# IgA antibodies to myeloperoxidase in patients with eosinophilic granulomatosis with polyangiitis (Churg-Strauss)

E. Oommen[1,14], A. Hummel[2], L. Allmannsberger[3], D. Cuthbertson[4], S. Carette[5], C. Pagnoux[5], G.S. Hoffman[6], D.E. Jenne[3], N.A. Khalidi[7], C.L. Koening[8], C.A. Langford[6], C.A. McAlear[9], L. Moreland[10], P. Seo[11], A. Sreih[9], S.R. Ytterberg[12], P.A. Merkel[9], U. Specks[2], P.A. Monach[1,13] for the Vasculitis Clinical Research Consortium

*[1]Section of Rheumatology, Boston University School of Medicine, MA; [2]Div. of Pulmonology, Mayo Clinic College of Medicine, Rochester, MN, USA; [3]Helmholtz Zentrum Muenchen, Munich, Germany; [4]Dept. of Biostatistics, University of South Florida, FL, USA; [5]Div. of Rheumatology, Mount Sinai Hosp., Toronto, ON, Canada; [6]Center for Vasculitis Care and Research, Cleveland Clinic, Cleveland, OH, USA; [7]Div. of Rheumatology, St. Joseph's Healthcare, McMaster Univ., Hamilton, ON, Canada; [8]Div. of Rheumatology, Univ. of Utah, Salt Lake City, UT; [9]Div. of Rheumatology and Clinical Immunology, Univ. of Pennsylvania, Philadelphia, PA; [10]Div. of Rheumatology, Univ. of Pittsburgh, PA; [11]Div. of Rheumatology, Johns Hopkins Univ., Baltimore, MD; [12]Div. of Rheumatology, Mayo Clinic College of Medicine, Rochester, MN; [13]Rheumatology Section, VA Boston Healthcare System, Boston, MA, USA; [14]Current position: Div. of Pulmonary, Allergy, Critical Care and Sleep Medicine, Emory Univ. School of Medicine, Atlanta, GA, USA.*

*Esha Oommen, MD; Amber Hummel, BA; Lisa Allmannsberger, PhD; David Cuthbertson, MS; Simon Carette, MD; Christian Pagnoux, MD; Gary S. Hoffman, MD Dieter E. Jenne, MD; Nader A. Khalidi, MD, FRCPC; Curry L. Koening, MD, MS; Carol A. Langford, MD, MHS; Carol A. McAlear, MA; Larry Moreland, MD; Philip Seo, MD, MHS; Antoine Sreih, MD; Steven R. Ytterberg, MD; Peter A. Merkel, MD, MPH; Ulrich Specks, MD; Paul A. Monach, MD, PhD*

*Please address correspondence to:*
*Dr Paul A. Monach,*
*Section of Rheumatology, Boston University School of Medicine, 72 East Concord St., E-533, Boston, MA 02118, USA.*
*E-mail: pmonach@bu.edu*

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*Funding and competing interests on page S101.*

## ABSTRACT

**Objective.** *To determine the prevalence of anti-myeloperoxidase (MPO) antibodies of IgA (IgA anti-MPO) isotype in patients with eosinophilic granulomatosis with polyangiitis (EGPA), and the association of the IgA antibodies with IgG anti-MPO and with disease activity.*

**Methods.** *Serum samples from patients with EGPA followed in a multicentre longitudinal cohort were tested by ELISA for the presence of IgA anti-MPO and IgG anti-MPO antibodies. Sera from 87 healthy controls were used to define a positive test. Sera from 168 patients with EGPA (298 samples) were tested. Frequencies of positive testing for IgA anti-MPO were compared between patients with active EGPA, patients in remission, and controls.*

**Results.** *IgA anti-MPO was detected in 10 of 168 (6%) patients with EGPA (11 of 298 serum samples) compared to 1 of 87 (1%) healthy controls (p=0.10). All 11 samples testing positive for IgA anti-MPO also tested positive for IgG anti-MPO. Ninety samples tested positive for IgG anti-MPO but negative for IgA. Samples taken during active EGPA were positive for IgA anti-MPO in 6/72 cases (8%), compared to 5/226 (2%) during remission (p=0.03). Among samples taken during moderate or high disease activity, 5/41 were positive (12%, p=0.01 compared to remission).*

**Conclusion.** *Although IgA anti-MPO antibodies are detectable in some patients with EGPA and may be detectable more frequently during active disease, their presence seems unlikely to provide information beyond what is obtained from conventional IgG anti-MPO.*

## Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA, Churg-Strauss) is a rare disease characterised by asthma, eosinophilia, eosinophilic inflammation, and necrotising vasculitis of small- and medium-sized vessels (1, 2). Because of clinical similarities of EGPA to granulomatosis with polyangiitis (Wegener's) and microscopic polyangiitis, two forms of vasculitis that are strongly associated with antineutrophil cytoplasmic antibodies (ANCA) (3), ANCA have also been tested in EGPA. Approximately 40% of patients with EGPA test positive for ANCA with specificity for myeloperoxidase (MPO) (4-6). In clinical practice anti-MPO antibodies are used to differentiate EGPA from other diseases, particularly idiopathic hypereosinophilic syndrome (HES), since biopsy proof of vasculitis to distinguish EGPA from HES is not always possible. Clinical manifestations of EGPA vary with ANCA status: ANCA-positive patients manifest more kidney or nerve involvement, and ANCA-negative patients have more cardiac disease (7).

Additional biomarkers that could aid in diagnosis or monitoring of disease activity in EGPA would be useful (8). The IgA subtype of ANCA could be of interest in EGPA because of involvement of the airway (sinusitis, rhinitis, asthma and bronchitis) in almost all patients, usually preceding the development of vasculitis. Additionally, IgA is a potent stimulant for eosinophil degranulation (9). ANCA of IgA isotype have been investigated in IgA vasculitis (Henoch-Schönlein Purpura) (10-12), autoimmune hepatitis and primary sclerosing cholangitis (13), ulcerative colitis (14, 15), cutaneous vasculitis (16), and neutrophilic dermatoses (17). In the only study in which antibodies to MPO or PR3 of IgA isotype were tested (in IgA vasculitis), only one patient tested positive (10). More recently, however, IgA

anti-PR3 was found in 30% of patients with GPA, particularly in patients with upper airway involvement, and with evidence of neutrophil degranulation in response to IgA anti-PR3 stimulation (18).

The main goals of the current study were to determine the frequency of positive testing for IgA anti-MPO among patients with EGPA in a large cohort, and to determine whether there was an association of IgA anti-MPO titre with current disease activity.

## Methods

*Patients and clinical data*

Serum samples and data from patients enrolled in the Vasculitis Clinical Research Consortium (VCRC) Longitudinal Study of EGPA were used**.** Patients were enrolled at 8 referral centers in the United States and Canada between 2006 and 2014 and returned quarterly or annually. Patients could be enrolled at any time after diagnosis of EGPA, independent of current disease activity or treatment. All patients fulfilled the 1990 American College of Rheumatology criteria for Churg-Strauss syndrome (19). Serum and data on specific clinical symptoms, summary scores of disease activity, and treatment status were collected at each visit. Summary scores included the physician global assessment (PGA) on a scale of 0–10; a categorical assessment of whether the patient was in remission or had active disease of low, moderate, or severe activity; the Birmingham Vasculitis Activity Score (BVAS), and BVAS modified for use in patients with Wegener's granulomatosis (BVAS/WG). Active asthma without other evidence of active EGPA was not considered to be active EGPA per the VCRC protocol. All patients were enrolled using protocols and informed consent forms approved by the institutional review boards (IRB) or ethics boards of all sites.

Volunteers without any medical problems ("healthy controls") were recruited at Boston University under a separate IRB-approved protocol.

*Study design*

298 serum samples from 168 patients with EGPA were selected for measurement of IgA anti-MPO antibodies. Two samples were used from most patients: one at study enrolment and one later. If enrolment occurred during remission, then the second sample was chosen during active EGPA, if available; for patients with multiple visits during active EGPA, the visit with the highest PGA was chosen. If enrolment occurred during active EGPA, then the second sample was chosen during remission. In 8 patients, no sample was available at enrolment, so a sample collected later was used. Remission visits were chosen so that the distribution of their timing following the baseline visit approximated that of the group in which the patients were enrolled during remission and had active disease later (quartiles of follow-up time were ≤7 months, 8–14 months, 15–35 months, and ≥36 months). Sera from 87 healthy controls were also assayed.

Pre-specified subgroups were defined by the presence of moderate-to-severe active disease (PGA ≥3), by moderate-to-severe active disease without current treatment, and by the involvement of different organ systems during the course of EGPA. PGA was the primary outcome measure to determine severity of disease, but the categorical assessment, BVAS, BVAS/WG, and grading of individual manifestations were also examined to ensure that all measures were consistent in the assessment of whether the patient had active disease or was in remission.

