Diagnostic Value of Distinguishing and Reporting Different Perinuclear ANCA (P-ANCA) Immunofluorescence Patterns

A Prospective Study

Susan B. Perel, MBBS, FRACP, FRCPA,1,2 Kerri M. Prain,1 Robert J. Wilson, MSc,1 Patrick G. Hogan, MBBS, PhD, FRACP, FRCPath,1,2 David Gillis, MBBS, FRACP, FRCPA,1,2 and Richard C. W. Wong, MBChB, FRACP, FRCP, FRCPA1,2

From the 1Division of Immunology, HSSA Pathology Queensland Central Laboratory, Royal Brisbane and Women’s Hospital, Herston, Queensland, Australia, and 2Department of Clinical Immunology, Princess Alexandra Hospital, Woolloongabba, Queensland, Australia.

Key Words: Antineutrophil cytoplasmic antibodies; P-ANCA; Fluorescent antibody technique; Vasculitis; Enzyme-linked immunosorbent assay; Antineutrophil cytoplasmic antibody–associated vasculitis; Granulomatosis with polyangiitis; Microscopic polyangiitis

ABSTRACT

Objectives: To investigate whether discriminating the classic perinuclear antineutrophil cytoplasmic antibody (P-ANCA) pattern from atypical P-ANCA and uninterpretable patterns improves the diagnostic utility of ANCA testing.

Methods: All ANCA requests (n = 3,544) referred to Pathology Queensland were analyzed prospectively over 4 months for P-ANCA pattern subtypes and myeloperoxidase (MPO)-ANCA/PR3-ANCA results and correlated with clinical, laboratory, and radiologic evidence of necrotizing small vessel vasculitis.

Results: Of the 436 perinuclear immunofluorescence-positive samples, 45 were classic P-ANCA, 163 were atypical P-ANCA, and 228 were antinuclear antibodies/uninterpretable. The classic P-ANCA pattern had a significantly stronger association with vasculitis (30/45) than atypical P-ANCA (2/163) (P < .0001) or ANA/uninterpretable patterns (8/228) (P < .0001). The combination of a classic P-ANCA pattern and positive MPO-ANCA/PR3-ANCA result was also more strongly associated with vasculitis than a positive MPO-ANCA/PR3-ANCA result in isolation (P = .003).

Conclusions: This study demonstrates that reporting different P-ANCA patterns (including ANA/uninterpretable patterns) provides additional diagnostic information to MPO-ANCA/PR3-ANCA results.

The antineutrophil cytoplasmic antibody (ANCA)–associated vasculitides are characterized by the presence of autoantibodies directed against constituents of neutrophil cytoplasmic granules. These conditions, which include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA), are characterized by the presence of systemic necrotizing small vessel vasculitis and are frequently associated with serious complications, including acute renal failure and potentially life-threatening diffuse alveolar hemorrhage.

The predominant autoantigens in these ANCA-associated vasculitides are myeloperoxidase (MPO) and proteinase 3 (PR3). Approximately 85% to 90% of patients with GPA have detectable ANCA, which are predominantly directed against PR3 (75%-80%) and occasionally MPO (10%-15%).2,3 Approximately 75% of patients with MPA have detectable...
ANCAs, which are more frequently directed against MPO (50%) than PR3 (25%).\textsuperscript{2,3} Antineutrophil cytoplasmic antibodies are also detectable in approximately 40% of patients with EGPA, usually directed against MPO.\textsuperscript{4}

The most commonly used screening test for ANCA is the indirect immunofluorescence (IIF) assay using ethanol-fixed neutrophils. This is followed by separate specific immunoassays that measure antibodies against PR3 and MPO for IIF-positive specimens.

