Myeloperoxidase-antineutrophil Cytoplasmic Antibodies (MPO-ANCA) and Proteinase 3-ANCA without Immunofluorescent ANCA Found by Routine Clinical Testing

Deepak A. Rao, Kevin Wei, Joseph F. Merola, William R. O'Brien, Samuel U. Takvorian, Paul F. Dellaripa, and Peter H. Schur

ABSTRACT. ***Objective.*** Concurrent testing for serum antineutrophil cytoplasmic antibodies (ANCA) by indirect immunofluorescence (IF) and by antiproteinase 3 (PR3)/antimyeloperoxidase (MPO) antibody assays may identify patients with PR3-ANCA or MPO-ANCA despite a negative IF (IF-negative MPO/PR3-positive); however, the significance of this result is not clear. We sought to determine whether IF-negative, MPO/PR3-positive results identified any cases of clinically meaningful systemic vasculitis at our institution.

Methods. We conducted a retrospective chart review of all IF-negative, MPO/PR3-positive patients identified at our institution over a 2-year period.

Results. Of the 2345 samples tested over 2 years, 1998 were IF-negative. Among these IF-negative samples, 49 samples (2.5%) derived from 38 patients tested positive for MPO-ANCA or PR3-ANCA. Only 1 IF-negative, MPO/PR3-positive patient was subsequently diagnosed with ANCA-associated vasculitis (AAV). Eleven IF-negative, MPO/PR3-positive patients (29%) had been previously diagnosed and treated for AAV, all with positive IF and antibody tests prior to treatment. Four patients had evidence of cutaneous vasculitis not attributed to AAV, while several of the remaining IF-negative, MPO/PR3-positive patients had other immunologic disorders, including systemic lupus erythematosus (5 patients) and inflammatory bowel disease (3 patients).

Conclusion. In this real-life cohort assayed simultaneously by IF and multiplexed bead assays, the detection of MPO-ANCA or PR3-ANCA without a positive IF rarely led to a new diagnosis of systemic vasculitis, and was more likely to occur in the context of a non-vasculitic inflammatory condition. Our results suggest that concurrent IF and MPO/PR3 testing may be of limited use in preventing a missed diagnosis of new-onset AAV. (First Release April 1 2015; J Rheumatol 2015;42:847–52; doi:10.3899/jrheum.140941)

Key Indexing Terms:
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Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) describes a set of related conditions including granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA), which are characterized by systemic vasculitis primarily targeting small blood vessels combined with the presence of ANCA in the serum. ANCA can be detected by immunofluorescence (IF) testing with a cytoplasmic pattern typically associated with GPA and a perinuclear pattern commonly associated with MPA. In AAV, the cytoplasmic ANCA pattern is usually a result of antibodies targeting the neutrophil protein proteinase 3 (PR3), while a perinuclear pattern results from antibodies binding to myeloperoxidase (MPO)[1,2]. Specific assays to detect PR3-ANCA and MPO-ANCA by ELISA or multiplexed bead assays are also routinely used diagnostically.

Comparisons of the use of IF and specific antibody assays for the diagnosis of AAV have frequently, though not uniformly, suggested that IF is more sensitive than MPO-ANCA and PR3-ANCA assays[3,4,5,6,7]. However, because of the low specificity of perinuclear ANCA (P-ANCA) and cytoplasmic ANCA (C-ANCA) IF patterns for AAV, MPO/PR3-ANCA assays may provide a better positive predictive value and likelihood ratio compared to IF, while the combination of the 2 demonstrates the best results[4,8].

From the Division of Rheumatology, Immunology, Allergy, Brigham and Women's Hospital; Harvard Medical School, Boston, Massachusetts, USA.

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D.A. Rao, MD, PhD, Fellow in Rheumatology; K. Wei, MD, PhD, Fellow in Rheumatology; W.R. O'Brien, Fellow in Rheumatology; S.U. Takvorian, MD, Resident in Internal Medicine; P.F. Dellaripa, MD, Assistant Professor of Medicine; P.H. Schur, MD, Professor of Medicine, Brigham and Women's Hospital; J.F. Merola, MD, Instructor, Harvard Medical School.

