primary results, have been reported elsewhere [16,17]. Briefly, RAVE was a multi-centre, randomized, double placebo-controlled trial that compared the efficacy and safety of rituximab with cyclophosphamide for remission induction in severe AAV. The RAVE study population consisted of 197 patients enrolled at eight centres in the United States and one centre in the Netherlands. All patients met 1994 Chapel Hill Consensus Conference definitions for the diagnosis of GPA or MPA, had positive serum assays for PR3-ANCA or myeloperoxidase (MPO)-ANCA at the time of enrolment, had active disease with a minimum BVAS/WG of 3 and had received glucocorticoids for no longer than 14 days prior to study screening. In addition, all patients were classified as having 'severe' AAV, defined as disease activity involving a vital organ and posing an immediate threat to that organ's function or to the patient's life.

### Patient evaluations and sample collections

Patients in WGET were evaluated during study visits that occurred at baseline, after 6 and 12 weeks, and then every 3 months until the trial ended [14]. RAVE participants were evaluated during study visits at baseline, and then on a regular schedule for at least 18 months [17]. At every study visit disease activity was measured using the BVAS/WG instrument, and serum samples were drawn in both WGET and RAVE. Serum samples were frozen and stored at  $-80^{\circ}\text{C}$ .

### ANCA assays

IgG ANCA assays had been performed previously in both study cohorts using standard immunofluorescence and enzyme immunoassays [4,5,17,18]. A novel capture enzyme-linked immunosorbent assay (ELISA) was designed to detect PR3-ANCA of the IgM isotype in patient serum. The capture ELISA used poly-His tagged recombinant PR3 (rPR3) [19] bound to nickel-coated plates to test for anti-rPR3 reactivity in serum samples diluted 1 : 20 in Tris-buffered saline (TBS) containing 0.5% bovine serum albumin (BSA). The presence of IgM antibodies bound to rPR3 were detected using a goat anti-human IgM ( $\mu$ -chain specific)-alkaline phosphatase antibody (Sigma-Aldrich, St Louis, MO, USA), diluted 1 : 20 000 in TBS buffer containing 0.5% BSA. The assay's cut-off value for a positive result was determined by performing the test on 37 samples drawn from patients who had tested negative previously for PR3-ANCA by standard clinical assays for IgG PR3-ANCA. All 37 of these samples were derived from waste material from the clinical immunology laboratory at Mayo Clinic, Rochester, Minnesota. In each instance, PR3-ANCA testing had been ordered by a

treating clinician, and the result had been negative. The cut-off value for a positive result for the novel IgM PR3-ANCA capture ELISA was set at 4 standard deviations (s.d.) above the mean of the assay's results from these 37 samples. This conservative cut-off value was chosen to minimize the problem of borderline positivity.

To assess the epitope specificity of IgM PR3-ANCA we used monoclonal antibodies with defined PR3 epitope specificity as capturing antibodies in capture ELISAs, as described previously for IgG ANCA detection [20-22]. Immulon strips were coated with the monoclonal antibodies mouse monoclonal proteinase 3 (PR3) antibody (MCPR3)-2, MCPR3-3 or MCPR3-7 prior to loading the rPR3 antigen. Bound IgM PR3-ANCA was detected as described above.

### Statistical analysis

Continuous variables are presented as median [interquartile ranges (IQR)] and categorical variables are presented as counts and percentages, unless specified otherwise. Associations between binary measures were performed with the use of Fisher's exact test, as appropriate, while associations between continuous variables were performed with Wilcoxon's rank-sum test. Statistical analyses were performed using JMP version 10.0.