A number of characteristic ANCA IIF staining patterns have been described to be strongly associated with systemic necrotizing vasculitis.\textsuperscript{2,5} The classic perinuclear ANCA (P-ANCA) staining pattern is characterized by perinuclear staining with nuclear extension (as defined by graduated shading from the perinuclear rim to the center of the nucleus with a lack of clear demarcation of the perinuclear staining and the nuclear rim) \textsuperscript{[Image 1A]} and is usually associated with antibodies directed against MPO.\textsuperscript{2,5} The classic cytoplasmic ANCA (C-ANCA) pattern is characterized by granular, cytoplasmic staining with central or interlobular accentuation\textsuperscript{6} \textsuperscript{[Image 1B]} and is usually associated with antibodies directed against PR3.\textsuperscript{2,5,6} The P-ANCA staining

pattern due to MPO-ANCA is actually an artifact of ethanol fixation and, if IIF is performed using formalin-fixed neutrophils, MPO-ANCA produces a C-ANCA pattern.7

A number of other ANCA IIF staining patterns have been described that are usually associated with inflammatory conditions other than systemic necrotizing vasculitis (eg, inflammatory bowel disease, chronic suppurative lung disease, some chronic infections, and rheumatoid arthritis).7,8,11 These patterns include the so-called atypical P-ANCA pattern, which can be defined as perinuclear immunofluorescence not meeting the criteria for classic P-ANCA as described above. In general, these atypical P-ANCA IIF patterns feature perinuclear staining without nuclear extension, usually in conjunction with sharply demarcated rim staining8,10. Image 1C. Sera that produce atypical P-ANCA staining patterns have been shown to contain antibodies that do not recognize MPO but are instead directed against a variety of other neutrophil antigens that include catalase, cathepsin G, elastase, lactoferrin, bactericidal/permeability-increasing protein, and lysozyme.2,3,5,9,12,13

It is also recognized that antinuclear antibodies (ANAs) can produce IIF staining patterns on ethanol-fixed neutrophils that may be indistinguishable from P-ANCA, hence emphasizing the need for concurrent testing for ANA.13-15 Image 1D. Unlike P-ANCA due to antibodies directed against MPO, P-ANCA that are directed against non-MPO antigens usually do not produce a cytoplasmic staining pattern on formalin-fixed neutrophils but become negative or retain their perinuclear staining pattern. For this reason, some authors have advocated performing IIF on both ethanol-fixed and formalin-fixed neutrophils to aid in the differentiation of these patterns.7,16 However, other authors have commented that retesting on formalin-fixed slides does not add any value to the standard approach of screening on ethanol-fixed slides and then proceeding to MPO/PR3-ANCA enzyme-linked immunosorbent assay (ELISA) testing for IIF-positive specimens. These authors have cited limitations in the use of formalin-fixed slides, including difficulties in pattern interpretation, poor reproducibility, increased labor, additional cost, and the potential to delay diagnosis.17 Another technique to help distinguish P-ANCA patterns in patients with necrotizing small vessel vasculitis from patients with inflammatory bowel disease, primary sclerosing cholangitis, and type 1 autoimmune hepatitis, involves DNase treatment of neutrophils used on methanol-fixed slides.18 However, this technique and the use of methanol (as opposed to ethanol) fixation would be impractical to implement in most diagnostic laboratories.

To our knowledge, no published prospective studies have examined whether reporting different P-ANCA IIF pattern subtypes provides additional diagnostic information compared with the results of the specific MPO/PR3-ANCA results. The 1999 International Consensus Statement on Testing and Reporting of Anti-neutrophil Cytoplasmic Antibodies does not require laboratories to distinguish between different types of P-ANCA patterns in their reporting of results.19,20 Instead, this document states that laboratories may report any type of perinuclear or granulocyte-specific nuclear fluorescence as “P-ANCA,” with the recipient of the ANCA report then being directed to refer to MPO/PR3-ANCA results.

Since the publication of the ANCA consensus statement, there has been increased awareness of the importance of different patterns and their clinical associations. A number of studies have clarified these patterns.5,8,10 Newer technologies, including automated slide processors, have improved the efficiency and standardization of ANCA IIF testing, and computer-aided image analysis has the potential to aid in the discrimination and reporting of immunofluorescence patterns.21,22

We therefore conducted a prospective study to investigate the clinical associations of the different P-ANCA IIF pattern subtypes—namely, the classic P-ANCA, atypical P-ANCA, and ANA/uninterpretable IIF patterns, alone and in combination with the MPO-ANCA/PR3-ANCA results. The primary aim of the study was to determine the association between different subtypes of the P-ANCA IIF pattern and underlying systemic necrotizing vasculitis, with the assumption that the classic P-ANCA staining pattern would have the strongest association. We also postulated that the combination of the P-ANCA subtype result and the MPO-ANCA/PR3-ANCA results would have a stronger correlation with systemic necrotizing vasculitis than the MPO-ANCA/PR3-ANCA results in isolation.