Address correspondence to Dr. P.H. Schur and Dr. P.F. Dellaripa, Brigham and Women's Hospital, 75 Francis St., Boston, Massachusetts 02115, USA. E-mail: pschur@partners.org, pdellaripa@partners.org

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In clinical practice, there is considerable variability in how IF and MPO/PR3-ANCA assays are used. The International Consensus Statement on the testing and reporting of ANCA recommended that all samples sent for diagnostic ANCA testing be evaluated by IF, and that samples with cytoplasmic fluorescence or nuclear fluorescence in a homogeneous or peripheral nuclear pattern be subsequently tested for MPO-ANCA and PR3-ANCA[9]. The consensus statement also states that optimally, all serum samples should be tested for MPO-ANCA and PR3-ANCA. One common clinical approach is to screen all serum samples by IF, and then test only IF-positive samples for MPO-ANCA and PR3-ANCA. Alternatively, some practitioners use specific antibody tests first, followed by reflex IF testing only on MPO/PR3-ANCA–positive samples[7,10], and in some specific cases, MPO/PR3-ANCA tests may be used alone[11].

Testing samples by IF and specific antibody tests simultaneously can identify IF-negative, MPO/PR3-positive patients who would otherwise be missed if only IF-positive samples were tested for MPO/PR3-ANCA. Prior reports have noted a small number of such IF-MPO/PR3–positive samples[4,12]. However, the clinical significance of this result is unclear because MPO/PR3-ANCA can also sometimes be detected in nonvasculitic conditions, including systemic lupus erythematosus (SLE) and inflammatory bowel disease (IBD)[13,14].

In our study, we sought to evaluate whether IF-negative, MPO/PR3-positive results identified any cases of clinically meaningful systemic vasculitis during 2 years of concurrent testing in a routine clinical setting.

MATERIALS AND METHODS

Our study was approved by the Brigham and Women's Hospital (BWH) Institutional Review Board. Results of all ANCA tests ordered through BWH between January 2011 and May 2013 were collected. These samples came from inpatients and outpatients at BWH and patients seen at associated outpatient centers. Throughout this period, all samples sent for evaluation for serum ANCA as part of clinical care, including for the evaluation of clinically suspected systemic vasculitis, monitoring of vasculitis disease activity, or any other indication, were included. All samples were evaluated by both IF and multiplex bead assays for MPO-ANCA and PR3-ANCA as part of routine clinical laboratory practice.

ANCA testing. IF was performed by the BWH Clinical Immunology laboratory. Serum samples were diluted 1:20 and incubated on ethanol-fixed human neutrophil substrate slides as per the manufacturer's method recommendations (INOVA). IF patterns were reported as cytoplasmic, perinuclear, atypical, or negative. Only cytoplasmic or perinuclear patterns were considered positive in our study. Atypical results were excluded from analyses. MPO-ANCA and PR3-ANCA were measured by multiplexed bead assays, a type of solid-phase immunoassay, performed at a commercial laboratory (ARUP Laboratories). An antibody level > 25 units/ml was considered positive. Previous analyses demonstrated very good concordance between results obtained on split samples over a broad range of levels, analyzed with this multiplexed bead assay with another commercial ELISA kit (INOVA) and with assays performed at the Massachusetts General Hospital (unpublished data).

Medical record review. The electronic Longitudinal Medical Record used by all sites from which samples were obtained was reviewed by 2 authors to determine the diagnoses for each IF-negative, MPO/PR3-positive patient. All IF-positive, MPO/PR3-positive patient charts were also reviewed, as well as charts of 50 IF-negative, MPO/PR3-negative patients matched by age and sex to the IF-negative, MPO/PR3-positive group. For IF-negative, MPO/PR3-positive patients, treatment with corticosteroids; disease-modifying antirheumatic drugs including methotrexate, sulfasalazine, hydroxychloroquine, azathioprine, cyclophosphamide, and rituximab; and exposure to cocaine were determined by manual electronic medical record review and by electronic queries using the Queriable Patient Inference Dossier (www.qpidhealth.com), a Web-based service that searches the electronic medical record for any queried term. Diagnosis of AAV was assigned according to the European Medicines Agency (EMA) vasculitis algorithm[15]. For disease designation with this algorithm, MPO/PR3 antibody levels > 25 units/ml were considered a positive MPO/PR3 result regardless of the IF result so that IF-negative, MPO/PR3-positive and IF-positive, MPO/PR3-positive patients could be compared using the same criteria.

Statistical analysis. Differences in anti-MPO and anti-PR3 antibody levels were evaluated using the Kruskal-Wallis 1-way analysis of variance with Dunn multiple comparisons test. A value of $p < 0.01$ was considered significant.

