Antineutrophil Cytoplasmic Antibodies: Historical perspectives and new advances

First described in 1982, antineutrophil cytoplasmatic antibodies (ANCA) represent a diagnostically important group of autoantibodies. Indirect immunofluorescence (IIF) on ethanol fixed human neutrophils was not only the first method described for the detection of ANCA, it is still proclaimed as the method of choice for the first line screening of sera from patients suspected to suffer from ANCA associated vasculitis (AAV). The labor-intensive methodology inherent to IIF prompted new technological advancements such as automated digital image analysis. Such technology allows for standardization, reduction of hands-on time, and facilitation of case review.

While low sensitivity and specificity of early enzyme immunoassays (ELISAs) caused much controversy, recent advances, such as the development of second and third generation capture ELISAs offered significant improvements in diagnostic accuracy. Ongoing innovations in the field of antigen specific solid phase immunoassays have continuously improved the analytical and clinical performance characteristics of these assays, supporting both diagnosis and follow-up. The recently developed chemiluminescent immunoassays (CIA) offer results in 30 minutes while allowing for high sensitivity and specificity. These advances have helped to facilitate new opportunities in the formerly unresolved associations of ANCA, such as the specific detection of PR3-ANCA in ulcerative colitis.

This issue of the INOVA Newsletter covers historical perspectives from thirty years ago to the current advances available today.

I hope you will enjoy reading!
An introduction to ANCA-associated vasculitis

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Discovery of ANCA

Antibodies to neutrophil cytoplasmic components (ANCA) were first described in 1982 by Davies et al. in some patients with necrotizing glomerulonephritis (FNGN) and symptoms of systemic vasculitis.1 In 1985 van der Woude et al. reported the strong association of ANCA producing a diffuse granular cytoplasmic staining pattern (cANCA) on ethanol-fixed neutrophils and granulomatosis with polyangiitis (formerly Wegener’s granulomatosis (WG)); a few years later, ANCA, producing a perinuclear fluoroscopic pattern (pANCA) on the same cellular substrate were described in patients with idiopathic necrotizing crescentic glomerulonephritis (iNCGN) and microscopic polyangiitis (MPA).1 (Figure 1a. and Figure 1b.)

The method available at that time to detect the cANCA and pANCA pattern was the indirect immunofluorescence test (IIF) on normal human ethanol-fixed leukocytes.2 Over 30 years since its first use, IIF remains the desired method to screen samples in patients suspected of vasculitis.

Function of target antigens-PR3 and MPO

The target antigens of ANCA are located in the primary granules of neutrophils and have antibacterial properties. PR3 and MPO are recognized in most ANCA-positive small vessel vasculitides. PR3 is a weak cationic protein consisting of 228 amino acid residues (MW 29-30 kD), belonging to the trypsin family of serine proteases. PR3 is synthesized as a preproenzyme and subsequently processed in four steps into the mature form. It is stored in the azurophilic granules of neutrophils, but can also be found within the membrane of secretory vesicles, and also expressed at the plasma membrane. PR3 is physiologically inhibited by α1-antitrypsin.4 PR3 disintegrates tissue to allow the passage of neutrophils into an inflammatory focus and is also involved in neutrophil maturation. MPO is a 150 kD heterodimer peroxidase enzyme abundantly expressed in neutrophils. The enzyme is characterized by a powerful bactericidal activity, whose peroxidase activity is physiologically inhibited by ceruloplasmin.5

The identification and purification of PR3 and MPO antigens resulted in development of several immunoassays for the quantitative detection of antibodies specific for PR3 and MPO; the conventional enzyme-linked immunosorbent assays (ELISAs) and, more recently, methods based on different fully automated technologies.3

Figure 1a.) cANCA is largely due to the presence of autoantibodies targeting the serine protease proteinase-3 (PR3-ANCA). Figure 1b.) pANCA is caused by antibodies directed against many antigens, among which myeloperoxidase (MPO-ANCA) is the most frequent in primary systemic vasculitis.3
Pathogenesis of ANCA disease

Numerous in vitro studies demonstrate that both MPO-ANCA and PR3-ANCA are capable of activating neutrophils and monocytes through Fab’2 and Fc engagement, which initiates several signal transduction pathways (Figure 2). Activation of these leukocytes results in adhesion to endothelial cells, causing endothelial damage. Studies in animal models provide conclusive evidence that anti-MPO antibodies induce necrotizing and crescentic glomerulonephritis and systemic small vessel vasculitis. Anti-MPO antibodies in the absence of functional T cells are capable of causing glomerulonephritis and vasculitis, and the induction of this disease is dependent on neutrophils. This can be exacerbated by a variety of cytokines, is dependent on activation of the alternative pathway of complement, and is abrogated by inhibition of the alternative pathway and by anti-C5 receptor antibodies. These studies provide a basis for exploring novel therapeutic strategies.

ANCA classification

ANCA are the serological hallmarks of idiopathic systemic vasculitis, and the term ANCA-associated vasculitis (AAV) has been used to collectively name those primary small vessel vasculitic syndromes in which circulating ANCA are commonly found (microscopic polyangiitis and its renal limited form, granulomatosis with polyangiitis, Churg-Strauss syndrome). This approach, adopted by the Chapel Hill International Consensus Conference (CHCC) and by the European Vasculitis Study Group (EUVAS), is supported by the striking clinical and histological similarities between the AAV, the widespread use of ANCA as a diagnostic marker, and the growing evidence of their pathogenetic potential.