Materials and Methods

Patient Selection and Sample Collection

The Immunology Laboratory of the HSSA Pathology Queensland Central Laboratory receives specimens from all public hospitals (adult and pediatric, including public outpatient departments) in the state of Queensland, Australia (population approximately 4.6 million), for ANCA testing. For this study, all routine requests for ANCA testing for a 4-month period (from January 8 to April 29, 2010) were evaluated. All specimens were screened on ethanol-fixed neutrophil slides (INOVA Diagnostics, San Diego, CA) at 1:40 dilution and ×400 magnification (Olympus BX41 microscope; Olympus, Tokyo, Japan), with fluorescein isothiocyanate F(ab’)2 goat anti–human immunoglobulin G (Fcγ fragment) as the antibody conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA). All specimens that produced a positive perinuclear immunofluorescence pattern, as defined by any staining of the perinuclear or nuclear region of the neutrophils, were included in the study.
All specimens were read independently by 2 authors (S.B.P. and K.M.P.) and 1 other reader at 3 serial dilutions (1:40, 1:160, and 1:640). The end-point titer was defined as the highest dilution at which the ANCA IIF staining pattern (see definitions below) was visible. All specimens were also tested on HEP-2000 slides (Immuno Concepts N.A., Sacramento, CA) at a single dilution (1:40) to determine whether a concurrent ANA was present. Readers were blinded to the clinical notes and any prior ANCA results at the time of reading. A subtype of the P-ANCA IIF pattern (see “Immunofluorescence Patterns”) was determined for each specimen by consensus between the 3 readers.

After a P-ANCA IIF pattern subtype had been assigned to each specimen, one author (S.B.P.) was unblinded to the clinical notes and any prior ANCA results. If duplicate specimens were received for the same patient over the study period, only the results from the chronologically earliest specimen were included in the subsequent analysis.

Immunofluorescence Patterns: Definitions of Different P-ANCA Subtypes

The following definitions for the different P-ANCA patterns had been developed and refined over time, through a process of review of the published literature and repeated discussions between scientific staff and immunopathologists in our laboratory.

Classic P-ANCA
Perinuclear staining accompanied by nuclear extension (ie, graduated shading from the perinuclear rim to the center of the nucleus) in more than 50% neutrophils in at least 2 different fields (at ×400) at the end-point titer.
Absence of a concomitant ANA (detected on an HEP-2000 slide).

Atypical P-ANCA
Any perinuclear staining pattern that was not classic P-ANCA or ANA, including perinuclear rim staining, with consistently sharp demarcation of the perinuclear rim staining from the adjacent nucleus, and staining that lacks nuclear extension.
Absence of a concomitant ANA (detected on a HEP-2000 slide).

Uninterpretable ANCA
The presence of an ANA detected on an HEP-2000 slide that was considered to potentially interfere with interpretation of a P-ANCA pattern on the ethanol-fixed neutrophil slide.

If an ANA was not visible on the HEP-2000 slide but there was significant staining of all visible eosinophil nuclei on the ethanol-fixed neutrophil slide (namely, granulocyte-specific ANA).

To ensure consistency in the interpretation of patterns, prior to the initiation of the study, the 3 readers received initial training and subsequent competency assessment in discriminating the various ANCA patterns (according to the above criteria).

Enzyme-Linked Immunosorbent Assay
All specimens with detectable P-ANCA staining (regardless of the particular staining subtype) underwent further testing for MPO-ANCA and PR3-ANCA (ORGENTEC Diagnostika, Mainz, Germany). In accordance with the manufacturer’s recommendations, MPO-ANCA/PR3-ANCA results of 6 IU/mL or more were reported as positive.

