

# Antineutrophil cytoplasmic antibodies (ANCA) testing: detection methods and clinical application

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## ABSTRACT

*Antineutrophil cytoplasmic antibodies (ANCA) are considered the diagnostic biomarker of some necrotising vasculitis such as granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and, to a lesser extent, eosinophilic granulomatosis with polyangiitis (EGPA).*

*According to the current recommendations, combining indirect immunofluorescence and proteinase 3 (PR3) and myeloperoxidase (MPO) antigen specific immunometric assays, in the proper clinical setting, assures the best diagnostic specificity. When such conditions are satisfied, ANCA are detected in up to 90% of patients with active generalised GPA and MPA and in about 40% of patients with EGPA.*

*Cytoplasmic ANCA (C-ANCA) with specificity for PR3 are usually found in patients with GPA whereas perinuclear ANCA (P-ANCA) in patients with MPA and EGPA. However, ANCA antigen specificity is more closely associated with disease phenotype and prognosis than clinical diagnosis. The clinical value of serial ANCA testing in monitoring disease activity is still debated. Recently, new promising developments in methodology and techniques (computer-based image analysis of immunofluorescence patterns, novel generation of PR3-/MPO-ANCA immunometric assays and multiplex technology) have been proposed but studies comparing the performances of the different assays are scarce.*

## Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are the serological markers of some idiopathic necrotising vasculitis, predominantly afflicting small (medium)-sized blood vessels, such as granulomatosis with polyangiitis

(GPA, previously called Wegener granulomatosis), microscopic polyangiitis (MPA) and, to a lesser extent eosinophilic granulomatosis with polyangiitis (EGPA, previously Churg-Strauss syndrome) (1-3). These vasculitides, in which circulating ANCA are commonly found, are generically named “ANCA-associated vasculitis” (AAV) (4).

ANCA were first detected by the immunofluorescence technique, using ethanol-fixed leukocytes or human purified neutrophils as cellular substrates (5-7). With the identification of the antigenic targets, antigen-specific ELISAs were developed (6). More recently, new techniques and methods, with the aim of improving diagnostic performance, have been introduced.

This paper will review ANCA detection methods and their clinical application.

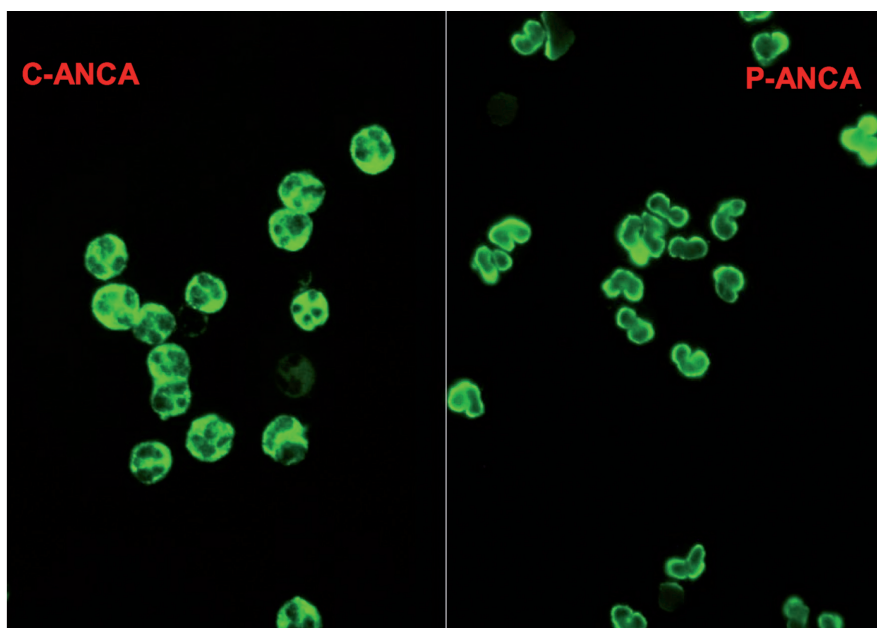
## Detection methods

### *Indirect Immunofluorescence Technique (IIFT)*

ANCA are classically detected by indirect immunofluorescence, using human ethanol-fixed neutrophils as cellular substrate. The slides are then incubated with an anti-human IgG antibody which is conjugated usually with fluorescein isothiocyanate. Two main fluoroscopic staining patterns can be identified: the diffuse, granular cytoplasmic (C-ANCA), and the perinuclear (P-ANCA). The former due to the presence of autoantibodies targeting the serine protease proteinase-3 (PR3-ANCA), while the latter caused by antibodies directed mainly against myeloperoxidase (MPO-ANCA) (Fig. 1) (5-7).