*IgA anti-MPO ELISA*

IgA anti-MPO and IgG anti-MPO antibodies were measured using capture ELISA assays based on the binding of recombinant human MPO (hMPO) to nickel coated ELISA plates via the poly-His tag of the recombinant hMPO antigen. Alkaline phosphatase-conjugated goat anti-human IgA (Sigma, A9669, 1:2,000 dilution) or goat anti-human IgG (Sigma, A9544, 1:10,000 dilution) secondary antibodies were used. All samples were tested in duplicate. Positive testing for IgA or IgG anti-MPO was defined as being >97.5% of the values among the 87 healthy controls.

Recombinant hMPO was expressed and purified as follows. The cDNA of hMPO was amplified by PCR from cDNA IMAGE clone BC130476 using the forward primer DJ3439 and reverse primer DJ3440. This PCR product was digested with *Sex*AI and *Nhe*I and subcloned into a pTT5 plasmid derivative at its *Sex*AI and *Avr*II sites (20). To enable secretion, the Igκ-chain secretion signal precedes the mature reading frame of human pro-MPO which starts with AAPAVL after cleavage of the natural 22 signal peptide residues. The C-terminus of hMPO has been extended with an avi-tag (GLNDIFEAQKIEWHEA) and six histidine residues. The cDNA construct was transfected into HEK293 EBNA cells (Yves Durocher, National Research Council Canada, Montreal, Canada) using polyethylenimine-DNA complexes. Cells were grown in suspension in serum-free Free-Style™ 293 expression medium (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1% Pluronic and G416 (25 μg/ml) at 37°C and 8% $CO_2$. After 3-4 days of expression, cell-culture supernatants were filtered, concentrated fivefold, and dialysed against binding buffer (20 mM $Na_2HPO_4$; 450 mM NaCl; 20 mM imidazole, pH 7.5) at 4°C. The dialysed solution was then loaded onto a HisTrap HP column (GE Healthcare). After washing with binding buffer, bound proteins were eluted with a linear imidazole gradient from 20 mM to 1 M imidazole in 20 mM $Na_2HPO_4$, 450 mM NaCl, pH 7.5. The fraction containing the monomeric proform of hMPO was dialysed against 20 mM $Na_2HPO_4$; 450 mM NaCl, pH 7.5, and kept frozen until use.

*Statistical analysis*

Proportions were compared using Fisher's exact tests.

## Results

*Patient characteristics and disease activity*

The demographic and clinical characteristics of the patients with EGPA are shown in Table I. The 87 controls included 36 men and 51 women, median age 42 (interquartile range 29–57, full range 21–77).

Among the 168 patients tested, 61 had 1 sample during active disease and 1

**Table I.** Characteristics of the EGPA cohort.

| | |
|---|---|
| Age at diagnosis, years: median (IQR, range) | 50 (40-60, 16-81) |
| Sex: number female (%, of 168) | 94 (56%) |
| Disease duration at enrolment, months: median (IQR, range) | 19 (4-50, 0-358) |
| Manifestations during course of EGPA: number (%, of 168) | |
| Constitutional | 139 (83%) |
| Musculoskeletal | 91 (54%) |
| Cutaneous | 97 (58%) |
| Otolaryngological | 149 (89%) |
| Pulmonary | 158 (94%) |
| Neurological | 120 (71%) |
| Cardiac | 41 (24%) |
| Gastrointestinal | 27 (16%) |
| Renal | 26 (15%) |

IQR: interquartile range.

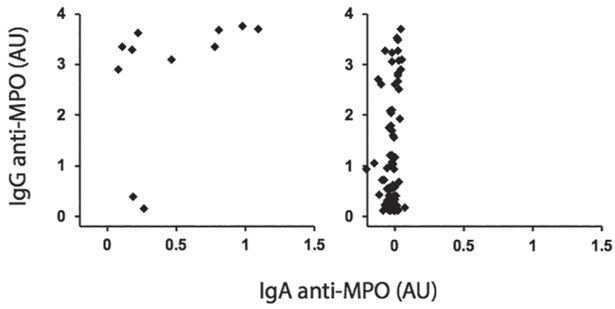


**Fig. 1.** Concentrations of IgA anti-MPO antibodies and IgG anti-MPO antibodies in individual samples. AU: absorbance units in ELISA assays. Left panel: all samples that tested positive for IgA; all of them also tested positive for IgG. Right panel: all samples that tested positive for IgG but negative for IgA. Together, these data show a poor correlation between IgA and IgG anti-MPO concentrations, supporting the conclusion that positive IgA anti-MPO testing is not merely a result of cross-reaction with high concentrations of IgG anti-MPO.

during remission; 68 had 2 samples during remission; 2 had 2 samples during active disease; and 37 had only one sample, 7 during active disease and 30 during remission. Enrolment occurred at a median of 19 months after diagnosis (interquartile range 4-50), and second samples were obtained a median of 13 months after the first samples (interquartile range 7-30). Patients were being treated with prednisone and/or other systemic immune-suppressive drugs at the time of collection of 272 of 297 (92%) samples for which data on treatment were available. PGA scores during active disease were 1–2 in 29 cases, 3–5 in 30 cases, 6–10 in 11 cases, and not recorded in 2 cases.