Clinical Information
The clinical information from all P-ANCA–positive specimens was followed for up to 6 months to help establish a clinical diagnosis. This clinical information was obtained from histology reports and chart notes and by direct correspondence with treating clinicians. A diagnosis of ANCA-associated vasculitis was determined by kidney or lung/upper airway biopsy specimen results that demonstrated histologic evidence of necrotizing small vessel vasculitis, in accordance with the Chapel Hill Consensus Criteria.23 If biopsy specimen results were unavailable or if biopsy was not performed, a diagnosis of probable ANCA-associated vasculitis was made based on a combination of available clinical history and other laboratory/radiology results. These included evidence of pulmonary involvement (infiltrates, cavitations, or diffuse alveolar hemorrhage) and/or renal involvement (active urinary sediment or renal failure) and a clinical history indicating previous or current treatment for presumed vasculitis with immunosuppressive drugs (eg, cyclophosphamide).

Statistical Analysis
Statistical analysis was performed using GraphPad QuickCalcs 2013 (GraphPad Software, San Diego, CA). P values were calculated using the Fisher exact test, with a statistically significant difference defined as a P value of less than .05. The modified Wald method was used for the calculation of 95% confidence intervals (CIs).

Results

Patient Characteristics
Of the 3,544 requests for ANCA testing received by the HSSA Pathology Queensland Central Laboratory between January 8, 2010, and April 29, 2010, a total of 487 (13.7%) specimens demonstrated perinuclear or nuclear immunofluorescence on screening and were therefore included for analysis. Fifty-one specimens were determined to be duplicate
specimens and were therefore excluded, leaving 436 specimens from different patients for subsequent analysis. None of the 51 duplicate specimens had differing patterns to the primary specimens.

Approximately 96% of specimens were referred from public hospitals in Queensland, with the remaining 4% referred from private medical practitioners. Most of the 436 specimens received were from the specialties of internal medicine (29.0%), nephrology (18.0%), gastroenterology (14.2%), and rheumatology/immunology (8.3%), with the remaining 30.5% of specimens coming from other specialties (data not shown). The range of indications for ANCA testing on the request forms is shown in Table 1.

Forty (9.2%) of 436 patients had clinical or laboratory evidence of necrotizing vasculitis, of whom 4 were newly diagnosed within the study period. Histology results were available for most patients and included renal (n = 29), upper airway (n = 2), lung (n = 2), and skin (n = 1) biopsy specimens that demonstrated the presence of necrotizing small vessel vasculitis. The diagnosis of necrotizing vasculitis in the remaining 6 patients was based on clinical notes and/or correspondence with the treating clinician. The male to female ratio of these patients was 1.2:1. The mean age of men with vasculitis was 65.7 years (range, 49-85 years), which was significantly older (P = .01) than that of the female patients with vasculitis (mean age, 55.2 years; range, 26-78 years).

Indirect Immunofluorescence Results

The classic P-ANCA pattern was observed in 45 (10.3%) of 436 specimens, the atypical P-ANCA pattern in 163 (37.4%) of 436 specimens, and the positive ANA/uninterpretable pattern in 228 (52.3%) of 436 specimens. Most specimens reported as having a positive ANA/uninterpretable pattern were due to homogeneous (60.1%), homogeneous/nucleolar (19.7%), or speckled (10.2%) ANA patterns. Approximately 8.0% of uninterpretable specimens were due to other ANA patterns, and 2.0% were due to the presence of a granulocyte-specific ANA. In 203 (89.0%) of 228 specimens, the presence of significant staining of all visible eosinophil nuclei on ethanol-fixed neutrophil slides was associated with the presence of a positive ANA on HEp-2000 slides.

Correlation Between Different Indirect Immunofluorescent P-ANCA Patterns and Necrotizing Vasculitis

The classic P-ANCA pattern demonstrated a highly statistically significant association with underlying necrotizing vasculitis compared with the atypical P-ANCA (P < .0001) and ANA/uninterpretable (P < .0001) patterns. Specifically, approximately 30 (66.7%) of 45 classic P-ANCA–positive patients had clinical/laboratory evidence of necrotizing vasculitis (95% CI, 52.0%-78.9%). In comparison, only approximately 2 (1.2%) of 163 patients with an atypical P-ANCA pattern had clinical/laboratory evidence of necrotizing vasculitis (95% CI, 0.1%-4.7%), with the other 161 patients having a wide range of nonvascular inflammatory conditions.

