IgM Antibodies to Proteinase 3 in Granulomatosis with Polyangiitis and Microscopic Polyangiitis

Clain JM[1], Hummel AM[1], Stone JH[2], Fervenza FC[1], Hoffman GS[3], Kallenberg CGM[4], Langford CA[3], McCune WJ[5], Merkel PA[6], Monach PA[7], Seo P[8], Spiera RF[9], St. Clair EW[10], Ytterberg SR[1], and Specks U[1] for the WGET and RAVE-ITN Research Groups.

Author Affiliations:
[1]Mayo Clinic and Foundation, Rochester, MN, USA
[2]Massachusetts General Hospital, Boston, MA
[3]Cleveland Clinic Foundation, Cleveland, OH
[4]University of Groningen, Groningen, the Netherlands
[5]University of Michigan, Ann Arbor, MI
[6]University of Pennsylvania, Philadelphia, PA
[7]Boston University Medical Center, Boston, MA
[8]Johns Hopkins University, Baltimore, MD
[9]Hospital for Special Surgery, New York, NY
[10]Duke University, Durham, NC

Short Title: IgM PR3-ANCA in GPA and MPA

***Corresponding author and reprints request*:**
Ulrich Specks, M.D.
Division of Pulmonary and Critical Care Medicine,
Mayo Clinic and Foundation, 200 First Street SW, Rochester, MN 55905
Phone: 507 284 2301; Fax: 507 284 4521
e-mail: specks.ulrich@mayo.edu

Keywords:
Antineutrophil cytoplasmic antibodies
ANCA-associated vasculitis
Granulomatosis with polyangiitis
Microscopic polyangiitis
Immunoglobulin M
Alveolar hemorrhage

Abbreviations:
ANCA – antineutrophil cytoplasmic antibodies
AAV – antineutrophil cytoplasmic antibody-associated vasculitis
GPA – granulomatosis with polyangiitis
MPA – microscopic polyangiitis
EGPA – eosinophilic granulomatosis with polyangiitis
PR3 – proteinase 3
MPO – myeloperoxidase
WGET – Wegener's Granulomatosis Etanercept Trial
RAVE - Rituximab versus Cyclophosphamide for AAV Trial
BVAS/WG - Birmingham Vasculitis Activity Score for Wegener Granulomatosis

SUMMARY

Antineutrophil cytoplasmic antibodies (ANCA) appear to play an important role in the pathogenesis of ANCA-associated vasculitis (AAV). Yet, 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 IgM isotype often serve as markers of recent infection, and IgM ANCA have previously been identified in patients with AAV, though the frequency and clinical relevance of IgM ANCA is not well established. We sought to better characterize IgM ANCA by creating a novel ELISA assay for IgM antibodies to proteinase 3 (IgM 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 with a trend toward a higher rate of alveolar hemorrhage (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 hemorrhage was observed among those with IgM PR3-ANCA (45.3% vs. 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.

INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA) -associated vasculitides (AAV) are defined by characteristic clinical and histopathologic features and the presence of ANCA in most patients(1). However, patients with AAV present heterogeneously, to the extent that three separate clinicopathologic 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 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. Yet, 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. Yet, ANCA alone are not sufficient for the disease process to play out in full in humans(3-5). Moreover, the specific triggers of a primary ANCA immune response, such as infections, and the mechanisms subsequently leading 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 hemorrhage(6, 10). However, these associations and their clinical relevance were questioned by others(7, 11, 12). Since these early reports on IgM ANCA were derived from rather small cohorts, and the theory of infections as triggering cofactors 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 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, and so there is substantial heterogeneity between the two groups; second, the separation of the two cohorts provided the opportunity to test and validate associations identified in our study of the first cohort.

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 multicenter, 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 8 centers 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 2 of the 5 modified American College of Rheumatology criteria for the classification of Wegener granulomatosis, and (2) there was evidence of disease activity within 28 days of enrollment, 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 multicenter, 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 8 centers in the United States and one center 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 MPO-ANCA at the time of enrollment, 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 three 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 -80º C.

ANCA Assays

IgG ANCA assays had previously been performed in both study cohorts using standard immunofluorescence and enzyme immunoassays(4, 5, 17, 18). A novel capture 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 TBS buffer containing 0.5% BSA. The presence of IgM antibodies bound to rPR3 were detected using a goat anti-human IgM (μ-chain specific)-alkaline phosphatase antibody (Sigma-Aldrich), 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 previously tested negative 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 4 standard deviations 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 previously described for IgG ANCA detection(20-22). Immulon strips were coated with the monoclonal

antibodies 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 otherwise specified. 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 rank sum test. Statistical analyses were performed using JMP 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 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) (**Figure 1**).

The organ systems involved by GPA did not differ according to IgM PR3-ANCA status, but there was a notable trend toward more frequent alveolar hemorrhage among those who tested positive for IgM PR3-ANCA. Alveolar hemorrhage was diagnosed at baseline in 8 of the 27 patients (29.6%) who tested positive for IgM PR3-ANCA, as 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 hemorrhage was diagnosed in 8 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 hemorrhage at the baseline visit, further IgM PR3-ANCA assays were performed on serum samples from all available subsequent study visits, as summarized in **Figure 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 8 patients had tested positive for IgM PR3-ANCA at the baseline visit. On follow-up, only 1 of the 8 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 reemerged in 4 of the 8 patients.

Among the 4 WGET patients who were diagnosed with alveolar hemorrhage 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 1 of the 4 patients, the IgM PR3-ANCA epitope target appeared to remain constant over time, but epitope variability was apparent in serial samples from the remaining 3 patients. The longitudinal results from 2 of the 4 patients are represented in **Figure 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 **Figure 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 does not simply follow the rise and fall of the overall ANCA level. The second point highlighted in **Figure 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 further investigate the most interesting observation made in the WGET cohort: the potential association between the presence of IgM PR3-ANCA and the diagnosis of alveolar hemorrhage. 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 (27.9%) were diagnosed with alveolar hemorrhage 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 as compared to those who tested negative (**Figure 4**). 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 hemorrhage was diagnosed nearly three times more frequently among those who tested positive for IgM PR3-ANCA as compared to those who tested negative (45.3% vs. 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 as 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 hemorrhage. 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. Yet, 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 quite convincingly. For example, in Guillain-Barré Syndrome, in which acute gastroenteritis caused by Campylobacter jejuni frequently precedes the development of the syndrome's neurologic manifestations, structural similarities and antibody cross-reactivity have been identified with respect to Campylobacter jejuni's lipopolysaccharide and components of peripheral nerves(24-26).

