# ANCA Diagnostics in Clinical Practice: New Developments

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## 1. Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) are the hallmark of the so-called ANCA-associated vasculitides (AAV) (Jennette & Falk, 1997). These primary small-vessel vasculitides comprise granulomatosis with polyangiitis (GPA, previously referred to as Wegener's granulomatosis) (Falk et al, 2011), the Churg-Straus syndrome (CSS), microscopic polyangiitis (MPA), and renal-limited necrotizing crescentic glomerulonephritis (NCGN). These disease entities can be discriminated based on systemic symptoms of disease (GPA, CSS, and MPA) versus clinical manifestations that are restricted to the kidneys (NCGN). The systemic diseases can further be subdivided by the presence (GPA and CSS) or absence (MPA) of granulomatous lesions in the airways, while GPA and CSS can be distinguished based on the presence of asthmatic manifestations and/or eosinophilia in CSS, but not in GPA. Classification criteria for these diseases have been defined by the American college of rheumatology (ACR; Fries et al, 1990) and the Chapel Hill consensus conference (Jennette, 1994). The presence of ANCA, however, is not part of these criteria which are primarily based on clinical manifestations and histopathology as observed in biopsies obtained from the affected tissues. More recently a novel consensus methodology for the classification of AAV was developed and validated for epidemiological studies (Watts et al, 2007). Importantly, the latter classification criteria incorporated the ANCA status of the patient.

By definition ANCA are directed to the cytoplasmic components of neutrophilic granulocytes. In particular the constituents of the granules appear to be the antigenic targets. With respect to AAV the serine protease (PR)3 and myeloperoxidase (MPO) are the most important autoantigens. ANCA were originally detected by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils. According to the international consensus on ANCA detection four patterns have to be distinguished (Savige et al, 1999). First, the *classical* (C-)ANCA is characterized by a granular, cytoplasmic fluorescence with central or interlobular accentuation; second, a diffuse flat cytoplasmic fluorescence without interlobular accentuation may be referred to as atypical C-ANCA. In clinical practice, however, this pattern is not distinguished by many clinical laboratories. Third, the *perinuclear* (P-)ANCA is characterized by perinuclear staining, with or without nuclear extension. Reading of the P-ANCA pattern may be hampered by the presence of interfering antinuclear antibodies (ANA). Finally, if a combination of cytoplasmic and perinuclear staining occurs, this is called atypical ANCA. The perinuclear staining pattern actually is an artefact, since formalin-fixation produces a cytoplasmic staining pattern, indistinguishable

from C-ANCA on ethanol-fixed neutrophils. Recently, we reviewed the history of ANCA detection (Cohen Tervaert & Damoiseaux, 2009). It is more than 50 years ago that ANCA were first described in relation with chronic, inflammatory diseases, like rheumatoid arthritis, ulcerative colitis and chronic autoimmune hepatitis. Only in 1985 the association with vasculitis, in particular GPA, was noticed. In these patients IIF revealed a C-ANCA and the antigen recognized proved to be PR3. In a number of GPA cases, however, the sera revealed a P-ANCA and this staining pattern appeared to be more prevalent in MPA and CSS. The antigen recognized in the AAV patients with P-ANCA proved to be MPO. The autoantibodies reactive to these two catalytic enzymes are referred to as PR3-ANCA and MPO-ANCA, respectively. The relation between C-ANCA and PR3-ANCA, and especially between P-ANCA and MPO-ANCA is far from perfect. In particular in chronic, inflammatory diseases other than AAV, the ANCA are directed to multiple other constituents of the neutrophilic granules (like cathepsin G, catalase, α-enolase, elastase, lysozyme, lactoferrin, azurocidin, and bactericidal permeability-increasing protein) and in these cases the P-ANCA is not the result of reactivity with MPO. Hence, the consensus on ANCA detection is to screen sera with IIF and to identify the antigen-specificity with assays specific for PR3 and MPO (Savige et al, 1999). Preferentially, all sera should even be analysed by both IIF and antigen-specific assays.

In this chapter we will first discuss the technologies used for detection of ANCA, including IIF and antigen-specific assays. Next, the international consensus on ANCA testing will be discussed in light of the new technical developments. Finally, we will discuss novel ways of reporting results to the clinic to facilitate the interpretation. These issues will all be dealt with only in terms of the diagnostic phase of AAV; ANCA detection in relation to follow-up of AAV or in light of other disease entities, will not be discussed.