MPO-ANCA/PR3-ANCA ELISA Results and Correlation With Necrotizing Vasculitis

Positive results were obtained on MPO-ANCA/PR3-ANCA testing by ELISA in 62 (14.2%) of 436 specimens.

Table 1

<table>
<thead>
<tr>
<th>Indication for Ordering ANCA Test</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal renal function</td>
<td>55 (12.6)</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>45 (10.3)</td>
</tr>
<tr>
<td>Previous positive ANCA result‡</td>
<td>43 (9.9)</td>
</tr>
<tr>
<td>Central nervous system syndromes§</td>
<td>39 (8.9)</td>
</tr>
<tr>
<td>Lung pathology</td>
<td>32 (7.3)</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>25 (5.7)</td>
</tr>
<tr>
<td>Connective tissue disease screen</td>
<td>24 (5.5)</td>
</tr>
<tr>
<td>Thrombophilia screen</td>
<td>15 (3.4)</td>
</tr>
<tr>
<td>Skin rash or cutaneous vasculitis</td>
<td>15 (3.4)</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>11 (2.5)</td>
</tr>
<tr>
<td>Not stated</td>
<td>84 (19.3)</td>
</tr>
<tr>
<td>Other§</td>
<td>48 (11.0)</td>
</tr>
<tr>
<td>Total</td>
<td>436 (100)</td>
</tr>
</tbody>
</table>

ANCA, antineutrophil cytoplasmic antibody.

‡ Includes patients with known necrotizing vasculitis.

§ Includes stroke, delirium, seizures, and cerebral vasculitis.

* Includes fever, weight loss, lethargy, arthralgias, myalgias, and cytopenias.

Table 2

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Classic P-ANCA, No. (%)</th>
<th>Atypical P-ANCA, No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotizing vasculitis</td>
<td>30* (66.7)</td>
<td>2* (1.2)</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>2 (4.0)</td>
<td>37 (22.7)</td>
</tr>
<tr>
<td>Connective tissue disease</td>
<td>2* (4.0)</td>
<td>8* (4.9)</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>0</td>
<td>2 (1.2)</td>
</tr>
<tr>
<td>Infection/sepsis</td>
<td>1 (2.0)</td>
<td>17 (10.4)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>0</td>
<td>9 (5.5)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (6.0)</td>
<td>27 (16.6)</td>
</tr>
<tr>
<td>Diagnosis unknown</td>
<td>7 (16.0)</td>
<td>61 (37.4)</td>
</tr>
<tr>
<td>Totals</td>
<td>45 (100)</td>
<td>163 (100)</td>
</tr>
</tbody>
</table>

ANCA, antineutrophil cytoplasmic antibody; P-ANCA, perinuclear antineutrophil cytoplasmic antibody.

* Classic P-ANCA vs atypical P-ANCA (Fisher exact test, P < .0001).

‡ Rheumatoid arthritis (n = 1) and seronegative arthritis (n = 1).

§ Rheumatoid arthritis (n = 6), seronegative arthritis (n = 1), and systemic lupus erythematosus (n = 1).
Of these, 39 (62.9%) were MPO-ANCA positive, 20 (32.3%) were PR3-ANCA positive, and 3 (4.8%) were MPO-ANCA/PR3-ANCA positive.

Thirty (48.3%) of 62 patients with a positive ELISA result (either PR3-ANCA or MPO-ANCA) had a diagnosis of necrotizing vasculitis. The other 31 (51.6%) patients with positive PR3-ANCA and/or MPO-ANCA results did not have evidence of necrotizing vasculitis but instead had a variety of conditions such as inflammatory bowel disease (n = 10), connective tissue disorders (n = 4), infection (n = 3), drug-induced vasculitis (n = 3), autoimmune hepatitis (n = 1), and other conditions (n = 11).