In AAV, infections have not definitively been tied 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, though 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 hemagglutinins following exposure to Mycoplasma pneumoniae(39, 40). Along these lines, the finding of an association between the presence of IgM PR3-ANCA and alveolar hemorrhage supports the possibility of an infectious trigger, as we would speculate that alveolar hemorrhage 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 enrollment criteria of the two trial protocols differed enough for patients in the WGET cohort on average being less acutely ill 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 enrollment 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 enrollment; that is, the IgM response in some WGET patients could have been "missed".

The potential to miss the coming-and-going of the IgM ANCA is an important point that has previously been highlighted in the literature. Certain original descriptions of IgM ANCA suggested a strong correlation with alveolar hemorrhage(6, 10). However, other investigators cast doubt on this association by reporting cases in which IgM ANCA were identified, but no alveolar hemorrhage or other pulmonary manifestations were present(7, 11, 12). Some argued that the correlation between alveolar hemorrhage and the identification of IgM ANCA was not meaningful, but attributable to the rapidity with which those who experience alveolar hemorrhage seek medical attention(12). That is, investigators are simply less likely to miss IgM ANCA in a patient with alveolar hemorrhage as 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 hemorrhage 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 hemorrhage 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 hemorrhage 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. Our focus on PR3-ANCA has several reasons. First, we wanted to keep the two cohorts as similar as possible. Second, 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 multifaceted, 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 etiology and varied clinical presentations of AAV. As shown here, IgM PR3-ANCA are found transiently in a sizable 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 hemorrhage, 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.

ACKNOWLEDGEMENTS

The WGET trial was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH N01-AR92240) and the Office of Orphan Products, FDA (grant FD-R-001652) The RAVE trial was supported by the National Institute of Allergy and Infectious Diseases (N01-AI-15416; protocol no. ITN021AI), Genentech, Inc. and Biogen IDEC, Inc. (study medications and partial funding). At the Mayo Clinic and Foundation, the trials were supported by a Clinical and Translational Science Award from the National Center for Research Resources (NCRR; RR024150); at Johns Hopkins University, by grants from the NCRR (RR025005) and career development awards (K24 AR049185, to Dr. Stone, and K23 AR052820, to Dr. Seo); and at Boston University, by a Clinical and Translational Science Award (RR 025771), grants from the National Institutes of Health (M01 RR00533) and a career development

award (K24 AR02224, to Dr. Merkel), and an Arthritis Foundation Investigator Award (to Dr. Monach). This study was supported by the Seventh Framework Programme (261382) and funds from the Mayo Foundation (to Dr. Specks).

CONFLICT OF INTEREST

None of the authors report financial or commercial conflicts of interest.

TABLES

Table 1 – Patient characteristics.

Clinical Characteristic	WGET Cohort* (n=180)	RAVE Cohort** (n=129)	P-value
Age (mean ± SD)	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 ± SD)	7.0 ± 3.4	8.0 ± 3.2	0.01
Alveolar hemorrhage	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)

Table 2 – Disease characteristics by IgM PR3-ANCA status, 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)	

FIGURE LEGENDS

Figure 1 – Disease severity, as measured by BVAS/WG score, at baseline visit in WGET cohort, by IGM PR3-ANCA status.

Figure 2 – Change in IgM PR3-ANCA over time in patients with alveolar hemorrhage in WGET cohort

Figure 3 – Longitudinal results of IgG PR3-ANCA ELISA, IgM PR3-ANCA ELISA, and IgM PR3-ANCA epitope binding pattern assays for two patients who were diagnosed with alveolar hemorrhage at baseline and who tested positive for IgM PR3-ANCA at multiple time points during the WGET study. Panels A and B display the results of ELISA assays for IgG PR3-ANCA (panel A) and IgM PR3-ANCA (panel B) performed on sera drawn from patient 1 across longitudinal study visits. Panels D and 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. Panel C displays 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. Panel F displays results of the same assays performed on sera drawn from patient 2. 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. Panels C and F highlight that the IgM PR3-ANCA epitope binding site appears to change over time for patient 1 (panel C), but not for patient 2 (panel F).

Figure 4 – Disease severity, as measured by BVAS/WG score, at baseline visit in RAVE cohort, by IGM PR3-ANCA status.