The target proteins PR3 and MPO are both localised in the azurophile (primary) granules of polymorphonuclear cells and monocytes, from where they can be exposed on the cellular surface as a result of various inflammatory stimuli (5,

Competing interests: none declared.



**Fig. 1.** Patterns of antineutrophil cytoplasmic antibodies (ANCAs).

**A:** Cytoplasmic ANCA pattern on ethanol-fixed neutrophils obtained from a patient with Wegener's granulomatosis and anti-proteinase 3 antibodies.

**B:** Perinuclear ANCA pattern on ethanol-fixed neutrophils obtained from a patient with microscopic polyangiitis and anti-myeloperoxidase (anti-MPO) antibodies.

7, 8-9). Ethanol treatment causes solubilisation of the granule membranes, thus allowing mobilisation of the content. Because of the different pI, PR3 and MPO behave differently in response to this chemical, with the latter, being a strongly cationic molecule, redistributing towards opposite-charged areas such as the perinucleus (5, 7-9).

In the last few years, automated fluorescent microscope systems that acquire, store, and display high resolution digital images obtained on IIF slides, have been developed. Digital images can be viewed but also stored for further analysis. Software programs provide tools to support the operator's decision making such as negativity, positivity, and pattern interpretation.

Automatic reading of ANCA slides is a very promising technique but needs further improvement to overcome some

interpretation problems. Moreover, evaluation in multicentre studies, before being introduced in routine laboratories, is warranted.

#### Antigen-specific immunometric assays

##### – First generation ELISA tests

After the antigens targeted by autoantibodies generating the P-ANCA and C-ANCA patterns were identified, the first ELISAs were developed using purified native PR3 and MPO as antigens (8-9). These test kits used simple absorption coating methods and the purity of the antigens was quite variable (6). Most tests lacked comparability of results and correlation towards IIF methods (10-11). The limited sensitivity of these first generation tests was attributed to the absorption immobilisation process resulting in masking and deformation of epitopes (12-14).

#### – Second and third generation tests

The low sensitivity of the first generation ELISAs led to the development of novel approaches to ANCA testing. The so called second generation ANCA assays used capture molecules, mostly monoclonal antibodies to bind the antigens (PR3 and MPO) to the surface without causing changes to the epitopes structure (Fig. 2) (12-14). These ELISAs showed a significant increase in sensitivity and proved to be superior to the direct binding ANCA assays (12-14).

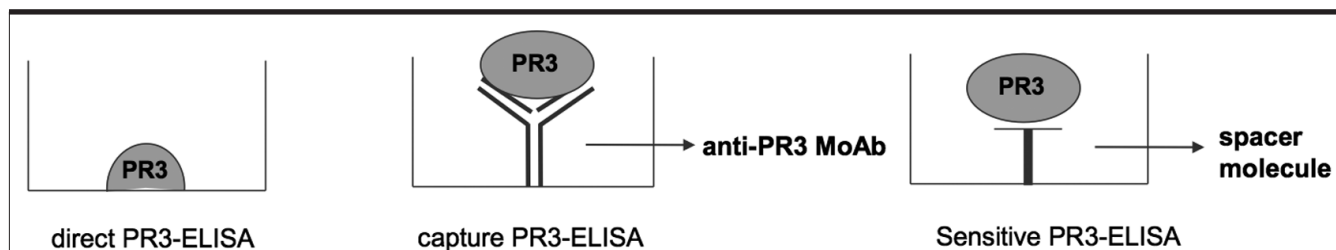
Third generation assays are based on "anchor" techniques to immobilise the antigens (Fig. 2) (15). The antigens are bound to plastic using anchor-molecules which are attached to the surface of the ELISA plate. This method should provide a better accessibility of epitopes improving the diagnostic performance.