Names of the common forms of vasculitis have been recently revised so that the eponyms such as WG and CSS have been changed with granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA), respectively. The new disease nomenclature system adopted by the 2012 CHCC for defining small vessel vasculitis is listed in Figure 3.

After the standardization of the methods for ANCA detection and the evaluation of their clinical application was agreed upon, a document produced by an international consensus of experts was published, with suggestions for the correct ANCA testing and reporting.

Figure 2. Pathogenesis of ANCA-associated vasculitis

Figure 3. The Chapel Hill Consensus nomenclature defines 10 primary vasculitides based on vessel size.
An introduction to ANCA-associated vasculitis...Continued

**ANCA testing**

The International Consensus Statement on Testing and Reporting of ANCA was created to enhance the diagnostic usefulness of ANCA testing in patients suspected of having vasculitis. Testing for ANCA in the presence of certain clinical indications (Table 1) yields a high positive predictive value. Guidelines stipulate testing samples with IIF on ethanol fixed leukocytes (or purified neutrophils) and PR3/MPO by solid phase assay such as ELISA (Table 2). Testing all IIF positive samples by ELISA produces sensitivities of 73% and 67% for GPA and MPA, respectively, and a diagnostic specificity of 99%.

*Table 1. Indications for ANCA testing*

<table>
<thead>
<tr>
<th>Clinical indication for ANCA testing</th>
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<tbody>
<tr>
<td>Glomerulonephritis, especially rapidly progressive glomerulonephritis</td>
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<tr>
<td>Pulmonary hemorrhage</td>
<td></td>
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<tr>
<td>Cutaneous vasculitis</td>
<td></td>
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<tr>
<td>Multiple lung nodules</td>
<td></td>
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<td>Chronic destructive disease of the upper airways</td>
<td></td>
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<tr>
<td>Long standing sinusitis or otitis</td>
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<tr>
<td>Subglottic tracheal stenosis</td>
<td></td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td></td>
</tr>
<tr>
<td>Retro-orbital mass</td>
<td></td>
</tr>
</tbody>
</table>

*Other possible indications for ANCA testing*

| Pulmonary fibrosis, with systemic features |  |
| Episcleritis, uveitis, retinal vasculitis, with systemic features |  |

*Table 2. Summary of the international consensus statement on testing of ANCA*

<table>
<thead>
<tr>
<th>Minimum requirements</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Perform IIF on all sera; 10% of ANCA-positive sera are detected only by IIF</td>
<td></td>
</tr>
<tr>
<td>Samples with IIF positivity should immediately be tested for both PR3 and MPO ELISA</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Optimal requirements</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>IIF titration should be performed for sera positive only by IIF</td>
<td></td>
</tr>
<tr>
<td>The inclusion of the most recent positive serum in the IIF or ELISA studies may be useful in demonstrating a change in antibody level</td>
<td></td>
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</tbody>
</table>

**In conclusion:**

- ANCA are the serological hallmarks of idiopathic systemic vasculitis, and the term AAV has been used to collectively name those primarily small vessel vasculitic syndromes in which circulating ANCA are commonly found.

- Names of the common forms of vasculitis have been recently revised so that the eponyms such as Wegener’s granulomatosis and Churg Strauss syndrome have been changed with granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA), respectively.

- The International Consensus Statement on Testing and Reporting of ANCA recommends screening by ANCA by IIF and to confirm any positivity by both PR3 and MPO ELISA.

**References**

Diagnostic methods for ANCA: past and present

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Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are one of the most common causes of small vessel vasculitis and are diagnostic hallmarks of microscopic polyangiitis (MPA), Wegener’s Granulomatosis, more recently referred to as granulomatosis with polyangiitis (GPA), and Churg Strauss syndrome, now called eosinophilic granulomatosis with polyangiitis (EGPA).

ANCA were first reported in 1982 by Davies et al. and investigated to a greater extent in 1985 by van der Woude et al. Myeloperoxidase (MPO) and proteinase 3 (PR3) were soon discovered as major antigenic targets for ANCA in neutrophils. After these antigens were described, continual developments were made to improve associated diagnostic tests. The importance of serological testing cannot be underestimated in patients suffering from vasculitis. These individuals must be swiftly diagnosed and proper therapy must be administered to avoid renal failure.

The initial screening method for these autoantibodies is, according to the International Consensus Statement on Testing and Reporting of ANCA, IIF on fixed neutrophils of human origin. The result of an initial IIF screening is verified by ELISA. The requirement of sample batching for ELISA has now driven innovation to random access test systems with reduction in assay times. Systems combining random access and chemiluminiscent immunoassay (CIA) technology have been developed and offer drastically reduced assay times of just 30 minutes.