## Results

### IgM PR3-ANCA in the WGET cohort

Serum samples from the baseline visit were available for all 180 patients enrolled in WGET. The key clinical characteristics of this cohort are summarized in Table 1. A full description of the cohort's baseline clinical and disease

**Table 1.** Patient characteristics

Clinical characteristics	WGET cohort* (n = 180)	RAVE cohort** (n = 129)	P-value
Age (mean ± s.d.)	49.9 ± 15.3	49.8 ± 14.8	0.95
Gender (% male)	60.0%	58.1%	0.74
Race			
White, non-Hispanic	92.2%	93.0%	0.79
Black, non-Hispanic	1.7%	2.3%	
Other	6.1%	4.6%	
Disease onset			
New-onset	44.1%	37.2%	0.20
Recurrent	55.9%	62.8%	
BVAS-WG (mean ± s.d.)	7.0 ± 3.4	8.0 ± 3.2	0.01
Alveolar haemorrhage	17.8%	27.9%	0.04
Major renal involvement	35.0%	44.2%	0.12

\*Derived from the Wegener's Granulomatosis Etanercept Trial (WGET) [14].

\*\*Derived from the Rituximab *versus* Cyclophosphamide for AAV trial (RAVE) [17].

characteristics has been published elsewhere [14]. The cohort included patients with both severe (71.1%) and limited or non-severe (28.9%) GPA, and patients in whom enzyme immunoassay testing for IgG PR3-ANCA had been either positive (72.8%) or negative (27.2%).

Twenty-seven of the 180 WGET baseline samples (15.0%) tested positive for IgM PR3-ANCA. IgM PR3-ANCA were detected among subjects with both limited and severe disease as well as with both newly and previously diagnosed GPA, but only among those who had been found to have IgG PR3-ANCA by standard clinical assays (Table 2).

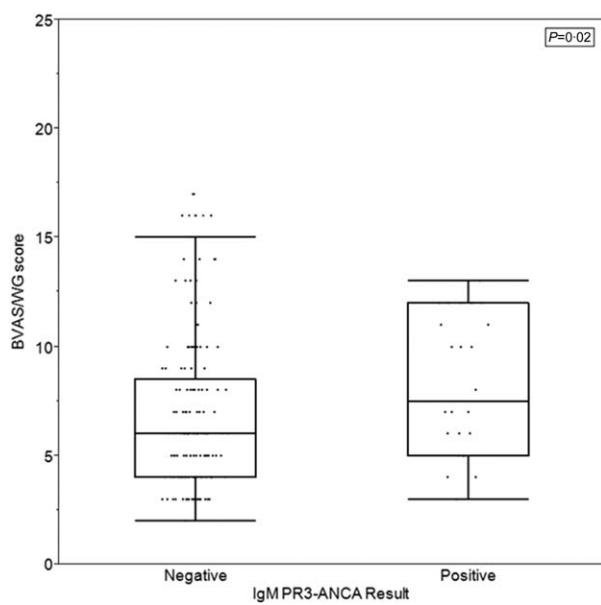
The disease activity at baseline was higher among patients who tested positive for IgM PR3-ANCA [median (IQR) BVAS/WG of 7.5 (5-12)] compared to those who tested negative [BVAS/WG of 6 (4-8.5); P = 0.02] (Fig. 1).

The organ systems involved by GPA did not differ according to IgM PR3-ANCA status, but there was a notable trend towards more frequent alveolar haemorrhage among those who tested positive for IgM PR3-ANCA. Alveolar haemorrhage was diagnosed at baseline in eight of the 27 patients (29.6%) who tested positive for IgM PR3-ANCA, compared to 24 of the 153 patients (15.7%) who tested negative (P = 0.10). This trend was also seen when the analysis was restricted to the 128 WGET patients with severe GPA, who are more likely to have detectable ANCA [18]. Among the 128 patients with severe GPA, alveolar haemorrhage was diagnosed in eight of the 19 patients (42.1%) with a positive IgM PR3-ANCA test result and 24 of the 109 patients (22.0%) who tested negative (P = 0.08).