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Figure 1

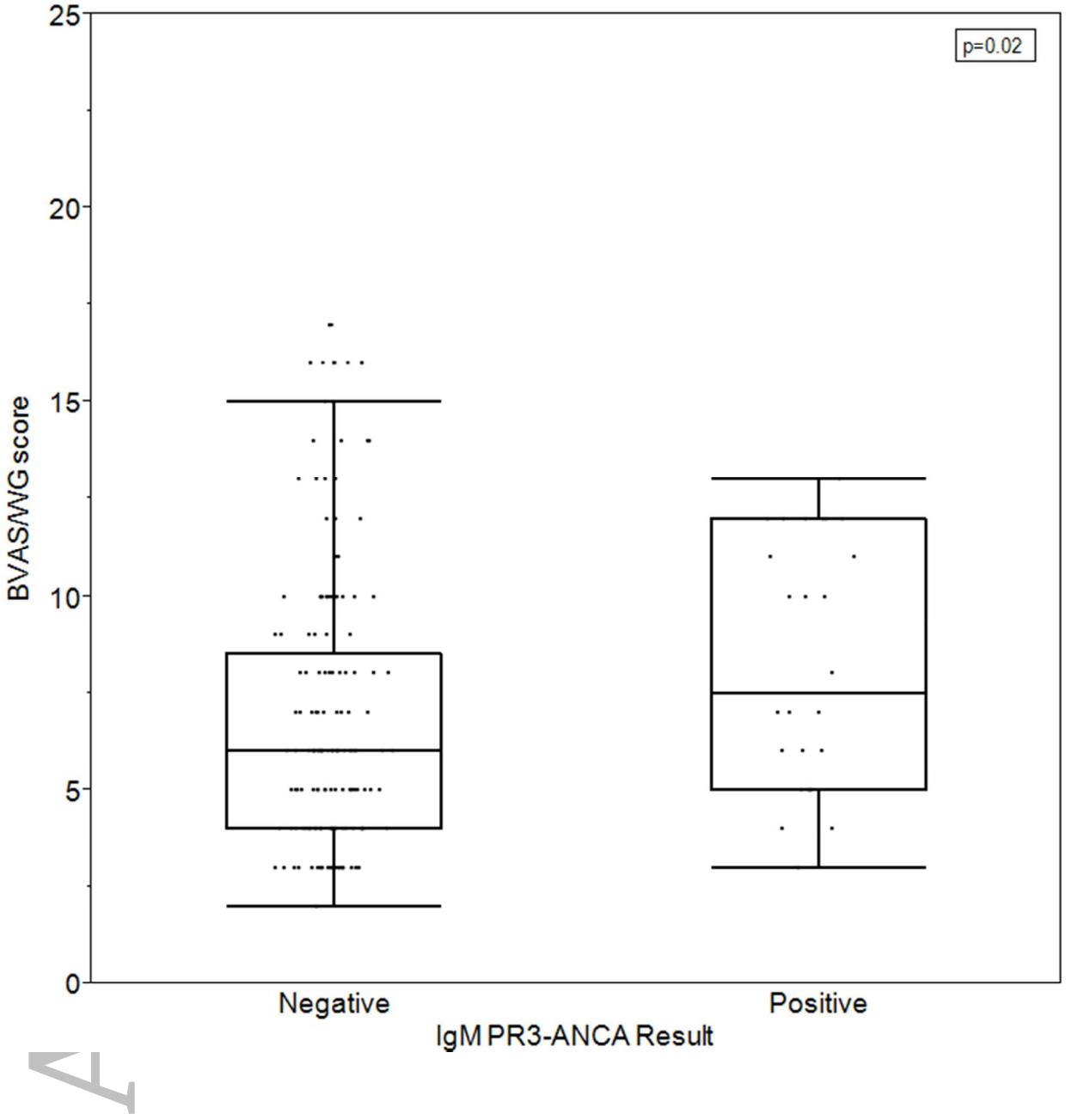


Figure 2