First generation ELISA Tests

After the target proteins for pANCA and cANCA were described, the first ELISA was developed using purified native PR3 and MPO antigens of various origin. These assays used simple direct coating methods and the purity of the antigens often was not very high. Most of all the tests lacked comparability of results and did not show strong correlation to IIF methods. This lack of sensitivity was the result of different epitopes on the PR3 antigen being blocked by the direct binding process. The lack of standardization led to the International Consensus Statement on Testing and Reporting of ANCA by a combination of ELISA and IIF test methods.

Second and third generation tests

Second generation ANCA tests utilize a capture ligand, generally consisting of a monoclonal antibody directed towards a specific epitope. This method allows the protein to retain its three dimensional structure while providing significantly higher sensitivity compared to first generation tests. Protein modification such as biotinylation led to third generation ELISAs, also aimed to prevent protein distortion. Spacer molecules are attached to PR3 antigen and the complex binds to the surface of the ELISA plate by streptavidin coupling. This method further increased the sensitivity and specificity and showed improved correlation with IIF.

Other ELISA methods

An alternative approach to achieve sensitivity comparable to IIF is a direct coated ELISA using native purified PR3 in mixture with a recombinant human PR3 antigen. This method purportedly offers significantly higher sensitivity compared to first generation direct coated ELISA tests and second generation capture ELISA. A promising performance analysis of this method was published by Damoiseaux et al. in 2009. However, a recent study presented at the 16th International Vasculitis & ANCA Workshop in Paris did not show a significantly higher diagnostic performance of this approach.
Lateral flow and multiplex assays

Historically, lateral flow assays (LFA) were regarded as fast, but lacking sensitivity compared to ELISA tests.20-22 The development of liquid antigens for LFA has significantly improved their diagnostic performance. The sensitivity of LFA is comparable to that of third generation ELISA tests with a result time of 20 minutes.12,13 Since LFA is based on a universal IgG with the antigens added in solution, this test system can accommodate multiple analytes. Presenting the antigen in the liquid phase prevents potential epitope masking, as seen in some solid phase assays.

Multiplex technology allows for the detection of multiple antibodies in a single assay run. The antigen antibody reaction is visualized using fluorescent dyes and is read by two lasers, one identifying the bead and the second exciting the fluorophore coupled to the conjugate. This technology allows detection of up to 100 different analytes in only one reaction. For the diagnosis of ANCA, tests are available which simultaneously measure antibodies against PR3 and MPO. The sensitivity for PR3 is comparable to first generation solid phase ELISA, when a cut off setting optimized for 100% specificity is used.23

BIO-FLASH®- Rapid Response Chemiluminescent Analyzer

The QUANTA Flash® PR3 and MPO assays (INOVA Diagnostics San Diego, CA) are novel CIAs performed on the BIO-FLASH® instrument using native purified antigens from human neutrophils (Figure 1). The CIAs are designed for the fully automated BIO-FLASH instrument, containing a luminometer as signal detector, as well as all the hardware and liquid handling accessories necessary to perform the assay. Native PR3 purified from human neutrophils is covalently bound to paramagnetic particles. Native MPO purified from human neutrophils is bound to paramagnetic particles via an anchor molecule to improve immunoreactivity.

Patient serum is prediluted by the BIO-FLASH instrument with sample buffer in a small disposable plastic cuvette. Small amounts of the diluted patient serum, the beads, and the assay buffer are all combined into a second cuvette, mixed, and then incubated for 9.5 minutes at 37 °C. The magnetized beads are sedimented and washed repeatedly. The isoluminol conjugate is oxidized when sodium hydroxide and peroxide solutions (“Triggers”) are added to the cuvette, and the flash of light produced from this reaction is measured as relative light units (RLUs) by the BIO-FLASH optical system.

The RLU:s are proportional to the amount of isoluminol conjugate that is bound to the human IgG, which is in turn proportional to the amount of autoantibodies bound to the antigen on the beads. Based on the results of running two calibrators, an instrument specific Working Curve is created, which is used to calculate chemiluminescent units (CUs) for each sample.

Figure 1. a.) Principle of the novel chemiluminescent immunoassays.14 Paramagnetic beads are coupled with native PR3 or MPO. The beads are then incubated with diluted patient samples. After 9.5 min incubation unbound antibodies are removed by washing. Anti-human IgG isoluminol conjugate (Tracer) is added and binds immobilized antibodies. After another 9.5 min incubation unbound Tracer is removed by washing. b.) Trigger 1 and Trigger 2 are injected and emerging light is measured. After injection of Trigger 1 and Trigger 2, the luminescence is measured as relative luminescence units (RLU).
Table 1. Agreement between QUANTA Lite ELISA and QUANTA Flash CIA

<table>
<thead>
<tr>
<th>Kappa = 0.92</th>
<th>QUANTA Lite PR3</th>
<th>Percent agreement (95% confidence)</th>
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<tr>
<td></td>
<td>Positive</td>
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<td>QUANTA Flash® PR3 CIA</td>
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*all samples were from GPA patients

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<th>QUANTA Lite MPO</th>
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*3 samples from MPA and 3 from GPA patients

Table 2. Agreement between QUANTA Flash and IIF

<table>
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<td>Negative</td>
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<tr>
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<tr>
<td></td>
<td>Negative</td>
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*5 samples from GPA and 1 from MPA patient

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<td>QUANTA Flash® MPO CIA</td>
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<tr>
<td></td>
<td>Negative</td>
<td>2#</td>
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<tr>
<td></td>
<td>Total</td>
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*3 samples from MPA and 2 from GPA patients #1 sample from MPA and 1 from GPA patients

Comparison of QUANTA Lite ELISA and QUANTA Flash CIA

Seventy one samples (41 GPA and 30 MPA, N=71) were used to compare the PR3 and MPO ANCA QUANTA Flash CIA with QUANTA Lite® PR3 and MPO ELISA. The positive agreement of the PR3 CIA compared to ELISA was 100%, the negative agreement 92.5%, and the overall agreement 95.8% (Table 1). The positive percent agreement of the MPO CIA compared to the ELISA was 100%, the negative agreement 85.7%, and the overall agreement 91.5% (Table 1).