**Clinical Associations of Combined IIF and ELISA Results**

A classic P-ANCA pattern in combination with a positive MPO-ANCA/PR3-ANCA result was more likely to be associated with necrotizing vasculitis than a positive MPO-ANCA/PR3-ANCA result, irrespective of the corresponding subtype of P-ANCA pattern (82.8% vs 48.4%; \( P = .003 \))

Of the 16 patients with an atypical P-ANCA IIF pattern and a positive MPO-ANCA/PR3-ANCA result, only 1 (6.3%) had clinical/laboratory evidence of necrotizing vasculitis. Five (29.4%) of 17 patients with an uninterpretable ANCA pattern...

**Table 3**

**Clinical Diagnoses for Patients With Positive MPO-ANCA/PR3-ANCA ELISA Results**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>MPO-ANCA Positive</th>
<th>PR3-ANCA Positive</th>
<th>MPO-ANCA and PR3-ANCA Positive</th>
<th>Total ELISA Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotizing vasculitis</td>
<td>29</td>
<td>1</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Connective tissue disease</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Drug-induced ANCA</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Not known</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>20</td>
<td>3</td>
<td>62</td>
</tr>
</tbody>
</table>

ANCA, antineutrophil cytoplasmic antibody; ELISA, enzyme-linked immunosorbent assay; MPO, myeloperoxidase; PR3, proteinase 3.

a Systemic lupus erythematosus (n = 1) and rheumatoid arthritis (n = 1).

b Rheumatoid arthritis (n = 1) and seronegative arthritis (n = 1).

c Cerebral tuberculosis (n = 1) and chronic hepatitis B with *Strongyloides* infection (n = 1).

d Subacute bacterial endocarditis with *Staphylococcus* bacteremia (n = 1).

f Propothiouracil (n = 1).

† Hydralazine (n = 2).

**Table 4**

**P-ANCA Immunofluorescence Pattern Subtypes, MPO/PR3-ANCA Results, and Corresponding Proportions of Patients With Necrotizing Vasculitis**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No./Total No. (%)</th>
<th>95% Confidence Interval, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIF pattern</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic P-ANCA²⁺ᵇᶜ</td>
<td>30/45 (66.7)</td>
<td>52.0-78.9</td>
</tr>
<tr>
<td>Atypical P-ANCA²⁺</td>
<td>2/163 (1.2)</td>
<td>0.0-4.7</td>
</tr>
<tr>
<td>ANA/uninterpretable</td>
<td>8/228 (3.5)</td>
<td>1.7-7.0</td>
</tr>
<tr>
<td>All P-ANCA pattern subtypes²⁺ᵈ</td>
<td>40/436 (9.2)</td>
<td>7.0-12.3</td>
</tr>
<tr>
<td>IIF pattern and MPO/PR3-ANCA results</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic P-ANCA with positive MPO/PR3</td>
<td>24/29 (82.8)</td>
<td>65.0-92.9</td>
</tr>
<tr>
<td>Atypical P-ANCA with positive MPO/PR3</td>
<td>1/16 (6.3)</td>
<td>0.0-30.3</td>
</tr>
<tr>
<td>ANA/uninterpretable with positive MPO/PR3</td>
<td>5/17 (29.4)</td>
<td>13.0-53.4</td>
</tr>
<tr>
<td>All P-ANCA patterns with positive MPO/PR3³⁺</td>
<td>30/62 (48.4)</td>
<td>36.4-60.6</td>
</tr>
</tbody>
</table>

ANA, antinuclear antibody; ANCA, antineutrophil cytoplasmic antibody; IIF, indirect immunofluorescence; P-ANCA, perinuclear ANCA; MPO, myeloperoxidase; PR3, proteinase 3.

² Classic P-ANCA vs atypical P-ANCA (\( P < .0001 \)).

³ Classic P-ANCA vs ANA/uninterpretable (\( P < .0001 \)).

⁴ All P-ANCA patterns = classic P-ANCA, atypical P-ANCA, and ANA/uninterpretable patterns.

© American Society for Clinical Pathology

*Am J Clin Pathol* 2013;140:184-192

DOI: 10.1309/AJCP4Y8ADMKOSCXV
and a positive MPO-ANCA/PR3-ANCA result had clinical/laboratory evidence of necrotizing vasculitis (Table 4).