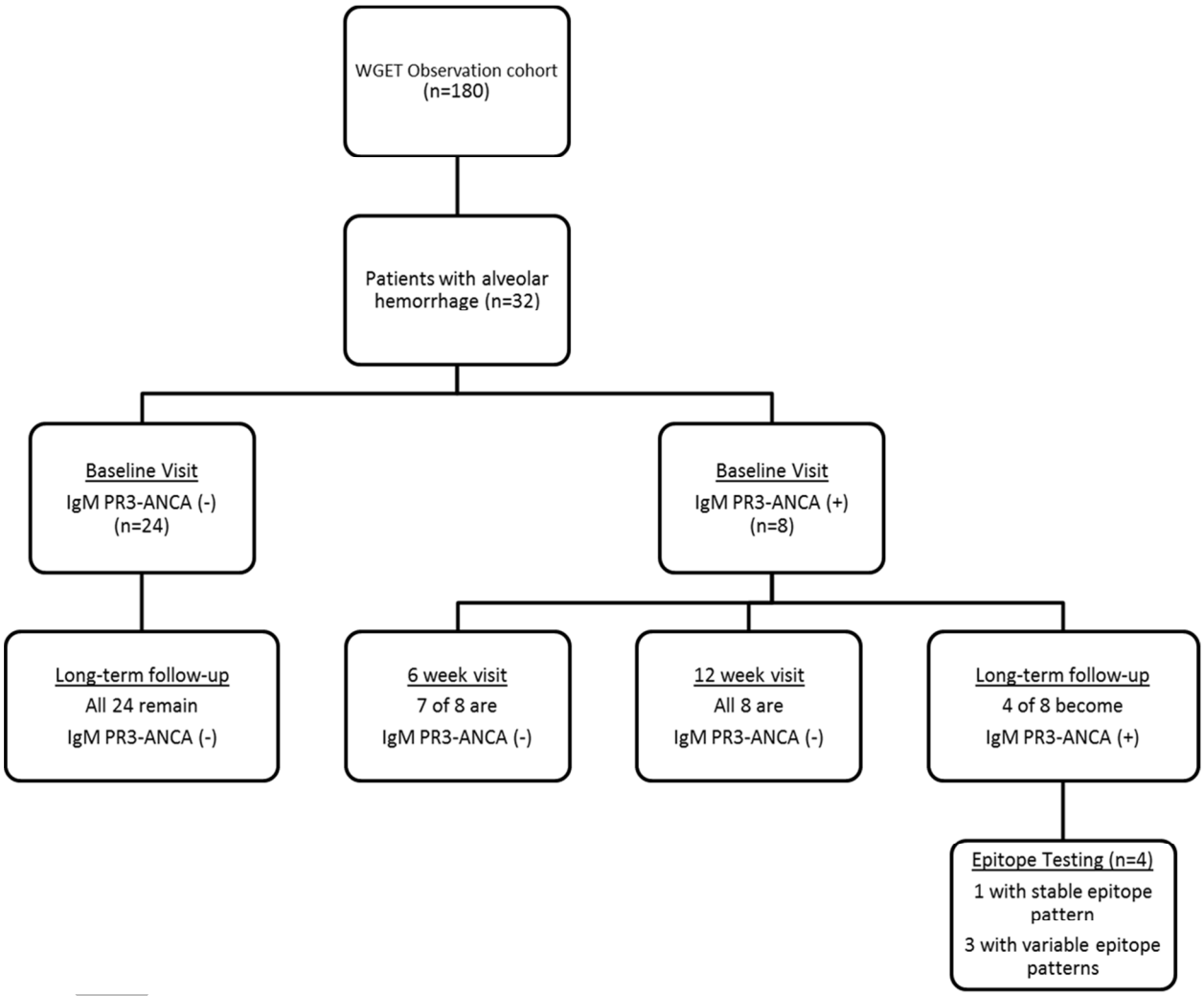


Figure 3

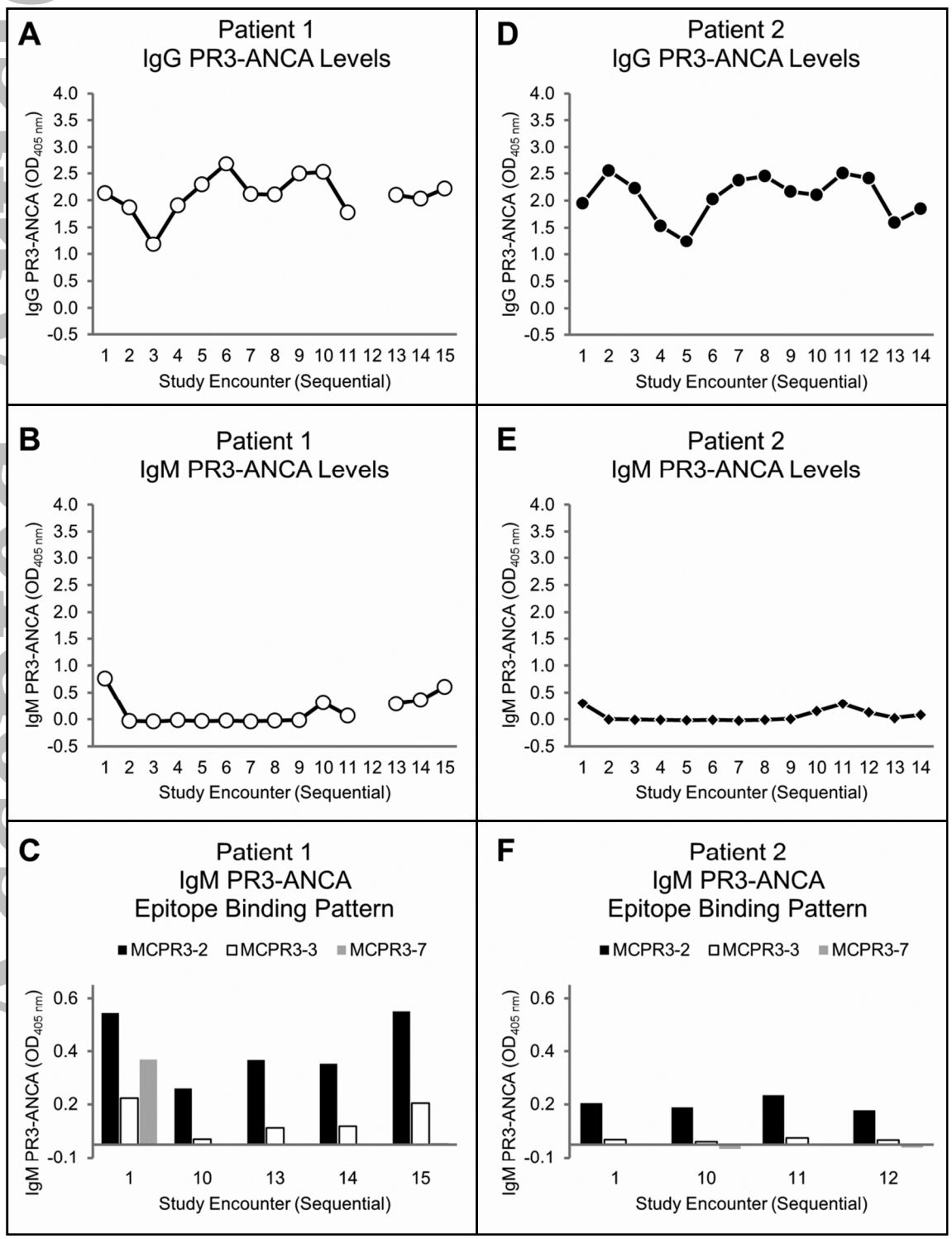
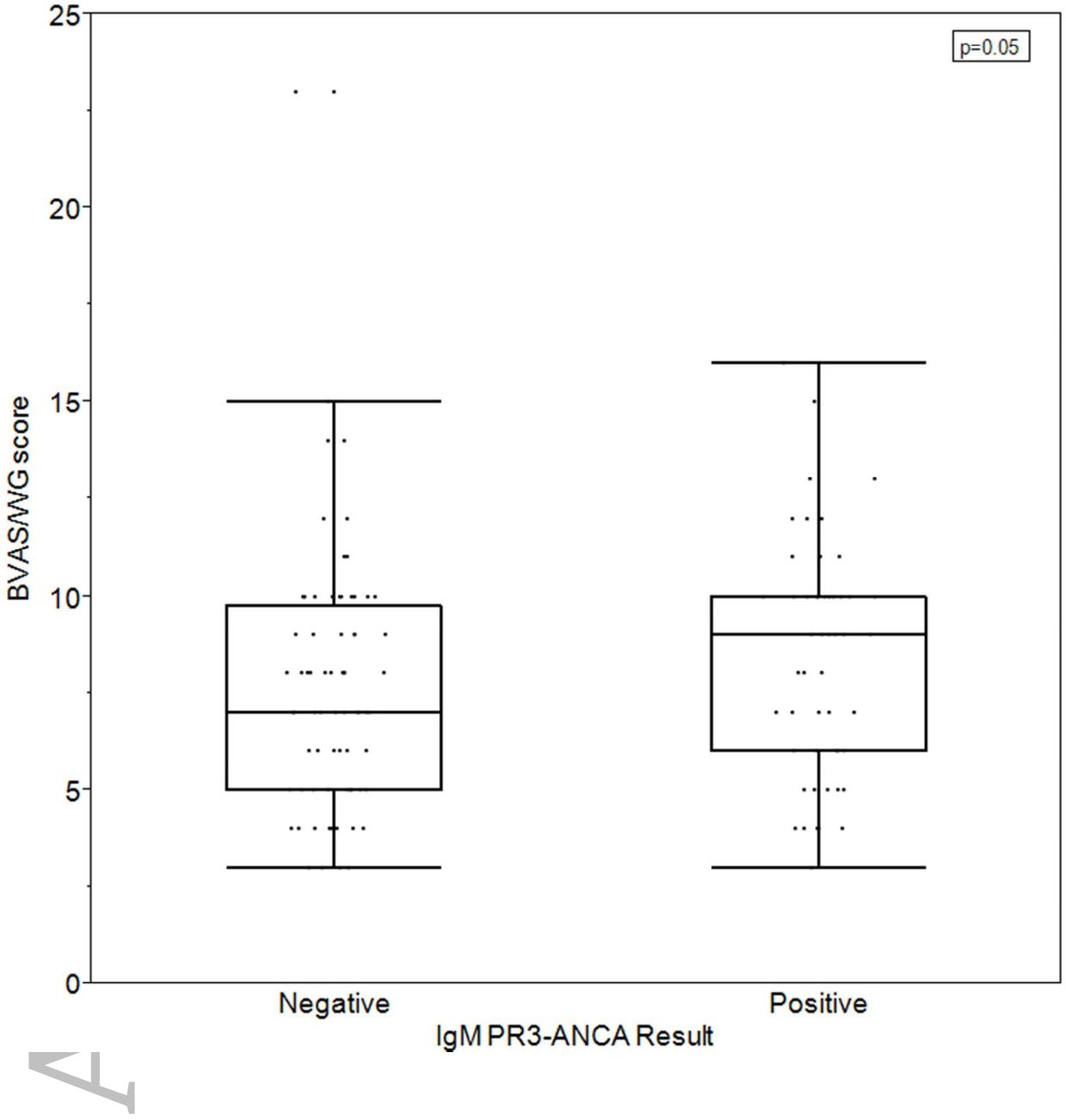