Among the 32 WGET patients who had been diagnosed with alveolar haemorrhage at the baseline visit, further IgM PR3-ANCA assays were performed on serum samples from all available subsequent study visits, as summarized in Fig. 2. Twenty-four of these 32 patients had tested negative for IgM PR3-ANCA at the baseline visit, and the test remained negative for all 24 patients at all follow-up visits. The remaining eight patients had tested positive for IgM

**Table 2.** Disease characteristics by immunoglobulin (Ig)M proteinase 3-anti-neutrophil cytoplasmic antibodies (PR3-ANCA) status, Wegener's Granulomatosis Etanercept Trial (WGET) cohort

	IgM PR3-ANCA (+) (n = 27)	IgM PR3-ANCA (-) (n = 153)	P-value
IgG PR3-ANCA, n (%)			
• Positive	27 (100)	121 (79)	0.005
• Negative	0 (0)	32 (21)	
Timing of diagnosis, n (%)			
• New diagnosis	16 (59)	64 (42)	0.10
• Previous diagnosis	11 (41)	89 (58)	
Disease severity, n (%)			
• Severe	19 (70)	109 (71)	1.0
• Limited	8 (30)	44 (29)	



**Fig. 1.** Disease severity, as measured by BVAS/WG score, at baseline visit in WGET cohort, by immunoglobulin (Ig)M proteinase 3-anti-neutrophil cytoplasmic antibodies (PR3-ANCA) status.

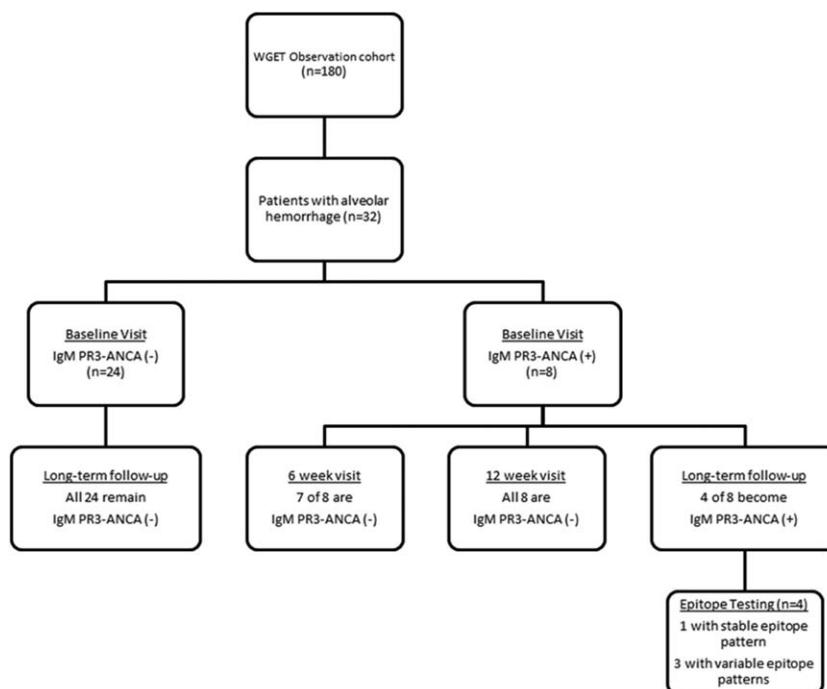
PR3-ANCA at the baseline visit. At follow-up, only one of the eight patients had a persistently positive IgM PR3-ANCA test at the week 6 visit, and that patient's test converted to negative at the week 12 visit. Subsequently, IgM PR3-ANCA re-emerged in four of the eight patients.

