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The significance of MPO-ANCA and PR3-ANCA without immunofluorescent ANCA found by routine clinical testing

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Abstract

Objective—Concurrent testing for serum antineutrophil cytoplasmic antibodies (ANCA) by indirect immunofluorescence (IF) and by anti-proteinase-3 (PR3)/anti-myeloperoxidase (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 2345 samples tested over 2 years, 1998 were IF-negative. Amongst these IF-negative samples, 49 samples (2.1%), 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 SLE (5) and inflammatory bowel disease (3).

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 utility in preventing a missed diagnosis of new onset AAV.

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Keywords

Antineutrophil cytoplasmic antibodies; vasculitis; outcomes

INTRODUCTION

AAV describes a set of related conditions including granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) that are characterized by systemic vasculitis primarily targeting small blood vessels, combined with the presence of ANCA in the serum. ANCA can be detected by 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 due to antibodies targeting the neutrophil protein proteinase-3 (PR3, PR3-ANCA), while a perinuclear pattern results from antibodies binding to myeloperoxidase (MPO, MPO-ANCA)(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 utility 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 (3–7). However, because of the low specificity of P-ANCA and 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).

In clinical practice, there is considerable variability in how IF and MPO/PR3-ANCA assays are employed. The International Consensus Statement on the testing and reporting of ANCAs recommended that all samples sent for diagnostic ANCA testing be evaluated by IF, and that samples with cytoplasmic fluorescence, or nuclear fluorescence in a homogenous 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 utilize 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 be otherwise missed if only IF-positive samples were tested for MPO/PR3-ANCA. Prior reports have noted a small number of such IF-MPO/PR3-positivesamples (4, 12); however, the clinical significance of this result is unclear, as MPO/PR3-ANCA can also sometimes be detected in non-vasculitic conditions, including SLE and IBD (13, 14).

In this 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

This 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 come from inpatients and outpatients at BWH and patients seen at associated outpatient centers. Through 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, San Diego, CA). IF patterns were reported as cytoplasmic, perinuclear, atypical, or negative. Only cytoplasmic or perinuclear patterns were considered positive in this 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, Salt Lake City, UT). 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, San Diego, CA) and with assays performed at Massachusetts General Hospital (Boston, MA) (unpublished data).

Medical record review

The electronic Longitudinal Medical Record (LMR), used by all sites from which samples were obtained, was reviewed by 2 authors to determine 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 anti-rheumatic drugs (DMARDs) 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 Queriable Patient Inference Dossier (QPIDTM, qpidthealth.com, Boston, MA), a web-based service that searches the electronic medical record for any queried term. Diagnosis of AAV was assigned according to the European Medical Agencies 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 one-way analysis of variance with Dunn's 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

2345 samples were sent for evaluation of serum ANCA at Brigham and Women's Hospital between January 2011 and May 2013. Of these, 1998 (85%) had a negative IF, 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% (49/1998) of IF-negative samples, derived from 38 patients, contained MPO-ANCA or PR3-ANCA. By comparison, 62% (123/200) of IF-positive samples contained MPO-ANCA and/or PR3-ANCA.

As demonstrated in Table 1, IF positivity was frequently, but not uniformly, detected in MPO/PR3-positive samples. When considered together, 65% (123/188) of MPO/PR3-positive samples had a positive IF, 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.

Amongst 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 IF but an MPO-ANCA at a high level (127 units). Due to 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 European Medicines Agency 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 is 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), 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 one 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.

Several non-vasculitic conditions, including SLE (5), IBD (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 identified in the same timeframe. 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). Non-vasculitic conditions were also observed in this group, including SLE (6), rheumatoid arthritis (1), IgA 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 non-vasculitic conditions, this data set of ANCA tests ordered to evaluate patients for the possibility of AAV indicates that an isolated PR3-ANCA or MPO-ANCA positive result is 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 employed 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 this 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, connective tissue disease (7, 8, 12, 19); however, there is little data reported on 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 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 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 due to 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 due to 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 one 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. The 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 captured 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 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 conclusion, 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 is 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, re-evaluation of such a strategy would be reasonable.

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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 greater than 25 IU/mL. * $p < 0.05$.

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.

Table 1

IF Pattern	Negative	PR3	MPO	MPO+PR3	Total
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

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.
Abbreviations: corticosteroids (CS), cyclophosphamide (CYC), azathioprine (AZA), methotrexate (MTX).

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

Table 3

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

Age	Sex	MPO	PR3	Condition
40	M	0	63	Demyelinating polyneuropathy
51	M	2	59	Hashimoto's thyroiditis, chronic sinusitis
45	F	0	47	Cocaine use
20	M	0	37	Henoch Schonlein 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 vs Host Disease
61	M	0	29	IBD
73	M	0	27	Chronic obstructive pulmonary disease, breast cancer
79	F	0	26	Chronic lymphocytic colitis, 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	Ankylosing spondylitis, drug-induced SLE
28	F	50	2	SLE
65	F	45	0	Rheumatoid arthritis, idiopathic progressive polyneuropathy
23	M	38	2	SLE
49	M	36	5	Desquamative interstitial pneumonia
79	F	36	0	Chronic obstructive pulmonary disease
72	F	31	0	Hypoadosteronism, sinusitis
51	F	29	1	Relapsing polychondritis, leukocytoclastic vasculitis

* lost to follow up before workup completed