Discussion

To date, most published studies on ANCA IIF pattern subtypes have grouped all atypical patterns together as “atypical ANCA” or “x-ANCA.” However, the 1999 International Consensus Statement recommended that atypical P-ANCA patterns should be included within the “P-ANCA” category rather than separately as “atypical P-ANCA,” “atypical ANCA,” or “x-ANCA.” In contrast, this statement recommends that laboratories should distinguish and report the different C-ANCA patterns, since this differentiation has been shown to be clinically useful, albeit in a study with a nonprospective design. Since we believed that categorizing atypical P-ANCA patterns as “P-ANCA” significantly reduced the diagnostic value of a P-ANCA result, we conducted this prospective study to examine whether reporting different subtypes of P-ANCA IIF patterns provides additional diagnostic information compared with combining all P-ANCA patterns as simply “P-ANCA.” This study clearly demonstrates that the differentiation of classic from atypical perinuclear ANCA immunofluorescence patterns significantly improves the clinical utility of the IIF test, regardless of MPO-ANCA/PR3-ANCA results, in terms of the association with underlying necrotizing small vessel vasculitis.

Over the 4-month period in our large public diagnostic laboratory, the classic P-ANCA pattern was associated much more closely with necrotizing small vessel vasculitis than the atypical P-ANCA pattern (66.7% vs 1.2%; $P < .0001$) or ANA/uninterpretable patterns (66.7% vs 3.5%; $P < .0001$). Furthermore if all 3 subtypes of “perinuclear” ANCA patterns (ie, classic P-ANCA, atypical P-ANCA, and ANA/uninterpretable) were combined, the correlation with vasculitis would be significantly weaker, with only 9.2% of patients having clinical/laboratory evidence of necrotizing small vessel vasculitis ($P < .0001$). These findings strongly suggest that the absence of a requirement in the 1999 International Consensus Statement to distinguish and report the different P-ANCA patterns is suboptimal and significantly reduces the diagnostic value of a positive P-ANCA IIF result. This would bring the recommendation related to distinguishing the different P-ANCA patterns in line with the existing recommendation to distinguish between the different C-ANCA patterns.

As an alternative to requiring laboratories to differentiate the different P-ANCA patterns, the 1999 International Consensus Statement currently recommends that users refer to MPO-ANCA/PR3-ANCA results for further clarification. Some authors have even suggested that MPO/PR3-ANCA should be performed in place of IIF as the initial screening test in patients suspected of having necrotizing small vessel vasculitis. However, it is recognized that MPO-ANCA and PR3-ANCA ELISA assays have performance issues, including varying sensitivities and specificities between kits, and problems with reproducibility. The sensitivity issues are greatest for patients with low titer antibodies, such as those with treated or inactive vasculitis. Indirect immunofluorescence is still recognized as the more sensitive screening test for vasculitis, and a number of studies have demonstrated that the combined approach of using IIF with MPO-ANCA/PR3-ANCA has superior specificity compared with using either test method alone. Our study supports this approach further demonstrates that the combination of the P-ANCA IIF pattern subtype and MPO-ANCA/PR3-ANCA results is clearly superior to interpreting the MPO/PR3-ANCA results in isolation. Therefore, there is additional diagnostic value in differentiating and reporting the different subtypes of the P-ANCA pattern in conjunction with MPO-ANCA/PR3-ANCA results. To aid clinicians, our laboratory provides the following interpretative comment with all atypical P-ANCA results, which also refers to correlation with the results of the MPO-ANCA and PR3-ANCA assays: “Atypical ANCA is not characteristically associated with small vessel vasculitis but may be found in a wide variety of non-vasculitic inflammatory and infectious diseases. Please refer to PR3-ANCA and MPO-ANCA results.”