Among the four WGET patients who were diagnosed with alveolar haemorrhage at baseline and who tested positive for IgM PR3-ANCA at multiple time-points during

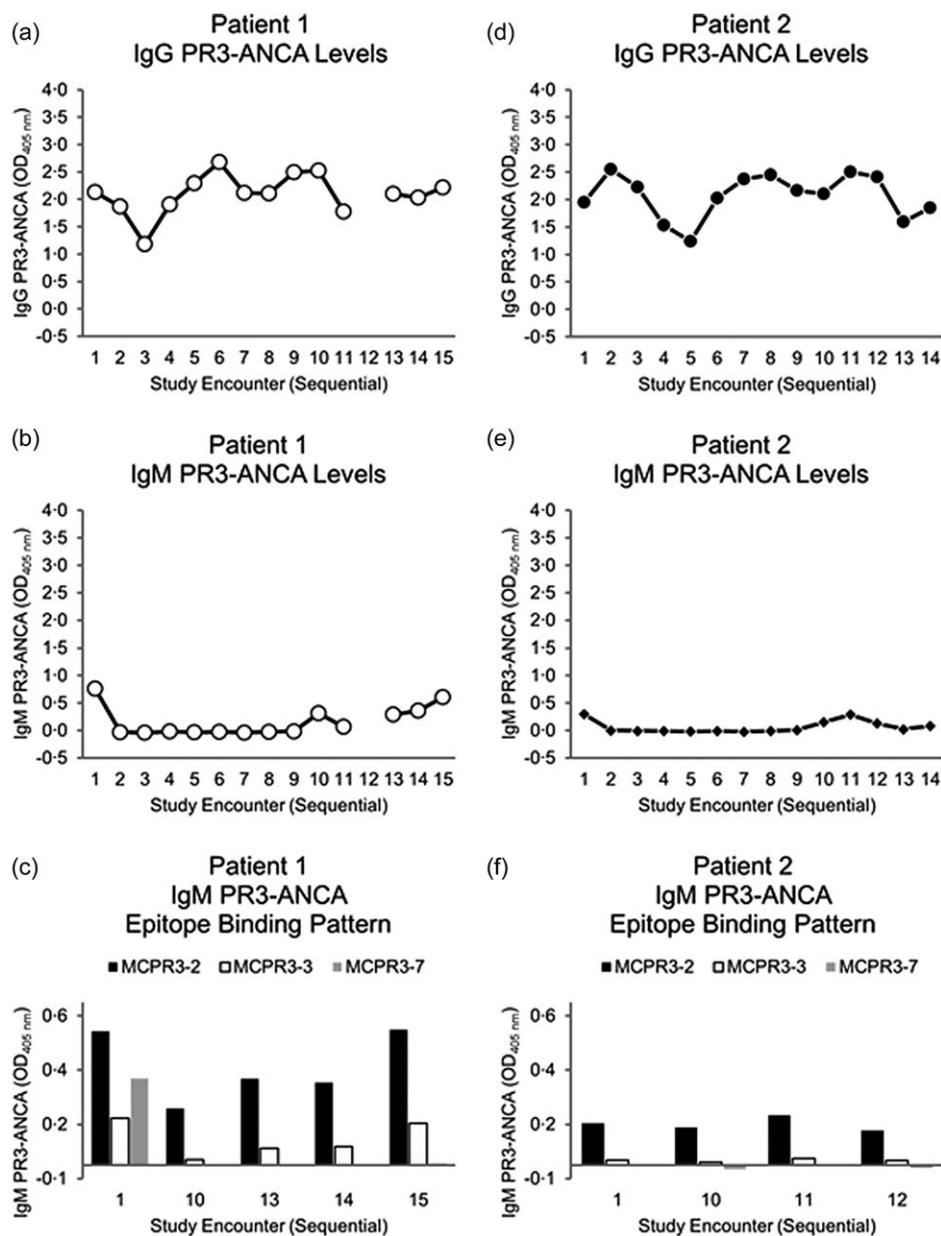
the study, the target epitopes of the identified IgM PR3-ANCA were studied further. In one of the four patients, the IgM PR3-ANCA epitope target appeared to remain constant over time, but epitope variability was apparent in serial samples from the remaining three patients. The longitudinal results from two of the four patients are represented in Fig. 3, which displays the capture ELISA results for both IgG and IgM PR3-ANCA at each sequential study encounter, and shows the epitope binding pattern of the IgM PR3-ANCA for each instance at which it is detected. Two key points are highlighted in Fig. 3. First, the longitudinal ELISA results show that the waxing and waning of IgM and IgG PR3-ANCA levels appear independent. That is, the emergence and disappearance of IgM PR3-ANCA in an individual do not simply follow the rise and fall of the overall ANCA level. The second point highlighted in Fig. 3 is that the epitope binding pattern of the IgM PR3-ANCA can change (as seen in patient 1) or remain stable (as seen in patient 2) over time.