Figure 4



IgM Antibodies to Proteinase 3 in Granulomatosis with Polyangiitis and Microscopic Polyangiitis

Clain JM[1], Hummel AM[1], Stone JH[2], Fervenza FC[1], Hoffman GS[3], Kallenberg CGM[4], Langford CA[3], McCune WJ[5], Merkel PA[6], Monach PA[7], Seo P[8], Spiera RF[9], St. Clair EW[10], Ytterberg SR[1], and Specks U[1] for the WGET and RAVE-ITN Research Groups.

Author Affiliations:
[1]Mayo Clinic and Foundation, Rochester, MN, USA
[2]Massachusetts General Hospital, Boston, MA
[3]Cleveland Clinic Foundation, Cleveland, OH
[4]University of Groningen, Groningen, the Netherlands
[5]University of Michigan, Ann Arbor, MI
[6]University of Pennsylvania, Philadelphia, PA
[7]Boston University Medical Center, Boston, MA
[8]Johns Hopkins University, Baltimore, MD
[9]Hospital for Special Surgery, New York, NY
[10]Duke University, Durham, NC

Short Title: IgM PR3-ANCA in GPA and MPA

***Corresponding author and reprints request*:**
Ulrich Specks, M.D.
Division of Pulmonary and Critical Care Medicine,
Mayo Clinic and Foundation, 200 First Street SW, Rochester, MN 55905
Phone: 507 284 2301; Fax: 507 284 4521
e-mail: specks.ulrich@mayo.edu

Keywords:
Antineutrophil cytoplasmic antibodies
ANCA-associated vasculitis
Granulomatosis with polyangiitis
Microscopic polyangiitis
Immunoglobulin M
Alveolar hemorrhage

Abbreviations:
ANCA – antineutrophil cytoplasmic antibodies
AAV – antineutrophil cytoplasmic antibody-associated vasculitis
GPA – granulomatosis with polyangiitis
MPA – microscopic polyangiitis
EGPA – eosinophilic granulomatosis with polyangiitis
PR3 – proteinase 3
MPO – myeloperoxidase
WGET – Wegener's Granulomatosis Etanercept Trial
RAVE - Rituximab versus Cyclophosphamide for AAV Trial
BVAS/WG - Birmingham Vasculitis Activity Score for Wegener Granulomatosis

SUMMARY

Antineutrophil cytoplasmic antibodies (ANCA) appear to play an important role in the pathogenesis of ANCA-associated vasculitis (AAV). Yet, 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 IgM isotype often serve as markers of recent infection, and IgM ANCA have previously been identified in patients with AAV, though the frequency and clinical relevance of IgM ANCA is not well established. We sought to better characterize IgM ANCA by creating a novel ELISA assay for IgM antibodies to proteinase 3 (IgM 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 with a trend toward a higher rate of alveolar hemorrhage (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 hemorrhage was observed among those with IgM PR3-ANCA (45.3% vs. 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.

INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA) -associated vasculitides (AAV) are defined by characteristic clinical and histopathologic features and the presence of ANCA in most patients(1). However, patients with AAV present heterogeneously, to the extent that three separate clinicopathologic 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 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. Yet, 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. Yet, ANCA alone are not sufficient for the disease process to play out in full in humans(3-5). Moreover, the specific triggers of a primary ANCA immune response, such as infections, and the mechanisms subsequently leading 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 hemorrhage(6, 10). However, these associations and their clinical relevance were questioned by others(7, 11, 12). Since these early reports on IgM ANCA were derived from rather small cohorts, and the theory of infections as triggering cofactors 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 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, and so there is substantial heterogeneity between the two groups; second, 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 in 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 multicenter,

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 8 centers 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 2 of the 5 modified American College of Rheumatology criteria for the classification of Wegener granulomatosis, and (2) there was evidence of disease activity within 28 days of enrollment, 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 multicenter, 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 8 centers in the United States and one center 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 MPO-ANCA at the time of enrollment, 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 three 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 -80º C.

ANCA Assays

IgG ANCA assays had previously been performed in both study cohorts using standard immunofluorescence and enzyme immunoassays(4, 5, 17, 18). A novel capture 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 TBS buffer containing 0.5% BSA. The presence of IgM antibodies bound to rPR3 were detected using a goat anti-human IgM (μ-chain specific)-alkaline phosphatase antibody (Sigma-Aldrich), 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 previously tested negative 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 4 standard deviations 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 previously

described for IgG ANCA detection(20-22). Immulon strips were coated with the monoclonal antibodies 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 otherwise specified. 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 rank sum test. Statistical analyses were performed using JMP 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 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) (**Figure 1**).