#### – Other ELISA methods

A novel approach using a mixture of human purified PR3 (hn-PR3) plus recombinant PR3 (hr-PR3) from human source (HEK 293) as antigen in solid-phase, has been developed. The recombinant PR3 expression in human cells should assure the optimal post-translational processing and folding, with the conformational similarity to the native protein (16). It has been postulated that this method might have a significantly higher sensitivity compared to first generation and second generation capture methods but this needs to be confirmed.

#### – Chemiluminescent immunoassays

Chemiluminescent assays (CLIA) are significantly different from ELISA technology, as the antigen is covalently attached to the surface of bead particles. In CLIA, paramagnetic beads are coupled with native PR3 or MPO. After the beads are incubated with diluted patient



**Fig. 2.** Different coating strategies in the first generation ELISA, capture ELISA and anchor-ELISA.

serum and washed, anti-human IgG isoluminol conjugate antibody is added. The conjugate is oxidised when sodium hydroxide and peroxide solutions are added and the flash of light produced from this reaction is measured as relative light units. Unlike ELISA or addressable laser bead immunoassays platforms, the detection system uses a proprietary CLIA technology that affords a remarkably wider dynamic range, an entirely linear titration curve and more consistent inter- and intra-test reliability (17-20).

#### – *Bead-based flow cytometry assays*

The BioPlex 2200 is a fully-automated multiplex immunoassay platform that allows the measurements of several autoantibodies. The instrument combines the multi-analyte profiling technology with antigen-coated fluoromagnetic beads as solid phase, in an automated platform where sampling, processing and data reduction are performed automatically. A preliminary report found excellent relative sensitivity and relative specificity for the semi-quantitative detection of IgG autoantibodies to MPO, PR3 and GBM of the Bioplex autoimmune Vasculitis kit (21).

#### *Other assays*

– *Combination of IIF and bead assays*  
Rapid tests for the qualitative ANCA detection are also commercially available, mainly based on the “blotting” technology, allowing to look for the presence of PR3-ANCA and MPO-ANCA (22).

A new approach to combine screening for and confirmation of ANCA was presented recently. This new technology combines ethanol fixed neutrophils and the antigens PR3 and MPO coupled to different microbeads which are attached to different compartments of the IIF slide. The different reactivities can be identified by either the size of the respective bead, or the position in different compartments of the IIF slide's cavities (23).

The performances of such assays have not been addressed by large, rigorous and independent studies; besides, comparative evaluations with the standard methods, supporting their efficiency, have never been carried out (17).

#### – *Summary of methods for ANCA detection*

Despite several comparative studies, it remains debatable as to which methodology for ANCA detection provides the highest clinical accuracy for the diagnosis of small-vessel vasculitis (SVV). 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 (reviewed in 17). Lately, the new emerging technology of CLIA showed comparable or superior sensitivity compared to established assay types (18-20).

A large number of studies have been made in order to evaluate the diagnostic performance of such novel formats for PR3-ANCA detection, most of them confirming a higher sensitivity with the same specificity, when compared to the traditional direct assays (18-20).

It is worth pointing out that direct comparison of the absolute data from different published series is difficult because of the different selection criteria for AAV patients and controls.

#### **Clinical application**

##### *Appropriateness of the requests*

The widespread use of ANCA screening has made the occurrence of false positive results an emerging topic.

Laboratory tests should always be ordered appropriately: this pre-condition is extremely important when looking for extremely rare diseases like AAV (estimated annual incidence 10–20/million) (24).

The laboratory contribution consists in confirming or excluding the diagnostic hypothesis, by using the best assays and technologies available. Indeed, the predictive values of any laboratory assay depend not only on the sensitivity and specificity of the given test, but also on the prevalence of the disease in the studied population. The predicted prevalence of an uncommon disease varies largely among patients with different clinical manifestations.

In other words, the clinical utility of ANCA testing results will be higher in those patients with high pre-test probability of having a small-vessel vasculitis, which means those whose clinical

picture suggests such diseases (Table I). In other clinical settings, the number of false positive and false negative results will exceed true positives and negatives; for these reasons a “gating policy” for ANCA testing has been suggested (25). In such report, the impact of the test ordering according to the guidelines would have decreased the test number by 23% and the false positive rate by 27%, with significant improvement in diagnostic accuracy and clinical implications (25).

The adherence to a “gating policy” for ANCA testing, coupled with close liaison between clinicians and laboratory specialists, does not result in either a missed or delayed diagnosis of AAV while makes ANCA detection more clinically relevant (25).