RESULTS

Concordance of IF results and MPO/PR3-ANCA measured by multiplex bead assays. There were 2345 samples sent for evaluation of serum ANCA at BWH between January 2011 and May 2013. Of these, 1998 had a negative IF (85%), 147 had an atypical IF, and 200 had a P-ANCA pattern, a C-ANCA pattern, or both. The IF and MPO/PR3 tests results for all samples are summarized in Table 1. Notably, 2.5% of IF-negative samples derived from 38 patients contained MPO-ANCA or PR3-ANCA (49/1998). By comparison, 62% of IF-positive samples contained MPO-ANCA and/or PR3-ANCA (123/200).

As demonstrated in Table 1, IF positivity was frequently, but not uniformly, detected in MPO/PR3-positive samples. When considered together, 65% of MPO/PR3-positive samples had a positive IF (123/188), while 26% of MPO/PR3-positive samples had a negative IF. MPO-ANCA were also found in some atypical IF samples, though PR3-ANCA were not.

Among samples with an MPO/PR3-ANCA, defined as > 25 units/ml, P-ANCA–positive samples had significantly higher MPO-ANCA antibody levels than did IF-negative or atypical samples (Figure 1). Similarly, C-ANCA–positive samples had significantly higher PR3-ANCA antibody levels than did IF-negative samples. Almost all of the samples with very high MPO-ANCA or PR3-ANCA levels had a positive IF, while antibody levels in the IF-negative samples clustered in the lower range.

Clinical conditions observed in the IF-negative, MPO/PR3-positive patient cohort. Systematic review of all 38 IF–negative, MPO/PR3-positive patients identified only 1 case in which a patient with an IF-negative, MPO/PR3–positive result was subsequently diagnosed with AAV. This patient had a history of several years of slowly progressive cough, plus intermittent arthritis and sinusitis, and rare epistaxis. Computed tomography imaging revealed pulmonary nodules and maxillary sinusitis. Initial ANCA testing reported a negative

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Table 1. Results of all ANCA tests performed at Brigham and Women's Hospital between January 2011 and May 2013. The number of samples with each result is shown. The number of independent patients with each result is shown in parentheses.

IF Pattern	Antibody Test Result				Total
	Negative	PR3	MPO	MPO + PR3	
Negative	1949 (1866)	29 (20)	20 (18)	0	1998 (1904)
Atypical	131 (119)	0	16 (11)	0	147 (130)
C-ANCA	37 (33)	41 (19)	3 (2)	1	82 (54)
P-ANCA	40 (37)	3 (2)	74 (44)	0	117 (83)
P-ANCA + C-ANCA	0	0	0	1	1
Total	2157 (2055)	73 (41)	113 (75)	2 (2)	2345

ANCA: antineutrophil cytoplasmic antibodies; IF: immunofluorescence; PR3: proteinase 3; MPO: myeloperoxidase; C-ANCA: cytoplasmic ANCA; P-ANCA: perinuclear ANCA.