The organ systems involved by GPA did not differ according to IgM PR3-ANCA status, but there was a notable trend toward more frequent alveolar hemorrhage among those who tested positive for IgM PR3-ANCA. Alveolar hemorrhage was diagnosed at baseline in 8 of the 27 patients (29.6%) who tested positive for IgM PR3-ANCA, as 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 hemorrhage was diagnosed in 8 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 hemorrhage at the baseline visit, further IgM PR3-ANCA assays were performed on serum samples from all available subsequent study visits, as summarized in **Figure 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 8 patients had tested positive for IgM PR3-ANCA at the baseline visit. On follow-up, only 1 of the 8 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 reemerged in 4 of the 8 patients.

Among the 4 WGET patients who were diagnosed with alveolar hemorrhage 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 1 of the 4 patients, the IgM PR3-ANCA epitope target appeared to remain constant over time, but epitope variability was apparent in serial samples from the remaining 3 patients. The longitudinal results from 2 of the 4 patients are represented in **Figure 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 **Figure 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 does not simply follow the rise and fall of the overall ANCA level. The second point highlighted in **Figure 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 further investigate the most interesting observation made in the WGET cohort: the potential association between the presence of IgM PR3-ANCA and the diagnosis of alveolar hemorrhage. 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 (27.9%) were diagnosed with alveolar hemorrhage 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 as compared to those who tested negative (**Figure 4**). 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 hemorrhage was diagnosed nearly three times more frequently among those who tested positive for IgM PR3-ANCA as compared to those who tested negative (45.3% vs. 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 as 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 hemorrhage. 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. Yet, 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 quite convincingly. For example, in Guillain-Barré Syndrome, in which acute gastroenteritis caused by Campylobacter jejuni frequently precedes the development of the syndrome's neurologic manifestations, structural similarities and antibody cross-reactivity have been identified with respect to Campylobacter jejuni's lipopolysaccharide and components of peripheral nerves(24-26).

In AAV, infections have not definitively been tied 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, though 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 hemagglutinins following exposure to Mycoplasma pneumoniae(39, 40). Along these lines, the finding of an association between the presence of IgM PR3-ANCA and alveolar hemorrhage supports the possibility of an infectious trigger, as we would speculate that alveolar hemorrhage 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 enrollment criteria of the two trial protocols differed enough for patients in the WGET cohort on average being less acutely ill 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 enrollment 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 enrollment; that is, the IgM response in some WGET patients could have been "missed".

The potential to miss the coming-and-going of the IgM ANCA is an important point that has previously been highlighted in the literature. Certain original descriptions of IgM ANCA suggested a strong correlation with alveolar hemorrhage(6, 10). However, other investigators cast doubt on this association by reporting cases in which IgM ANCA were identified, but no alveolar hemorrhage or other pulmonary manifestations were present(7, 11, 12). Some argued that the correlation between alveolar hemorrhage and the identification of IgM ANCA was not meaningful, but attributable to the rapidity with which those who experience alveolar hemorrhage seek medical attention(12). That is, investigators are simply less likely to miss IgM ANCA in a patient with alveolar hemorrhage as 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 hemorrhage 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 hemorrhage 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 hemorrhage 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. Our focus on PR3-ANCA has several reasons. First, we wanted to keep the two cohorts as similar as possible. Second, 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 multifaceted, 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 etiology and varied clinical presentations of AAV. As shown here, IgM PR3-ANCA are found transiently in a sizable 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 hemorrhage, 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.

ACKNOWLEDGEMENTS

The WGET trial was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH N01-AR92240) and the Office of Orphan Products, FDA (grant FD-R-001652) The RAVE trial was supported by the National Institute of Allergy and Infectious Diseases (N01-AI-15416; protocol no. ITN021AI), Genentech, Inc. and Biogen IDEC, Inc. (study medications and partial funding). At the Mayo Clinic and Foundation, the trials were supported by a Clinical and Translational Science Award from the National Center for Research Resources (NCRR; RR024150); at Johns Hopkins University, by grants from the NCRR (RR025005) and career development awards (K24 AR049185, to Dr. Stone, and K23 AR052820, to Dr. Seo); and at Boston University, by a Clinical and Translational Science Award (RR 025771), grants from the National Institutes of Health (M01 RR00533) and a career development

award (K24 AR02224, to Dr. Merkel), and an Arthritis Foundation Investigator Award (to Dr. Monach). This study was supported by the Seventh Framework Programme (261382) and funds from the Mayo Foundation (to Dr. Specks).

CONFLICT OF INTEREST

None of the authors report financial or commercial conflicts of interest.

TABLES

Table 1 – Patient characteristics.

Clinical Characteristic	WGET Cohort* (n=180)	RAVE Cohort** (n=129)	P-value
Age (mean ± SD)	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 ± SD)	7.0 ± 3.4	8.0 ± 3.2	0.01
Alveolar hemorrhage	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)

Table 2 – Disease characteristics by IgM PR3-ANCA status, 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)	

FIGURE LEGENDS

Figure 1 – Disease severity, as measured by BVAS/WG score, at baseline visit in WGET cohort, by IGM PR3-ANCA status.

Figure 2 – Change in IgM PR3-ANCA over time in patients with alveolar hemorrhage in WGET cohort

Figure 3 – Longitudinal results of IgG PR3-ANCA ELISA, IgM PR3-ANCA ELISA, and IgM PR3-ANCA epitope binding pattern assays for two patients who were diagnosed with alveolar hemorrhage at baseline and who tested positive for IgM PR3-ANCA at multiple time points during the WGET study. Panels A and B display the results of ELISA assays for IgG PR3-ANCA (panel A) and IgM PR3-ANCA (panel B) performed on sera drawn from patient 1 across longitudinal study visits. Panels D and 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. Panel C displays 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. Panel F displays results of the same assays performed on sera drawn from patient 2. 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. Panels C and F highlight that the IgM PR3-ANCA epitope binding site appears to change over time for patient 1 (panel C), but not for patient 2 (panel F).

Figure 4 – Disease severity, as measured by BVAS/WG score, at baseline visit in RAVE cohort, by IGM PR3-ANCA status.