##### *Diagnostics performance*

Several studies, including large prospective ones, have shown that ANCA have a high diagnostic value for AASV, provided that a correct methodology is used in the relevant clinical setting (6, 26-28).

Results from these studies have demonstrated that ANCA, detected by the immunofluorescence technique (C-ANCA or P-ANCA), are a sensitive marker for the so-called AAV, with sensitivity ranging from 80 to more than 90%. Unfortunately, immunofluorescence has a low specificity (80% or less), which is mainly caused by positive P-ANCA in disease controls, such as ulcerative colitis, autoimmune hepatitis, sclerosing cholangitis, rheumatoid arthritis). P-ANCA, in disease controls, can be also caused by the interference of anti-nuclear antibodies, especially in systemic lupus erythematosus patients. C-ANCA (with specificity for bactericidal permeability increasing protein, BPI) have been reported in infectious diseases and are frequently found in cystic fibrosis (6, 17, 26-28).

The use of immunometric assays with purified antigens improves the specificity up to 98% or more, with a slight loss in sensitivity (6, 17, 26-28).

Antigen specificity (PR3 or MPO) does not effectively differentiate among the different AAV, however C-ANCA/PR3-ANCA are mainly found in GPA,

**Table I.** Clinical indications for ANCA testing.

- Glomerulonephritis, especially Rapidly Progressive Glomerulonephritis
- Pulmonary hemorrhage, especially pulmonary-renal syndrome
- Cutaneous vasculitis with systemic features
- Multiple lung nodules
- Chronic destructive disease of the upper airways
- Long-standing sinusitis or otitis
- Subglottic tracheal stenosis
- Mononeuritis multiplex or other peripheral neuropathy
- Retro-orbital mass
- Pulmonary fibrosis, with systemic features
- Episcleritis, uveitis, retinal vasculitis, with systemic features

while P-ANCA/MPO-ANCA are more prevalent in MPA, and EGPA. ANCA are detected in 70–90% of active, generalized GPA, but only in about 40–50% of the loco-regional forms (6).

#### *ANCA and clinical phenotype*

Recent developments have shown that ANCA specificity is more closely associated with disease phenotype and prognosis than clinical diagnosis (29). In particular, as expected, patients with kidney-limited disease or any form of vasculitis without radiographic or histologic proof of granulomatous inflammation were more likely to have MPO ANCA, and those with the most compelling evidence for necrotising granulomatous inflammation were most likely to have PR3 ANCA (29). Moreover, ANCA specificity was predictive of relapse, with PR3 ANCA-positive patients almost twice as likely to relapse as those with MPO ANCA (HR 1.89 [95% CI 1.33–2.69],  $p=0.0004$ ), and ANCA specificity had the best predictive model fit compared to the Chapel Hill Consensus Conference (CHCC) and European Medicines Agency (EMA) systems (29).

These findings are in line with the results of a genomewide association study, performed in AAV, which has demonstrated striking differences in genetic association between granulomatosis with polyangiitis and microscopic polyangiitis that were not driven by the clinically defined syndromes but by the underlying autoantibody specificity (30). Specifically, both the *HLA* and *SERPINA1* associations with granulomatosis with polyangiitis are seen in patients with granulomatosis with polyangiitis who are positive for

proteinase 3 ANCA, not for myeloperoxidase ANCA, and can also be seen in the proteinase 3 ANCA subgroup of patients with microscopic polyangiitis. This clear association of genetic background with autoantibody specificity suggests that it might contribute to the clinical classifications of granulomatosis with polyangiitis and microscopic polyangiitis (30).

Churg–Strauss syndrome is classified among the so-called ANCA-associated systemic vasculitides because of the overlapping clinico-pathological features with the other AAV. However, while ANCA are consistently found in 70–95% of patients with GPA and MPA, their prevalence in CSS is sharply lower (around 40%). The main fluoroscopic pattern is perinuclear with antibodies to MPO.

CSS ANCA-positive patients are more likely than ANCA-negative patients to present with the typical clinico-pathological picture of the other small-vessel vasculitis and less likely to suffer from heart and non-haemorrhagic lung involvement (3).