Comparison of QUANTA Flash CIA with IIF

The results of the QUANTA Flash PR3 and MPO CIA were compared to IIF results. MPO-ANCA was analyzed vs. the presence of a pANCA pattern and PR3-ANCA vs. the presence of cANCA. In the entire cohort, 28 samples showed a cANCA pattern in IIF, and 34 were positive by CIA. For MPO-ANCA, 32 samples showed a pANCA pattern, and 35 were positive by CIA. The agreement with MPO-ANCA was 90.1% for CIA (Table 2). For PR3-ANCA the agreement was 91.5% for CIA (Table 2).
Diagnostic methods for ANCA: past and present...Continued

IN CONCLUSION

In conclusion:

- Many algorithms have been proposed as the best possible way to detect ANCA. The IIF screening on fixed neutrophils followed by verification of positive results remains the recommended method according to the International Consensus Statement on ANCA, Testing and Reporting.

- ELISA methods often are burdened with batching and relatively long result times. Accurate, rapid diagnosis followed by appropriate therapy is paramount to halting the deleterious effects of ANCA vasculitic disease. Many clinicians now desire assays which allow quick turnaround time along with diagnostic accuracy.

- QUANTA Flash PR3 and MPO CIAs are sensitive and specific assays for rapid detection (30 min) of PR3 and MPO ANCA, which fulfills the compelling need for a rapid detection system. Moreover, these CIAs allow quantitative detection of antibodies with a broad assay range.


Reference List


ANCA testing in emergency setting

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Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are a heterogeneous group of autoantibodies whose antigenic targets include proteinase-3 (PR3) and myeloperoxidase (MPO).\(^1\) Detection of these autoantibodies is useful for the diagnosis and follow-up of small vessel vasculitis such as granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA).\(^2\) These autoantibodies and their respective prevalence are reported in Table 1.\(^3\)

PR3 and MPO autoantibodies can be detected using various techniques and different multi step strategies.\(^4\) The first step is typically a screening test using an indirect immunofluorescence (IIF) assay on human neutrophils which have been fixed with ethanol. Autoantibodies against antigens other than PR3 and MPO can cause positive results with ANCA testing by IIF and the sensitivity of IIF is not perfect. Therefore confirmation and identification of PR3 and MPO ANCA antibodies is very important and can be obtained using different solid phase technologies such as immunodot, enzyme linked immunosorbent assay (ELISA), multiplex assays, or more recently by chemiluminescent immunoassay (CIA). Just as these autoantibodies are useful tools for the diagnosis of small vessel vasculitis, they are also useful markers for monitoring disease activity and therapy efficacy.\(^5,6\) Providing numerical laboratory results across a broad detection range is especially helpful for the management of these diseases. Furthermore, when patients present with critical acute symptoms having quantitative results can further aid in making a differential diagnosis between other conditions including infection.

Limitations of IIF and ELISA for certain patient subsets

Even though IIF assays are sensitive and widely accepted as the first step method to be used in routine practice for ANCA screening, they are time consuming for technicians and inappropriate in emergency settings. Moreover, some discrepancies between IIF and specific tests for identification of the target of these antibodies have been reported, mainly in patients undergoing immunosuppressive treatment. Like any IIF assay, wide intra- and inter-laboratory variability is also observed in ANCA testing. This is due to variability from a number of factors including; sources of neutrophils, neutrophil preparation and fixation, the conjugate reagent, the reading system used and finally the subjectivity and the expertise of the technician. Different PR3 and MPO preparations and various methodologies are used to detect these antibodies that specifically recognize targets with conserved conformational epitopes and overcome the challenges of IIF for patient monitoring.\(^7,8\)

Table 1. Prevalence of PR3 and MPO-ANCA in different groups of small vessel vasculitis.

<table>
<thead>
<tr>
<th>Condition</th>
<th>PR3 (%)</th>
<th>MPO (%)</th>
</tr>
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<tbody>
<tr>
<td>Granulomatosis with polyangiitis</td>
<td>66</td>
<td>24</td>
</tr>
<tr>
<td>Microscopic polyangiitis</td>
<td>26</td>
<td>58</td>
</tr>
<tr>
<td>Eosinophilic granulomatosis with polyangiitis</td>
<td>10</td>
<td>60</td>
</tr>
</tbody>
</table>
ANCA testing in emergency setting

ANCA associated vasculitides are chronic, multi-systemic disorders often affecting several organs, characterized by flares and remissions. However, despite this chronic nature, ANCA testing can be requested in emergency cases during acute stage flare-ups. Such testing is critical in order to quickly support a differential diagnosis and initiate appropriate immunosuppressive therapies in order to avoid irreversible organ damage. Additionally, the clinical manifestations of vasculitis can be severe including alveolar hemorrhage, glomerulonephritis, scleritis, and necrotizing sinusitis. Therefore, it is of the utmost importance to report ANCA results in a timely manner to aid in prompt diagnosis and treatment.