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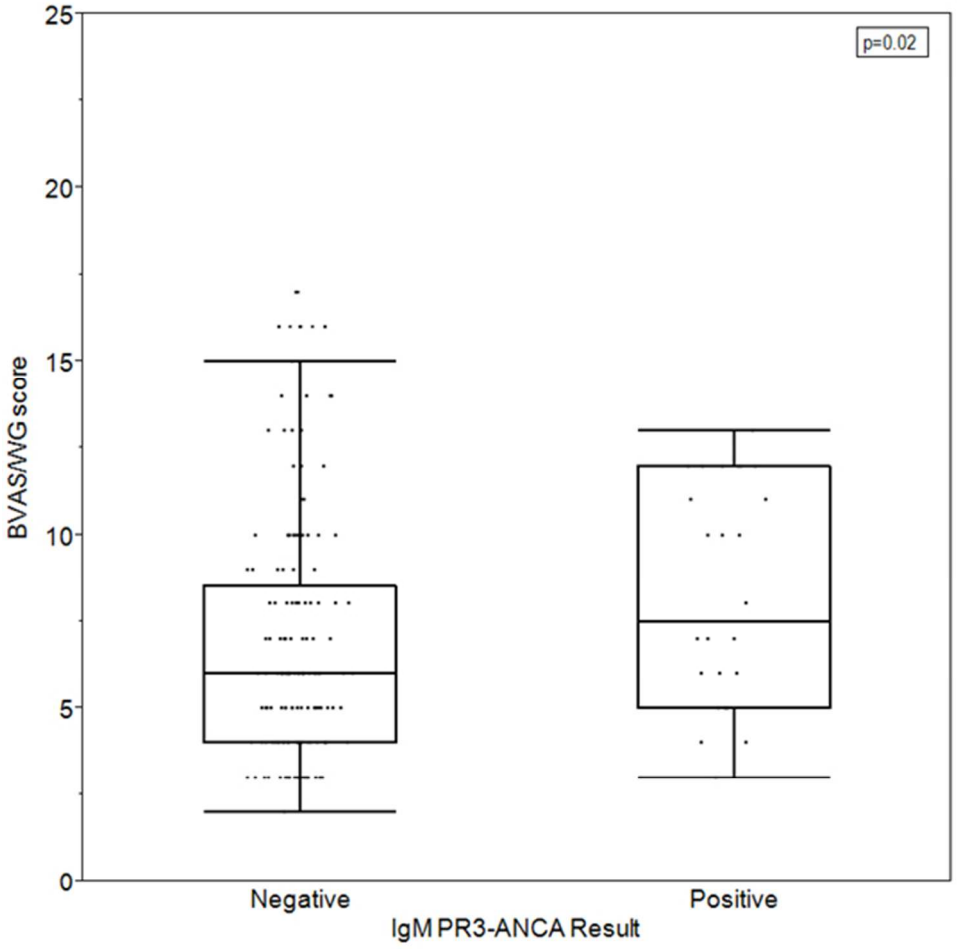

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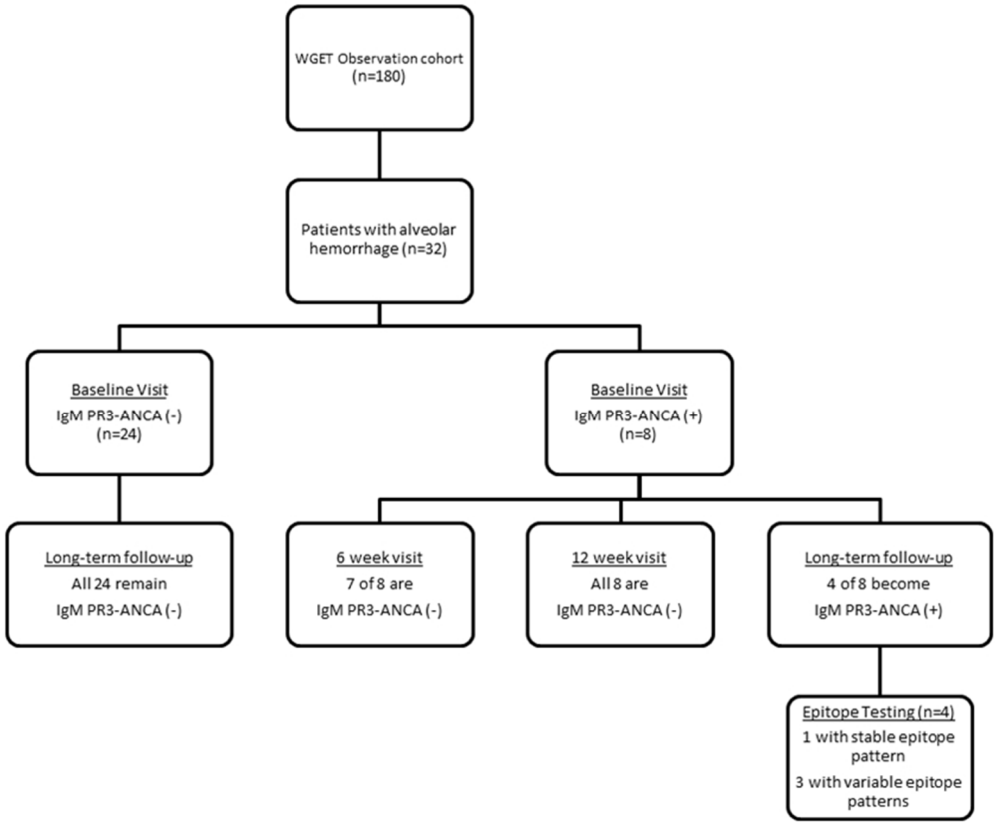