#### *ANCA-negative patients*

As previously stated, conventional serologic assays fail to detect ANCA in some patients with classic clinical and pathologic features of AAV. These patients are labelled as having ANCA-negative disease. If these patients are true ANCA-negative or ANCA-positive cases who are missed by the current techniques is not clear. Recently, autoantibodies from human and murine samples were epitope mapped using a highly sensitive epitope excision/mass spectrometry approach. This methodology led to the discovery of MPO-

ANCA in ANCA-negative disease that reacted against a sole linear sequence. The confounder for serological detection of these autoantibodies was the presence of a fragment of ceruloplasmin in serum, which was eliminated in purified IgG, allowing detection (31). If confirmed, these results when applied in clinical laboratories, should increase the diagnostic sensitivity of ANCA in AAV.

#### **ANCA testing in monitoring disease activity and predicting relapse**

Whereas the diagnostic value of ANCA in AAV has been widely recognised (6, 25–17), the role of ANCA in monitoring disease activity in patients with vasculitis is still controversial.

Tomasson *et al.* recently performed a meta-analysis on the value of ANCA measurements during remission to predict a relapse of AAV (32). The summary estimates for positive likelihood ratio (LR+) and negative likelihood ratio (LR-) of a rise in ANCA during remission on subsequent relapse of disease were 2.84 (95% CI 1.65, 4.90) and 0.49 (95% CI 0.27, 0.87), respectively. The summary estimates for LR+ and LR- of persistent ANCA during remission for subsequent disease relapse were 1.97 (95% CI 1.43, 2.70) and 0.73 (95% CI 0.50, 1.06), respectively. The authors concluded that among patients with AAV, a rise in or persistence of ANCA during remission is only modestly predictive of future disease relapse (32). These conclusions are, however, challenged by the limitations of the published papers in terms of low number of studies, differences in ANCA testing assays, time interval between measurements, relapse and ANCA increase definitions, patients population. Since there was such substantial between-study heterogeneity, further multicentre studies with standardised protocols are needed to solve the controversy.

#### **ANCA problems in clinical practice**

##### *Lack of standardisation of the current assays*

Years ago, repeated surveys clearly demonstrated that the performances of the widely utilised direct assay for MPO and PR3-ANCA detection were

not satisfying, with poor reliability in terms of sensitivity, specificity and diagnostic accuracy. Lately, new methods have been developed in order to partially overcome these pitfalls and improve the performance of the traditional ANCA assays.

Although there are a number of ongoing reports showing the expected superiority of the novel formats for MPO and PR3-ANCA detection, nevertheless the different brands show different performances.

Undoubtedly, the 2<sup>nd</sup> and 3<sup>rd</sup> generation assays, based on the indirect coating of the antigens to the support, due to the better exposition and accessibility of most epitopes, have in general increased sensitivity and reduced variations in comparison with the classic direct methods. However, extremely impressive data reports often refer to small cohorts or selected sub-populations, and cannot be generalised. The sensitivity of ANCA in AAV affected patients, detected by using the best performing kits and considering a specificity  $\geq 95\%$ , ranges between 70 and 90%, but the prevalence is lower (40–60%) in case of limited GPA (18).

The recent availability of MPO and PR3 reference sera should help in the standardisation efforts. However, to refer to a common reference material is far from assuring a comparison between results obtained using different tests, because other biological features play an important role, although it may be the first step towards an acceptable standardisation. It should be emphasised how difficult it is for clinicians to monitor response to treatment and to predict relapse, when consecutive serum samples are tested in laboratories using different assays.

### Conclusions

- ANCA are a very sensitive and specific marker for the so-called AAV (GPA, MPA and EGPA), providing that the correct methodology (indirect immunofluorescence plus PR3 and MPO antigen specific assays) in the proper clinical setting (appropriateness of the request) is applied.
- A positive ANCA result by itself, without clinical evidence and pos-

sibly histological confirmation, is not diagnostic for AAV. On the other hand, negative ANCA test results cannot completely rule out a diagnosis of AAV, since AAV without detectable ANCA do exist.

- In differential diagnosis with mimicking conditions, infectious diseases must be considered and ruled out.
- The predictive values of an ANCA test are significantly increased when guide-lines for ANCA testing request are applied.
- The second and third generation assays seem to have a higher sensitivity while maintaining a good specificity.
- ANCA levels are useful to monitor disease activity but of limited value in predicting relapses. Therefore, they should not be used, by themselves, to guide treatment.

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