Since relapse of ANCA associated vasculitis occurs in 30-60% of cases, ANCA testing is also required for follow-up. According to disease stage and activity, serial testing is required at intervals of several months (three to six months). Furthermore, ANCA testing is usually ordered to assess the side effects of immunosuppressive therapy, such as malignancies, or for differential diagnosis with undercurrent infectious diseases.

Rapid and accurate results using the BIO-FLASH® chemiluminescent analyzer

We have recently analyzed a chemiluminescence immunoassay developed on an automated analyzer for the detection of PR3 and MPO-ANCA. A retrospective study from 74 sera corresponding to 45 patients with ANCA associated vasculitis (32 GPA, 13 MPA) and 62 sera from healthy and control diseases (systemic lupus erythematosus, Sjögren's syndrome, healthy donors) was performed. Sera were tested using the QUANTA Flash® PR3 and MPO assays on the BIO-FLASH® chemiluminescent analyzer (INOVA Diagnostics). Results were compared to those obtained using routine multiplex assays Fidis™ Vasculitis Panel (BMD) performed using the Luminex analyser in our laboratory (Figure 1).

The sensitivity and the specificity of the QUANTA Flash assay was 78.4% and 83.4%, respectively. The sensitivity and the specificity of routine technique was 73% and 80.1%, respectively. A good agreement was observed for MPO (92.9% kappa =0.82) and for PR3 (96.4% kappa=0.92). Four samples were PR3-ANCA positive by QUANTA Flash and negative by multiplex. Eight samples were MPO-ANCA positive by QUANTA Flash and negative by multiplex. Eight samples were MPO-ANCA positive by multiplex and negative.

Figure 1. Comparison of PR3 and MPO-ANCA tested on BIO-FLASH and Fidis Vasculitis Panel. Units are expressed as Calculated Units (CU) and Luminex Units (LU/mL)
by QUANTA Flash (Figure 2). Those samples were from four patients with treated ANCA associated vasculitis (two GPA, one mononeuritis simplex and one alveolar haemorrhage) and four patients with inflammatory/autoimmune diseases (Grave’s disease, psoriatic rheumatism, giant cell arteritis and Sjögren’s syndrome). Four samples were PR3-ANCA positive by CIA and negative by multiplex, coming from patients with treated ANCA-associated vasculitis (two GPA, one MPA and one EGPA).

<table>
<thead>
<tr>
<th>Multiplex</th>
<th>PR3</th>
<th>Neg</th>
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<td>BIO-FLASH</td>
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</tr>
<tr>
<td>Pos</td>
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<tr>
<td>Total</td>
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<table>
<thead>
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<th>MPO</th>
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<td>Neg</td>
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<td>8</td>
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<td>Total</td>
<td>79</td>
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</table>

Figure 2. Concordance analysis for PR3 and MPO-ANCA tested on BIO-FLASH and Fidis Vasculitis Panel. Neg: negative; Pos: positive.

**Utility of BIO-FLASH in an emergency setting**

For assessment of practicability of the BIO-FLASH in an emergency setting, inexperienced residents at our hospital were recruited to evaluate the functionality and ease of use of BIO-FLASH. After a short briefing lasting 15 minutes, the residents were able to perform the analysis and retrieve results within 30 minutes. The QUANTA Flash PR3 and MPO assays on the BIO-FLASH system display good sensitivity and specificity combined with high agreement to routine methods. Moreover, the retrieval of results was rapid and easy to obtain, making the technique suitable for emergency settings as well as for the follow-up of patients in routine practice.

**IN CONCLUSION**

In conclusion:

- ANCA testing by IIF is an important first step when screening, positive results or cases of strong clinical suspicion should be run on a solid phase assay for confirmation.

- When monitoring disease activity or therapy efficacy, a methodology other than IIF should be used to allow for numerical results.

- Acute disease flares can be confounding and adequate testing methods in an emergency setting can impact patient outcomes.

- A method such as BIO-FLASH which is rapid, accurate, and can be performed by all levels of laboratory staff is ideally suited for urgent as well as monitoring requests for ANCA testing.

**References**

A new look at ANCA testing with NOVA View®

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Introduction

The International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA)\(^1,2\) states that the minimum requirements for ANCA testing is that “IIF should be performed on all sera from new patients, since 10% of ANCA positive sera in patients with Wegener’s granulomatosis or microscopic polyangiitis can be demonstrated only by IIF”\(^3\). (Wegener’s granulomatosis has recently been renamed as granulomatosis with polyangiitis (GPA)\(^3\)). In spite of these recommendations, laboratory strategy for the detection of ANCA varies across laboratories according to geographical areas, traditions and local experience. New antigen-specific solid phase assays are highly sensitive and specific\(^4\), but the performance characteristics of available assays vary widely\(^5,6\).