Recently, newer ELISA techniques, including capture and anchor ELISA, have been developed with reportedly improved sensitivity and specificity for the diagnosis of necrotizing small vessel vasculitis. This improved performance is thought to be due to better preservation of antigen conformational epitopes, which may be destroyed or blocked in a direct binding ELISA during attachment of antigens to the solid-phase ELISA plate. Capture (second-generation) ELISA technology employs a plate precoated with antibody to capture the antigen, while anchor (third-generation) ELISA uses a peptide linker to immobilize the antigen on the plate. Capture ELISA has been reported to be highly sensitive and specific for the detection of PR3-ANCA, with authors citing the overall diagnostic performance of this assay as being superior to IIF and direct binding ELISA. In some studies, anchor ELISA assays have demonstrated superior sensitivity for PR3-ANCA compared with direct binding and even capture ELISAs. Although these newer generation ELISA assays show promise, at this stage there are insufficient data, especially for MPO-ANCA, to recommend replacing the standard testing algorithm of screening on IIF followed by antigen-specific ELISA with a single-step capture/anchor ELISA. Recently, an ANCA Biochip mosaic (EUROPLUS, EUROIMMUN, Lübeck, Germany) has also been developed that incorporates immunofluorescence on ethanol-fixed and
formalin-fixed neutrophils, HEp-2 cells, and monkey liver with microdots for MPO and PR3 in the same incubation step. This is a qualitative assay that aims to help discriminate between MPO-ANCA/PR3-ANCA and non–MPO-ANCA/PR3-ANCA–associated ANCA IIF patterns. It is anticipated that this assay will be of most value as a quick screening test for patients with rapidly progressively renal and/or pulmonary disease. However, its higher cost compared with standard ethanol-fixed neutrophil slides may limit its use by laboratories as a routine screening assay for ANCA.

Our study had a number of limitations. First, some referral bias is present in our study population, with a relatively high proportion of requests (14% of total) coming from gastroenterologists for investigation of inflammatory bowel disease. Furthermore, during the 4-month period, a significant number of requests were follow-up tests for patients with known vasculitis, which inevitably increased the pretest probability for vasculitis in our study group. Despite this limitation, this study prospectively examined a cohort of consecutive specimens submitted for ANCA testing from all public hospitals in Queensland, Australia, and therefore reflects the spectrum of requests likely to be received by other large hospital-based diagnostic immunology laboratories.

Second, ANCA-associated vasculitis is known to be a rare disease. Consequently, only 4 newly diagnosed cases of necrotizing vasculitis (out of a total of 40 cases of confirmed vasculitis) were identified by our laboratory during the study period. We therefore cannot validly calculate the predictive value of different perinuclear ANCA patterns for a new diagnosis of necrotizing vasculitis, since most of the patients with vasculitis had been diagnosed for varying lengths of time prior to the study period.

Third, this study has the limitation that it was not feasible to obtain additional detailed clinical information on the more than 3,000 specimens that were ANCA negative over the study period. The paucity or absence of clinical notes on the majority of request forms also made it impossible to obtain a presumptive clinical diagnosis on these patients.

In conclusion, our study clearly demonstrates that the reporting of different P-ANCA patterns (including ANA/uninterpretable patterns) provides additional diagnostic information compared with the recommendation from the 1999 International Consensus Statement that does not require laboratories to distinguish between different P-ANCA patterns. The 1999 International Consensus Statement should therefore be updated to include an optimal recommendation to distinguish and report these different P-ANCA patterns and use this information in conjunction with the results of MPO-ANCA/PR3-ANCA testing. However, for this to occur in a consistent manner, universal definitions of classic and atypical P-ANCA patterns are required and would need to be applied by different laboratories in a standardized fashion.

An in-house program to regularly assess the competency of personnel who report ANCA patterns is also required, as has been implemented in our laboratory. The definitions of the different P-ANCA pattern subtypes developed in our laboratory could be evaluated for inclusion in a future revision of the International Consensus Statement for ANCA testing.

Address reprint requests to Dr Wong: Division of Immunology, HSSA Pathology Queensland Central Laboratory, Royal Brisbane and Women’s Hospital, Herston, Queensland, Australia, 4029; e-mail: Richard_Wong@health.qld.gov.au.

This study was presented in poster form at the 16th International Vasculitis & ANCA Workshop, Paris, France, April 14-17, 2013.

References