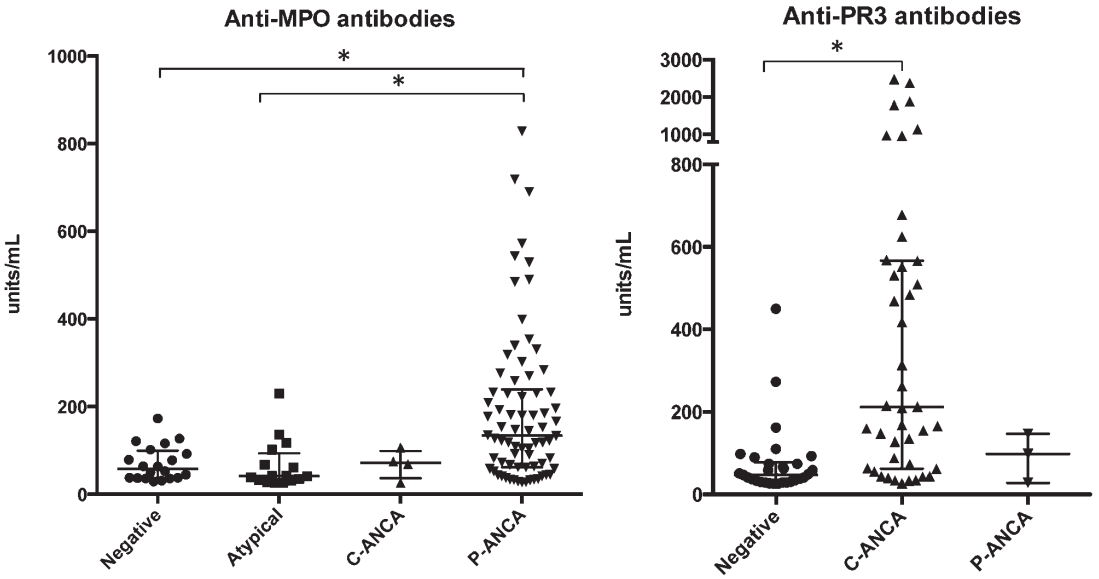

Figure 1. Anti-MPO and anti-PR3 antibody levels determined by multiplex bead assay for all IF-negative, atypical, C-ANCA, and P-ANCA samples with PR3-ANCA or MPO-ANCA > 25 IU/ml. * $p < 0.05$. MPO: myeloperoxidase; PR3: proteinase 3; IF: immunofluorescence; C-ANCA: cytoplasmic ANCA; P-ANCA: perinuclear ANCA; ANCA: antineutrophil cytoplasmic antibodies.

IF, but an MPO-ANCA at a high level (127 units). Because of a high clinical suspicion for AAV, ANCA testing was repeated 1 month later, this time revealing a P-ANCA and MPO-ANCA antibody of 117 units. Diagnosis of AAV was made according to the EMA vasculitis algorithm based on the presence of GPA surrogate markers (pulmonary nodules, chronic sinusitis), plus an MPO-ANCA[15].

Notably, 11 of the IF-negative, MPO/PR3-positive cases (29%) were patients who had been previously diagnosed with AAV and treated with standard therapies, all of whom had a positive IF and positive MPO-ANCA or PR3-ANCA prior to treatment (Table 2). Several patients had MPO-ANCA or PR3-ANCA levels well above the upper limit of normal despite a negative IF result. Although the number of patients was small, serial testing obtained for clinical care demonstrated different patterns of serologic conversion, including (1) IF converted from positive to negative while the MPO/PR3-ANCA remained positive (3 patients), (2) IF converted from positive to negative more rapidly than did the MPO/PR3-ANCA test (5 patients), and (3) after converting from IF-positive, MPO/PR3-positive to IF-negative, MPO/PR3-negative, MPO/PR3-ANCA positivity recurred before IF positivity (2 patients).

Twenty-six patients with an IF-negative, MPO/PR3-positive result did not have a history of confirmed AAV. Most of these patients had only 1 ANCA result in our electronic medical record. However, 10 patients had ANCA testing performed at BWH at least twice in the course of their clinical evaluation, of which 6 had the same IF-negative, MPO/PR3-positive result reproduced on repeat testing.

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Table 2. Patients with a diagnosis of AAV, previously IF-positive, MPO/PR3-positive, who then had an IF-negative, MPO/PR3-positive result after therapy.

Clinical Diagnosis	Age, yrs	Prior IF	Prior Antibody	Antibody Titer When IF-negative	Documented Therapies
MPA	60	P-ANCA	MPO+	MPO 102	CS
MPA	54	P-ANCA	MPO+	MPO 92	CS, CYC, AZA
CSS	79	C-ANCA	MPO+	MPO 37	CS, MTX
Drug-induced vasculitis	59	P-ANCA	MPO+	MPO 38	CS
GPA	72	C-ANCA	PR3+	PR3 59	CS, CYC, MTX, AZA
GPA	61	C-ANCA	PR3+	PR3 90	CS, RTX, AZA
GPA	52	C-ANCA	PR3+	PR3 93	CS, CYC, AZA
GPA	69	C-ANCA	PR3+	PR3 98	CS, RTX
GPA	53	C-ANCA	PR3+	PR3 110	CS, MTX
GPA	43	C-ANCA	PR3+	PR3 273	CS, RTX, MTX
GPA	57	C-ANCA	PR3+	PR3 33	CS, RTX, MTX

AAV: ANCA-associated vasculitis; IF: immunofluorescence; MPO: myeloperoxidase; PR3: proteinase 3; MPA: microscopic polyangiitis; CSS: Churg-Strauss syndrome; GPA: granulomatosis with polyangiitis; P-ANCA: perinuclear ANCA ; C-ANCA: cytoplasmic ANCA; CS: corticosteroids; CYC: cyclophosphamide; AZA: azathioprine; MTX: methotrexate; ANCA: antineutrophil cytoplasmic antibodies; RTX: rituximab.