Challenges of IIF method for detection of ANCA

The IIF method (whether it is used for the detection of antinuclear antibodies (ANA), ANCA, or organ-specific antibodies) occupies a special – not very popular – place in the laboratory. First, both slide preparation and slide reading requires significant manual processes, which is time consuming and prone to technical issues.

The manual nature of slide preparation and slide reading, coupled with handwritten transcription of results in the dark room is the primary source of sample mixups and erroneous result reporting. Furthermore, some (predominantly homogeneous) ANA samples may produce patterns identical to those produced by pANCA. This is one of the reasons why the interpretation of ANCA IIF images is challenging and requires the review of results on both ethanol and formalin fixed substrates.

After successful launch of the ANA module on NOVA View\(^*\), INOVA Diagnostics has developed a new application that allows the use of NOVA View automated fluorescent microscope for ANCA testing. NOVA View with ANCA software module and NOVA Lite ANCA reagents are used together as a system to bring ANCA IIF testing to a level that meets the expectations of modern autoimmune laboratories.

NOVA View® with ANCA module

The NOVA View\(^*\) system contains a fluorescent microscope with a motorized stage, LED light source, and a CCD (charge-coupled device) digital camera. The NOVA View software controls the microscope and the camera. The system acquires high resolution digital images of the substrate, archives them, and displays them on the computer screen for the user for review. NOVA View measures the light intensity of the images, and preliminarily categorizes the samples as negative or positive according to a pre-programmed cutoff. The NOVA View software also provides pattern recognition for positive samples based on software algorithm. The ability of the operator to manage (enlarge, overlay) the acquired digital images facilitates interpretation. Importantly, NOVA View does not report final results without confirmation from a trained technician.

The NOVA Lite\(^*\) Ethanol ANCA kit with DAPI and NOVA Lite Formalin ANCA kit with DAPI are enhanced versions of the NOVA Lite ANCA kits, that are IVD licensed for

*NOVA View is not available for sale in the US
Use of the NOVA Lite ANCA DAPI kits with QUANTA-Lyser and NOVA View simplifies and streamlines the IIF reading/interpretation workflow, by:

- Reducing hands-on time in the slide reading process.
- Supporting the interpretation of the samples with objective information.
- Eliminating transcription errors with barcoded slides.
- Separating image acquisition from image interpretation.
- Eliminating the need for darkroom.

manual use. Cell density in the wells was increased and standardized. The conjugate in the kit contains DAPI (4',6-diamidino-2-phenylindole), a blue fluorescent dye that stains the nuclei. As images are taken by NOVA View with both the DAPI and the FITC filters, every image is available in both “colors” which can be overlaid to facilitate pattern identification, and can be used to confirm the presence of cells on negative wells.

Each kit contains twenty, 12-well slides (Figure 1). Each slide contains a two-dimensional barcode that designates the slide type (ethanol- or formalin-fixed, 12-well), and has a unique identifier, allowing the identification of the slide and linking of patient information to the wells (Figure 1). These slides can be processed by automated slide processing instruments, such as QUANTA-Lyser®. QUANTA-Lyser is programmed to read IFA slide barcodes to confirm the substrate type, and to match patient information received from the laboratory information system (LIS) to a specific well location on the slide.

These unique features ensure sample traceability, and deliver positive patient identification for IFA processing and analysis. QUANTA Link®, a middleware software facilitates communication between the LIS, QUANTA-Lyser, and other lab instruments, thereby providing the foundation of a fully integrated autoimmune laboratory.

**ANCA module characteristics and user interface**

During slide reading, NOVA View analyzes at least 25 cells, and acquires at least three representative digital images. Very bright samples will undergo a second imaging, when images are captured with reduced (optimal) exposure time to facilitate pattern recognition. After finishing the imaging process, the measured LIU and the suggested result (negative/positive and pattern) are displayed for the user. NOVA View identifies nuclear and cytoplasmic staining patterns. In the case of non-characteristic or mixed patterns (such as in the presence of certain ANAs), NOVA View reports positive result with unrecognized patterns.
A new look at ANCA testing... Continued

Figure 2 shows a representative screen shot of a pANCA positive sample, as the user sees it. During the image review process, the operator can enlarge and overlay DAPI and FITC images (Figure 2). After review, the operator has two choices: either confirming the NOVA View suggested result, or revising it (regarding both negativity/positivity and pattern) and then confirming. The software contains five customizable buttons that the users can pre-program according to their local reporting nomenclature (for example, pANCA, cANCA, atypical ANCA, Possible ANA, or See Comment). For each sample, a comment can be entered that becomes part of the reported results. Results can be transferred to the LIS system or a patient report can be directly generated by the NOVA View.

QUANTA Link and the Multi-Analyte Screen

QUANTA Link can greatly enhance the IIF experience with the NOVA View, and facilitates ANCA interpretation. One important feature of QUANTA Link is the Multi-Analyte Screen. This feature brings together digital images generated on the same sample by various assays. In the case of ANCA, images obtained on ethanol-fixed and formalin-fixed substrates can be displayed next to each other, and if HEP-2 ANA results are available, this can also be added to the same screen. Having these images on the same screen at the same time allows the interpretation of ANCA IIF results with high accuracy. Figure 3 shows an example that was generated on a cANCA and speckled ANA double positive sera sample.