#### IgM PR3-ANCA in the RAVE cohort

The RAVE cohort was used to investigate further the most interesting observation made in the WGET cohort: the potential association between the presence of IgM PR3-ANCA and the diagnosis of alveolar haemorrhage. Serum samples from all 129 RAVE patients who had tested positive for IgG PR3-ANCA by standard clinical assays were included in the cohort. The key clinical characteristics of the cohort are summarized in Table 1. As a condition of inclusion in the RAVE trial, all patients had severe GPA or MPA. Thirty-six of the 129 PR3-ANCA-positive patients



**Fig. 2.** Change in immunoglobulin (Ig)M proteinase 3-anti-neutrophil cytoplasmic antibodies (PR3-ANCA) over time in patients with alveolar haemorrhage in the Wegener's Granulomatosis Etanercept Trial (WGET) cohort.

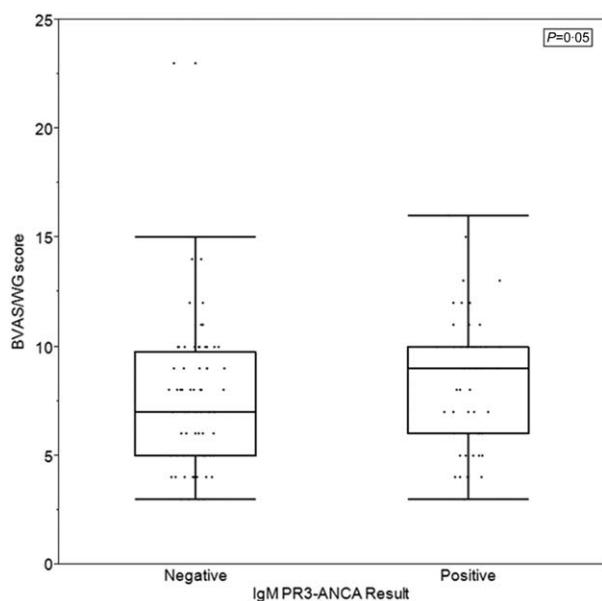


**Fig. 3.** Longitudinal results of immunoglobulin (Ig)M proteinase 3-anti-neutrophil cytoplasmic antibodies (PR3-ANCA) enzyme-linked immunosorbent assay (ELISA), IgM PR3-ANCA ELISA and IgM PR3-ANCA epitope binding pattern assays for two patients who were diagnosed with alveolar haemorrhage at baseline and who tested positive for IgM PR3-ANCA at multiple time-points during the Wegener's Granulomatosis Etanercept Trial (WGET) study. (a, b) The results of ELISA assays for IgG PR3-ANCA (a) and IgM PR3-ANCA (b) performed on sera drawn from patient 1 across longitudinal study visits; (d, e) similarly display longitudinal results of the same assays from sera drawn from patient 2. For both patients, fluctuations in the IgG PR3-ANCA levels and the IgM PR3-ANCA levels occur independently. (c) Results of the IgM PR3-ANCA epitope binding assays performed on sera drawn from patient 1, restricted to time-points at which the antibody was detected. (f) Results of the same assays performed on sera drawn from patient 2. Mouse monoclonal proteinase 3 (PR3) antibody (MCPR3)-2, MCPR3-3, and MCPR3-7 refer to the monoclonal antibodies used as capturing antibodies for the epitope binding assays. Changes in the pattern of results among these assays are suggestive of changes in the epitope binding site of the patient's IgM PR3-ANCA over time. (c, f) Highlight that the IgM PR3-ANCA epitope binding site appears to change over time for patient 1 (c), but not for patient 2 (f).