Several nonvasculitic conditions, including SLE (n = 5), IBD (n = 3), and other conditions were identified in patients with IF-negative, MPO/PR3-positive results (Table 3). Four of the patients had evidence of a cutaneous vasculitis not considered consistent with AAV. We compared these clinical conditions to those identified in the 68 patients with IF-positive, MPO/PR3-positive results detected in the same time frame. As expected, IF-positive, MPO/PR3-positive patients frequently carried a diagnosis of AAV (47/68, 69%). When comparing IF-positive, MPO/PR3-positive patients with IF-negative, MPO/PR3-positive patients, a negative IF test significantly reduces the likelihood of a diagnosis of AAV, with a negative predictive value of 97% and a negative likelihood ratio of 0.03 (95% CI 0.004–0.23). Nonvasculitic

Table 3. Conditions documented for patients with at least 1 IF-negative, MPO/PR3-positive result, and without a diagnosis of AAV.

Age, yrs	Sex	MPO, n	PR3, n	Condition
40	M	0	63	Demyelinating polyneuropathy
51	M	2	59	Hashimoto thyroiditis, chronic sinusitis
45	F	0	47	Cocaine use
20	M	0	37	Henoch-Schönlein purpura
42	F	1	35	Cutaneous papulonodular eruption with lymphocytic vasculitis
86	F	0	33	Pyoderma gangrenosum, COPD
63	F	3	31	SLE, breast cancer
20	F	0	30	IBD
47	F	1	29	Graft versus host disease
61	M	0	29	IBD
73	M	0	27	COPD, breast cancer
79	F	0	26	IBD, hydralazine exposure
54	M	0	26	Hepatitis C, hepatocellular carcinoma
23	F	121	2	SLE, autoimmune polyneuropathy
19	M	116	1	Retiform purpura*
92	F	79	0	Bronchiectasis, breast cancer
22	F	64	6	SLE, cutaneous vasculitis
50	F	63	1	Goodpasture disease
33	F	53	1	AS, drug-induced SLE
28	F	50	2	SLE
65	F	45	0	RA, idiopathic progressive polyneuropathy
23	M	38	2	SLE
49	M	36	5	Desquamative interstitial pneumonia
79	F	36	0	COPD
72	F	31	0	Hypoaldosteronism, sinusitis
51	F	29	1	Relapsing polychondritis, leukocytoclastic vasculitis

* Lost to followup before investigations completed. IF: immunofluorescence; MPO: myeloperoxidase; PR3: proteinase 3; AAV: ANCA-associated vasculitis; COPD: chronic obstructive pulmonary disease; SLE: systemic lupus erythematosus; IBD: inflammatory bowel disease; AS: ankylosing spondylitis; RA: rheumatoid arthritis; ANCA: antineutrophil cytoplasmic antibodies.

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conditions were also observed in this group, including SLE[6], rheumatoid arthritis[1], immunoglobulin A nephropathy[1], as well as chronic lung disease and malignancies.

Because patients tested for ANCA frequently have an inflammatory condition that prompts ANCA evaluation, we also examined diagnoses of 50 age and sex-matched controls with IF-negative, MPO/PR3-negative results. This group included 9 patients with interstitial lung disease, 5 with malignancies, 3 with renal disease, 1 with IBD, and none with SLE.

Although the small number of IF-negative, MPO/PR3-positive patients precludes rigorous statistical analyses of associated nonvasculitic conditions, this dataset of ANCA tests ordered to evaluate patients for the possibility of AAV indicated that an isolated PR3-ANCA or MPO-ANCA positive result was more likely to be found in the context of a non-vasculitic immunologic disorder than to lead to a new diagnosis of AAV.

DISCUSSION

Testing for the presence of ANCA is a key component of the diagnostic evaluation for AAV. However, significant variability exists in the strategies used to detect ANCA, including reflex testing (test by IF first, and if positive, test for MPO-ANCA and PR3-ANCA) and concurrent testing (test by IF and MPO/PR3-ANCA assays simultaneously). It is well established that isolated IF positivity, without an associated MPO-ANCA or PR3-ANCA, has a poor predictive value for AAV[4]. However, the significance of positive MPO-ANCA or PR3-ANCA in the absence of IF positivity is less clear.