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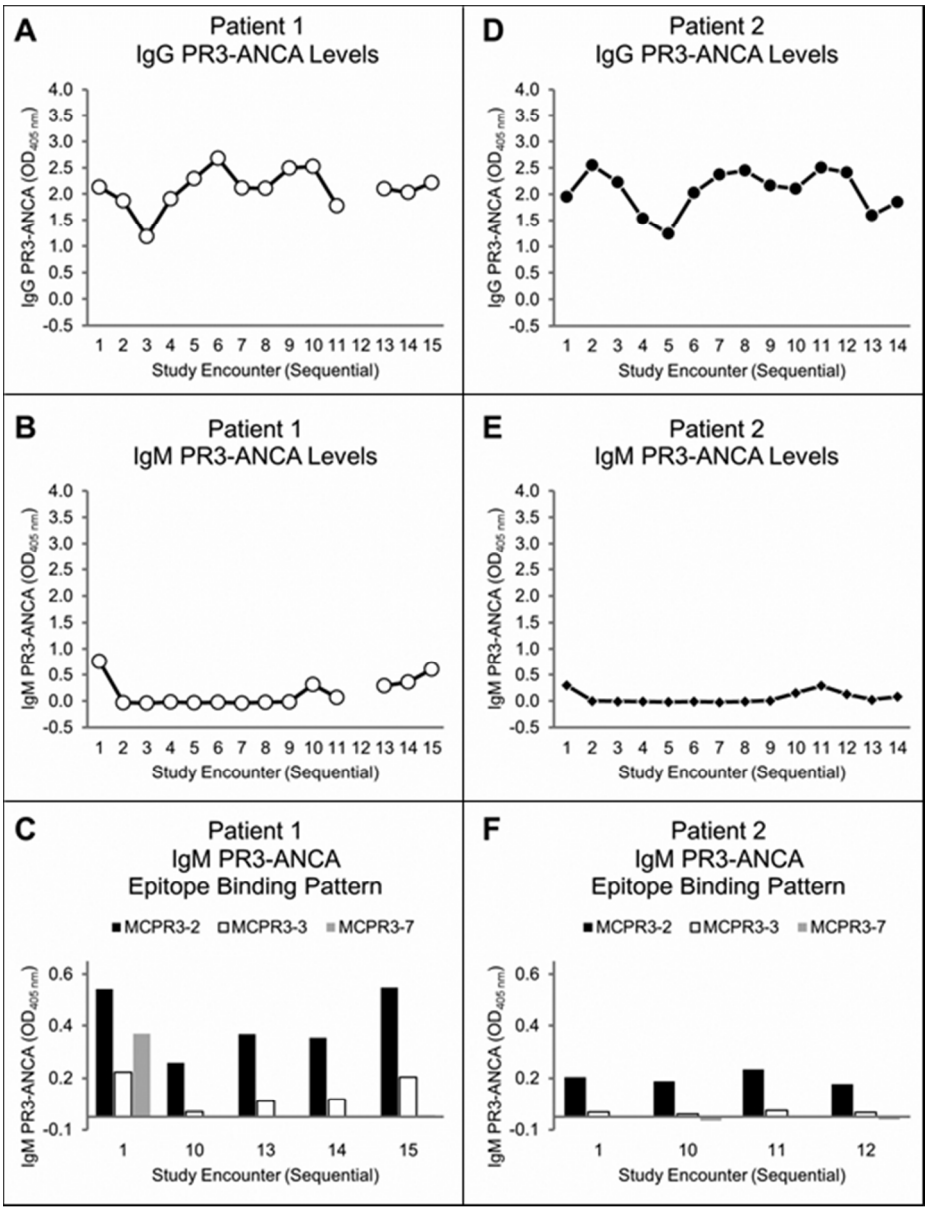

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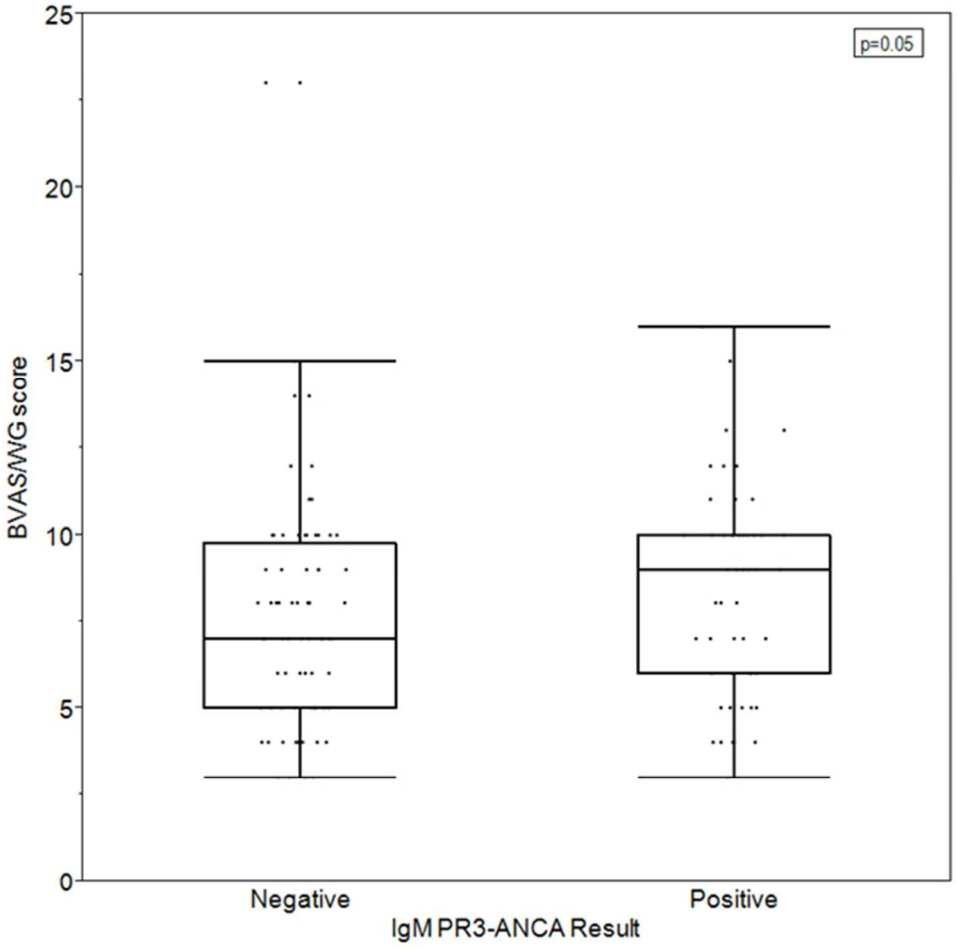

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