*IgA anti-MPO and IgG anti-MPO ANCA*

The ELISA for IgA anti-MPO was a technical success: the mean absorbance in controls was only 0.015 AU higher than buffer alone, and the cut-off for a positive test based on data from controls (0.082) was similar to that for IgG anti-MPO (0.100). Based on duplicate testing of all samples, the coefficient of variation was excellent at 5.4%.

IgA anti-MPO was detected in 10 of 168 (6%) patients with EGPA (11 of 298 samples) compared to 1 of 87 (1%, by definition) healthy controls ($p$=0.1). All 11 of the samples positive for IgA anti-MPO also were positive for IgG anti-MPO, and 90 additional samples were positive for IgG anti-MPO but negative for IgA. There was a poor correlation between titres for IgA or IgG anti-MPO in samples that tested positive for either or both (Fig. 1), indicating that testing positive for "IgA" was not merely an artifactual cross-reaction when IgG was present at high concentrations.

Samples taken during active EGPA were positive for IgA anti-MPO in 6 of 72 cases (8%), compared to 5 of 226 samples (2%) during remission ($p$=0.03). Five of 41 (12%) samples taken during moderate or high disease activity (PGA ≥3) were positive ($p$=0.01 compared to samples during remission). Among samples collected during moderate or high activity, only 2 patients were untreated; both samples were positive for both IgA and IgG anti-MPO.

Among 61 patients with paired samples during active EGPA and remission, IgA anti-MPO were at least 2-fold higher during active disease in 4 patients, at least 2-fold higher during remission in 1 patient, elevated at similar titre (less than 2-fold different) in 1 patient, and negative in both samples in 55 patients. These numbers were too small to draw conclusions about strength of association of IgA anti-MPO with disease activity compared to IgG anti-MPO, which were at least 2-fold higher during active disease in 15 patients, at least 2-fold higher during remission in 8 patients, elevated at similar titre in 9 patients, and negative in both samples in 29 patients.

No significant differences ($p$>0.1) in the rates of historical ENT, pulmonary, or skin involvement were detected between the IgA anti-MPO positive and negative patients, although the numbers of patients testing positive for IgA anti-MPO was too small to draw strong conclusions.

## Discussion

The aim of this study was to evaluate if IgA anti-MPO antibodies are detectable in patients with EGPA, particularly among patients without detectable IgG anti-MPO, and to determine whether presence of IgA anti-MPO correlates with current disease severity. These results suggest that, although IgA anti-MPO are detectable in some patients with EGPA and may be detectable more frequently during active disease, their presence seems unlikely to assist with diagnosis above what is obtained from IgG anti-MPO, or with disease activity above what is obtained from IgG anti-MPO, eosinophil counts, or inflammatory markers (21-23). Additionally, presence of IgA anti-MPO did not show a striking association with any particular disease manifestation, such as upper airway involvement.

The cohort studied was large for this rare disease. All the data were collected in a prospective, standardised fashion by investigators experienced in the evaluation of patients with EGPA. The IgA anti-MPO ELISA developed for this study was performed on both the patient and control groups in the same lab, which is highly experienced in assays for ANCA. The IgA anti-MPO as-

say had low background when tested in healthy controls and an excellent coefficient of variation (5%).

This study's main limitation is that most patients were being treated for EGPA at the time of serum collection, and only a small proportion of patients had high disease activity. When the sub-group of patients with higher scores of disease severity were analysed for IgA anti-MPO, a larger proportion tested positive, suggesting that this study might have underestimated the frequency of IgA anti-MPO in untreated patients with EGPA. However, the percentage of samples testing positive for IgG anti-MPO (101/298, 34%) was in the range seen in other studies in EGPA, and use of low-dose corticosteroids to control asthma could have underestimated the percentage of ANCA-positive patients in those studies also (2, 4, 5, 22). Above all, the fact that all IgA anti-MPO positive samples also tested positive for IgG anti-MPO (and not vice versa) suggests that IgA anti-MPO is not likely to have clinical utility above and beyond the standard IgG anti-MPO assay.

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