In our report of over 2 years of concurrent ANCA testing by IF and MPO/PR3 multiplexed bead assays in a real-life clinical setting, 2.5% all of samples were IF-negative and MPO/PR3-positive. The frequency of this result will vary with the specifics of test methods[3,6,16,17], but is within the range of prior reports[4,12,18]. IF-negative, MPO/PR3-positive results have been previously described in patients with conditions including inflammatory arthritis, IBD, and connective tissue disease[7,8,12,19]. However, there is little data reported on the significance of this result in routine clinical testing[4,12]. Stone, *et al* noted 5 IF-negative, MPO/PR3-positive patients out of 856 consecutive patients evaluated for AAV, but the clinical associations were not described[4]. Tsiveriotis, *et al* reported 17 IF-negative, MPO/PR3-positive patients out of 4786 outpatients concurrently tested, including 2 with connective tissue diseases, 4 with mixed/undefined disorders, and 11 with AAV; however, new diagnosis of AAV was not distinguished from previously treated AAV[12].

Our study is unique in that we evaluated the clinical characteristics of 38 patients with an IF-negative, MPO/PR3-positive result during routine clinical testing, constituting what we believe is the largest cohort of such patients identified in the context of real-world clinical practice. Only 1 patient with an IF-negative, MPO/PR3-positive result was subsequently diagnosed with AAV, suggesting that the likelihood of missing a case of AAV because of a false-negative or true-negative IF is low.

It has been suggested that MPO/PR3-ANCA testing alone may be reasonable in emergent clinical situations[11]. However, in our experience, 35% of PR3/MPO-positive samples did not have a positive IF, indicating that caution must be used when interpreting positive MPO/PR3-ANCA results in the absence of concurrent IF testing. Consistent with previous reports[5,12,13], we identified patients with SLE, IBD, and other inflammatory disorders with IF-negative, MPO/PR3-positive results. This phenomenon may be a result of a number of mechanisms, including antibody cross-reactivity in the multiplex bead assay, higher sensitivity of the multiplex bead assay, specific characteristics of the antigenic epitopes targeted, or technical variability[7,11].

IF-negative, MPO/PR3-positive results were also identified in some patients with AAV after treatment. The most common pattern among these patients was that after treatment, the MPO/PR3-ANCA level fell but remained positive until after the IF had converted from positive to negative. It appears that the MPO/PR3-ANCA multiplex bead assay demonstrated greater sensitivity than the IF ANCA test in these patients, although this has not been formally evaluated. These findings underscore the controversy and challenges associated with monitoring serial ANCA.

IF-negative, MPO/PR3-positive results were found in several patients without a prior diagnosis of AAV, but with non-vasculitic inflammatory conditions including SLE and IBD, consistent with prior reports that these conditions can yield MPO/PR3-ANCA[9,19]. Because various inflammatory signs or symptoms may prompt ANCA testing, we examined the frequency of these and other inflammatory conditions in a group of age/sex-matched patients found to be IF-negative, MPO/PR3-negative. This group also contained several patients with inflammatory disorders, although only 1 with IBD and none with SLE. A larger cohort of patients will be required to determine whether an IF-negative, MPO/PR3-positive result is statistically associated with specific immunologic disorders.

Our study has several limitations. Our study is retrospective in design, and determination of clinical diagnosis was limited to data available in the electronic medical record. It is possible that additional clinical events, including the development of systemic vasculitis, may have occurred that were not identified in the available electronic medical record. The total number of patients with an IF-negative, MPO/PR3-positive result is small, limiting the interpretation of clinical outcomes in this population. We do not have samples available to replicate test results and cannot formally evaluate the contribution of technical variability. Finally, as noted, the likelihood of an IF-negative, MPO/PR3-positive

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result may vary with the specifics of the tests used, and caution is required when attempting to apply these observations to results obtained through different methods.

In this unique experience of concurrent ANCA testing in routine clinical practice, the detection of either MPO-ANCA or PR3-ANCA without IF positivity rarely led to a new diagnosis of AAV and was more likely to be associated with a non-vasculitic inflammatory condition. Although the number of IF-negative, MPO/PR3-positive patients identified was small, these results suggest that a strategy of ANCA evaluation by routine concurrent IF and antibody testing offers minimal advantage over a strategy of reflex antibody testing of IF-positive samples, and in an era of limited resources, reevaluation of such a strategy would be reasonable.

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