(27.9%) were diagnosed with alveolar haemorrhage at the baseline visit.

The IgM PR3-ANCA assay was performed on serum samples obtained at the baseline visit. IgM PR3-ANCA

were detected in 53 of the 129 patients (41.1%). As had been observed in the WGET cohort, those who tested positive for IgM PR3-ANCA had higher levels of disease activity compared to those who tested negative (Fig. 4).



**Fig. 4.** Disease severity, as measured by Birmingham Vasculitis Activity Score for Wegener Granulomatosis (BVAS/WG) score, at baseline visit in the Rituximab *versus* Cyclophosphamide for AAV trial (RAVE) cohort, by immunoglobulin (Ig)M proteinase 3-anti-neutrophil cytoplasmic antibodies (PR3-ANCA) status.

The median (IQR) baseline BVAS/WG was 9 (6–10) among those who tested positive for IgM PR3-ANCA and 7 (5–9.75) among those who tested negative ( $P = 0.05$ ). As with the WGET cohort, the frequencies of newly diagnosed and recurrent AAV were similar among patients with and without IgM PR3-ANCA. The rate of newly diagnosed AAV was 35.8% among patients who tested positive for IgM PR3-ANCA and 38.2% among patients who tested negative ( $P = 0.85$ ).

Alveolar haemorrhage was diagnosed nearly three times more frequently among those who tested positive for IgM PR3-ANCA compared to those who tested negative (45.3 *versus* 15.8%;  $P < 0.001$ ). In contrast, the frequency of major renal disease at baseline was similar among patients who tested positive for IgM PR3-ANCA compared to those who tested negative (47.2 *versus* 42.1%;  $P = 0.59$ ).

## Discussion

With the use of a novel assay for IgM PR3-ANCA, we have shown that ANCA of the IgM isotype are present in certain patients with active AAV, and that the presence of IgM PR3-ANCA is associated with increased disease activity and with alveolar haemorrhage. To our knowledge, these are the largest cohorts on which IgM ANCA testing has been reported. Both the finding of IgM ANCA and the apparent association with the most acute respiratory manifestation of disease are of interest with respect to disease pathogenesis, as they suggest a possible link to infection.

Infections have often been implicated in the development of autoimmune diseases. However, their specific roles remain poorly defined. Many theories have connected autoimmune diseases to antecedent infections, with potential mechanisms including molecular mimicry, epitope spreading and the release of hidden antigens, among others [23]. In certain autoimmune diseases, the connection has been made convincingly. For example, in Guillain–Barré Syndrome, in which acute gastroenteritis caused by *Campylobacter jejuni* frequently precedes the development of the syndrome's neurological manifestations, structural similarities and antibody cross-reactivity have been identified with respect to *C. jejuni*'s lipopolysaccharide and components of peripheral nerves [24–26].

In AAV, infections have not been tied definitively to disease pathogenesis, but several observations have suggested that infectious agents may play an important role in the development of AAV and in its course. For instance, several but not all epidemiological studies have reported seasonal variations in the frequency of presentation of AAV, as would be expected in an infection-mediated disease [27–32]. In addition, the development of circulating ANCA during infections has been described worldwide, most notably in association with infectious endocarditis, although the clinical relevance of these antibodies remains unclear [33–35]. In terms of specific organisms, special consideration has been given to *Staphylococcus aureus*. Chronic nasal colonization with *S. aureus* has been reported to occur more frequently in patients with GPA than in healthy controls and, among those with GPA, nasal colonization with *S. aureus* has been associated with a higher risk of disease relapse [3,36].