Performance characteristics

During the establishment and validation of the ANCA module performance, the cut-off LIU was determined separately for ethanol- and formalin-fixed slides on 120 reference specimens.

• Precision for both substrate types was examined on three samples, run for five days in triplicate with two runs per day. All results were within one grade difference based on digital image reading.

• Accuracy was assessed by determining the endpoint titer of six positive samples based on LIU value, digital image reading, and manual image reading. Endpoints were within one dilution step from each other in each comparison.

• In a clinical validation study, samples from 80 healthy controls, 20 infectious disease patients, and 21 MPO/PR3 positive patients were assayed. Performance is shown in Table 1.
Table 1. Clinical performance of NOVA View with formalin-fixed and ethanol-fixed neutrophil substrate

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Formalin-fixed neutrophil substrate</th>
<th>Ethanol-fixed neutrophil substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative sensitivity based on LIU cutoff</td>
<td>76.2% (16/21)</td>
<td>90.5% (19/21)</td>
</tr>
<tr>
<td>Relative sensitivity based on digital image reading</td>
<td>90.5% (19/21)</td>
<td>95.2% (20/21)</td>
</tr>
<tr>
<td>Relative specificity based on LIU cutoff</td>
<td>100.0% (80/80)</td>
<td>96.3% (77/80)</td>
</tr>
<tr>
<td>Relative specificity based on digital image reading</td>
<td>100.0% (80/80)</td>
<td>90.0% (72/80)</td>
</tr>
<tr>
<td>Agreement between LIU-based NOVA View results and digital image reading</td>
<td>96.7% (117/121)</td>
<td>93.3% (112/120)</td>
</tr>
<tr>
<td>Agreement between digital image reading and manual (microscopic) reading</td>
<td>90.9% (110/121)</td>
<td>93.4% (113/121)</td>
</tr>
</tbody>
</table>

References


In Conclusion

In conclusion:

- NOVA View is an automated solution for autoimmune laboratories performing IIF assays. It simplifies and streamlines the IIF reading/interpretation workflow, and increases the safety of IIF testing by sample traceability.

- The newly developed ANCA module acquires high resolution digital images on ANCA slides, and provides the user with tools that facilitate image interpretation.

- The ANCA module showed reliable and reproducible performance during evaluation studies.
Anti-PR3 antibodies in ulcerative colitis

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Introduction

Antineutrophil cytoplasmic antibodies (ANCA) directed against proteinase 3 (PR3) or myeloperoxidase (MPO) are used in the diagnostic workup of small vessel vasculitis (SVV), such as granulomatosis with polyangiitis (GPA), and microscopic polyangiitis. Conventional screening for anti-PR3- and MPO-antibodies starts with the indirect immunofluorescence assay (IIF) on ethanol-fixed human neutrophils followed by confirmatory ELISA. Anti-PR3 antibodies generate a cytoplasmic staining pattern (cANCA) on ethanol-fixed human neutrophils, whereas anti-MPO antibodies generate a perinuclear staining (pANCA) pattern. Many laboratories also evaluate specimens on formalin-fixed human neutrophils.

Table 1. Prevalence of autoantibodies in ulcerative colitis and Crohn’s disease

<table>
<thead>
<tr>
<th>Marker</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>pANCA</td>
<td>40-60%</td>
<td>5-20%</td>
</tr>
<tr>
<td>ASCA IgG</td>
<td>5-10%</td>
<td>40-70%</td>
</tr>
<tr>
<td>ASCA IgA</td>
<td>5-10%</td>
<td>40-70%</td>
</tr>
<tr>
<td>PR3-ANCA</td>
<td>15-40%</td>
<td>0-10%</td>
</tr>
<tr>
<td>MZGP2</td>
<td>0-5%</td>
<td>20-30%</td>
</tr>
</tbody>
</table>

Biomarkers for inflammatory bowel disease

An atypical pANCA pattern, sometimes referred to as xANCA, is found in patients with inflammatory bowel disease (IBD), mainly in patients with ulcerative colitis (UC). When combined with anti-Saccharomyces cerevisiae antibodies (ASCA), atypical pANCA has been recommended as a way to help distinguish UC from Crohn’s disease (CrD). ASCA sero-positivity is a predominant feature of CrD, while atypical pANCA is a marker of UC. Both ASCA and ANCA have been reported to predict the development of IBD. Despite many studies, the specificity of ANCA in IBD remains poorly defined. The diagnosis of IBD including UC and CrD is largely based on endoscopic and histological assessment of the inflamed tissue. While several antibody tests can assist in the diagnosis of CrD including (ASCA) and pancreatic major zymogen granule protein 2 (MZGP2), the only serological biomarker for UC is atypical pANCA detected by IIF. However, IIF is time consuming and observer-dependent, has low throughput requiring highly-trained personnel, can generate significant variation and demonstrates limited specificity. IIF is also unable to provide information about ANCA antigen specificity. Several studies have aimed to identify the major target antigen of atypical pANCA in IBD, but major disease-specific target antigens are yet to be identified.