As with these observations, our finding of transient IgM PR3-ANCA in substantial numbers of patients can be seen as suggestive of a link between AAV and an infectious exposure. A short-lived rise in antigen-specific IgM antibodies is the expected initial humoral response to pathogen exposure. In theory, this initial IgM phase of the humoral response is particularly susceptible to self-cross-reactivity, as the majority of IgM antibodies are polyreactive, due to low affinity and high valency properties [37,38]. It is therefore possible that the IgM PR3-ANCA has been triggered by an infectious stimulus, analogous to the development of cold haemagglutinins following exposure to *Mycoplasma pneumoniae* [39,40]. Along these lines, the finding of an association between the presence of IgM PR3-ANCA and alveolar haemorrhage supports the possibility of an infectious trigger, as we would speculate that alveolar haemorrhage is more likely in the setting of respiratory inflammation secondary to an infectious insult. Future studies will be required to investigate the association between IgM PR3-ANCA and respiratory infections.

Beyond speculation, there are significant differences between the two trial cohorts to consider as they may explain differences in results. The enrolment criteria of the

two trial protocols differed enough for patients in the WGET cohort being less acutely ill, on average, than those in the RAVE cohort. This difference in acuity derives in part from differences in disease manifestations, as patients with non-severe disease were included in WGET but not in RAVE. It also derives from the fact that the time from the symptom onset of the disease episode leading to enrolment tended to be longer in WGET than in RAVE. As the IgM response is expected to be short-lived, it is possible that the difference in frequency of IgM PR3-ANCA observed in the two cohorts is due in part to the timing of study enrolment; that is, the IgM response in some WGET patients could have been 'missed'.

The potential to miss the coming-and-going of IgM ANCA is an important point that has been highlighted previously in the literature. Certain original descriptions of IgM ANCA suggested a strong correlation with alveolar haemorrhage [6,10]. However, other investigators cast doubt on this association by reporting cases in which IgM ANCA were identified, but no alveolar haemorrhage or other pulmonary manifestations were present [7,11,12]. Some argued that the correlation between alveolar haemorrhage and the identification of IgM ANCA was not meaningful, but attributable to the rapidity with which those who experience alveolar haemorrhage seek medical attention [12]. That is, investigators are simply less likely to miss IgM ANCA in a patient with alveolar haemorrhage compared to patients with other disease manifestations.

Our study, which includes considerably more patients than any previous examination of IgM ANCA, suggests that there is an increased frequency of alveolar haemorrhage among patients with IgM PR3-ANCA, but that the relationship is not absolute. We also observed patients with IgM PR3-ANCA who did not have pulmonary manifestations of AAV, as well as patients with alveolar haemorrhage who tested negative for IgM PR3-ANCA. It is therefore not surprising that prior reports with small numbers of patients found variable clinical correlations for IgM ANCA. However, we cannot discount the possibility that alveolar haemorrhage simply reduces the time from symptom onset to presentation, allowing for a higher probability of observing the transient IgM ANCA.

Our study has strengths and limitations. Among its strengths are the number of patients examined and the comprehensive capturing of clinical data. The most notable limitation is that we focused only on PR3-ANCA, for several reasons. First, we wanted to keep the two cohorts as similar as possible. Secondly, in contrast to IgM PR3-ANCA, samples that tested positive for IgM MPO-ANCA in the RAVE cohort did not test positive on confirmatory immunofluorescence testing (data not shown).

In conclusion, the pathogenesis of AAV is multi-faceted, involving a number of host factors and immune pathways. Insights into the origins and actions of ANCA, including IgM ANCA, may enhance understanding of the aetiology

and varied clinical presentations of AAV. As shown here, IgM PR3-ANCA are found transiently in a sizeable number of patients with active disease, both newly diagnosed and relapsing. The presence of IgM PR3-ANCA is associated with a higher degree of disease activity and with a higher rate of alveolar haemorrhage, which is the most acute and life-threatening manifestation of AAV. IgM PR3-ANCA could thus represent a link between infectious triggers and AAV disease activity.

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## Disclosure

None of the authors report financial or commercial disclosures.

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