Detection methods for PR3-ANCA

Over the last decade, a variety of different methodologies have been developed and commercialized for the detection of anti-PR3 antibodies, including IIF on human neutrophils, ELISA, line immunoassays (LIA), capture and anchor ELISAs as well as multiplex assays using native purified PR3 and, more recently, recombinant PR3 antigen. Despite several comparative studies, it remains debatable as to which methodology for anti-PR3 antibodies detection provides the
highest clinical accuracy for the diagnosis of GPA.\textsuperscript{19, 29} Several studies published over the last decade suggested that the sensitivity of both capture as well as novel anchor assays were superior to classical ELISA and even to IIF.\textsuperscript{19, 23-24, 30-31}

**PR3-ANCA as a marker for UC**

In contrast to historical data,\textsuperscript{32} more recent studies reported anti-PR3 antibodies in a significant percentage of IBD patients.\textsuperscript{27} This raises the possibility that akin to anti-PR3 antibodies as a marker for SVV, anti-PR3 antibodies may also be a marker for IBD. Recently it was suggested that anti-PR3 antibodies measured by a novel chemiluminescent immunoassay (CIA) on a random access auto-analyzer (BIO-FLASH\textsuperscript{33}) are useful in the differential diagnosis of UC and CrD.\textsuperscript{33} Our observation that anti-PR3 antibodies can be detected in sera from patients with IBD, with higher prevalence in UC vs. CrD patients, suggests that anti-PR3 antibodies testing could assist in discriminating UC from CrD, and in discriminating IBD from other gastrointestinal conditions. The terms “indeterminate colitis” or “IBD unclassified” (IBD-U) categorize patients in whom the diagnosis of UC or CrD is not clear.\textsuperscript{5, 17, 34} The differential diagnosis may be complicated in patients with irritable bowel syndrome, celiac disease, or other colorectal diseases, with features indistinguishable from those seen in patients with IBD.\textsuperscript{3}

Studies of anti-PR3 antibodies in IBD are limited and have been based on relatively small cohorts of UC patients.\textsuperscript{27, 35-36} When twelve anti-PR3 antibody assays were compared using 22 IBD sera, the reported prevalence of anti-PR3 antibodies ranged from 4% to 43%, raising concerns as to the reliability of the assays used in these studies.\textsuperscript{27, 35-36} The two assays with highest sensitivity (BINDAZYME Anti-PR3 39% and Rainbow ELISA PR3 43%) also showed the lowest specificity (88%). In a recent study it was found that anti-PR3 antibodies are found in patients with IBD, albeit at lower titers than in patients with GPA. This was demonstrated using three separate and independent fully automated platforms for autoantibody detection. The prevalence of the antibodies differed between the methods used, but this was mainly related to the way the manufacturer-specific cut-off was defined (focused on high specificity for GPA).
Anti-PR3 antibodies in ulcerative colitis...Continued

DIFFERENTIATION BETWEEN PR3-ANCA POSITIVE UC AND GPA PATIENTS

The fact that anti-PR3 antibodies are found in UC could be interpreted as compromising the specificity of anti-PR3 antibodies for GPA. Anti-PR3 antibodies in patients with GPA are often associated with a cANCA pattern on ethanol-fixed neutrophils, while in UC patients an atypical pANCA is most often observed. The latter is most likely explained by reactivity to other antigens that have been reported in the past to be associated with UC.

Interestingly, recent studies have described patients with overlapping features of UC and GPA. To what extent anti-PR3 antibody positive patients with UC will develop full-blown SVV over the course of their disease, needs to be assessed in large longitudinal studies. It is widely appreciated that different autoimmune diseases can overlap in some patients, which was recently described as poly-autoimmunity. Therefore, additional studies are required to determine whether there is an overlap between the two chronic inflammatory diseases. While GPA typically affects the upper respiratory tract and the kidneys, UC typically is an inflammatory disease limited to the colon. Although 10% of patients with SVV can present with ulcerations of the colon, isolated gastrointestinal tract involvement is frequently seen in ANCA-positive patients with SVV.

IN CONCLUSION

In conclusion:

- The only serological marker for UC is atypical pANCA detected by IIF. However, IIF is observer-dependent and is unable to provide information about ANCA antigen specificity.
- Anti-PR3 antibodies are present in a significant percentage of IBD patients and may be a marker of concurrent SVV-related disease.
- Anti-PR3 antibodies measured by BIO-FLASH CIA are useful in the differential diagnosis of UC and CrD.

REFERENCE LIST


ABBREVIATIONS

ASCA, anti-Saccharomyces cerevisiae antibody; AUC, Area under the curve; cANCA, cytoplasmic anti-neutrophil cytoplasmic antibodies; CIA, chemiluminescence assay; CrD, Crohn’s disease; CU, calculated units; GPA, granulomatosis with polyangiitis; IBD, inflammatory bowel disease; IIF, indirect immunofluorescence assay; LIA, line immunassay; LR, likelihood ratio; pANCA, perinuclear anti-neutrophil cytoplasmic antibodies ROC, Receiver Operating Characteristic; UC, ulcerative colitis; WG, Wegener’s granulomatosis.


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- Antinuclear antibodies: From past to present (No. 7)