## 2. Methods for ANCA detection

As mentioned above, ANCA are traditionally detected by IIF. For detection of autoantibodies by IIF serum samples are diluted and incubated with antigen substrate to allow specific binding of autoantibodies (Fig. 1) (Damoiseaux & Cohen Tervaert, 2005). The antigen substrate consists of neutrophilic granulocytes on glass slides that are prepared with a fixative to enable autoantibody binding. In stead of neutrophils it is advocated to be better to use total white blood cells in order to identify true neutrophil specific autoantibodies (Wiik, 1980). In most assays the serum is diluted ~20 times, but one should be aware that the test result of an IIF assay is not only dependent on the serum dilution, but also on the quality of the substrate, the conjugate, and the fluorescence microscope. The latter, however, is more standardized since the introduction of LED-technology as light source. After washing to remove non-specific antibodies, the substrate is incubated with an anti-human IgG antibody reagent conjugated to fluorescein isothiocyanate (FITC). The final three-part complex, consisting of fluorescent secondary antibody, human autoantibody, and antigen can be visualized with the aid of fluorescent microscopy enabling the distinction of different patterns, for instance C-ANCA or P-ANCA. Typical for ANCA detection by IIF is that the substrate, i.e. neutrophilic granulocytes, is fixed with ethanol. This results in (partial) destruction of the lipid bilayer of the cytoplasmic granules enabling the constituents to freely diffuse in the cytoplasm. The strongly cationic enzymes, including MPO, then bind to the negatively charged nuclear membrane, while less cationic enzymes, like PR3 (pI 9.1) remain within the cytoplasm. The artifactual P-ANCA pattern is best illustrated upon

fixation of the neutrophils with formalin. In that case the different constituents of the neutrophils are cross-linked and therefore remain within the cytoplasmic granules revealing a C-ANCA pattern. This holds at least for MPO, but other constituents in the granules may be sensitive to formalin-fixation and therefore loose reactivity. Reading of ANCA slides is subjective, labour intensive, and requires expertise of the technicians involved. Thus far, automation of reading ANCA slides is limited. We applied a method of image analyses as a novel approach for the quantification of ANCA levels during follow-up of AAV patients (Boomsma et al, 2003). This technique enabled ANCA quantification by IIF in a single serum dilution. No major differences were observed between image analysis and other techniques in their capacity to predict relapses of disease activity. Therefore, automated ANCA quantification by IIF should also be possible in the diagnostic stage. One step beyond quantification, however, is appropriate recognition of ANCA patterns. Several diagnostic companies have developed software for automated pattern recognition (Hiemann et al, 2009). The software may be fixed, or ready to include pattern information acquired during application of the device in clinical practice. The latter, self-learning device is, however, at risk of a gradual shift in the pattern recognition. Thus far, no studies have been published on automated ANCA pattern recognition.
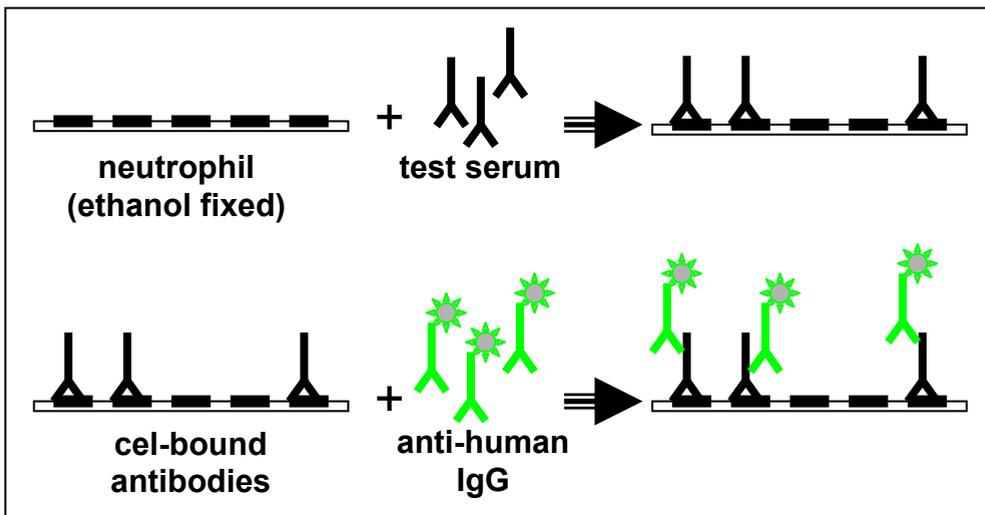


*Antineutrophil cytoplasmic antibodies (ANCA) are detected by indirect immunofluorescence (IIF). Slides of ethanol-fixed neutrophils are incubated with patient serum and visualized by FITC-labelled anti-human immunoglobulin G. Fluorescent microscopy may reveal distinct staining patterns.*
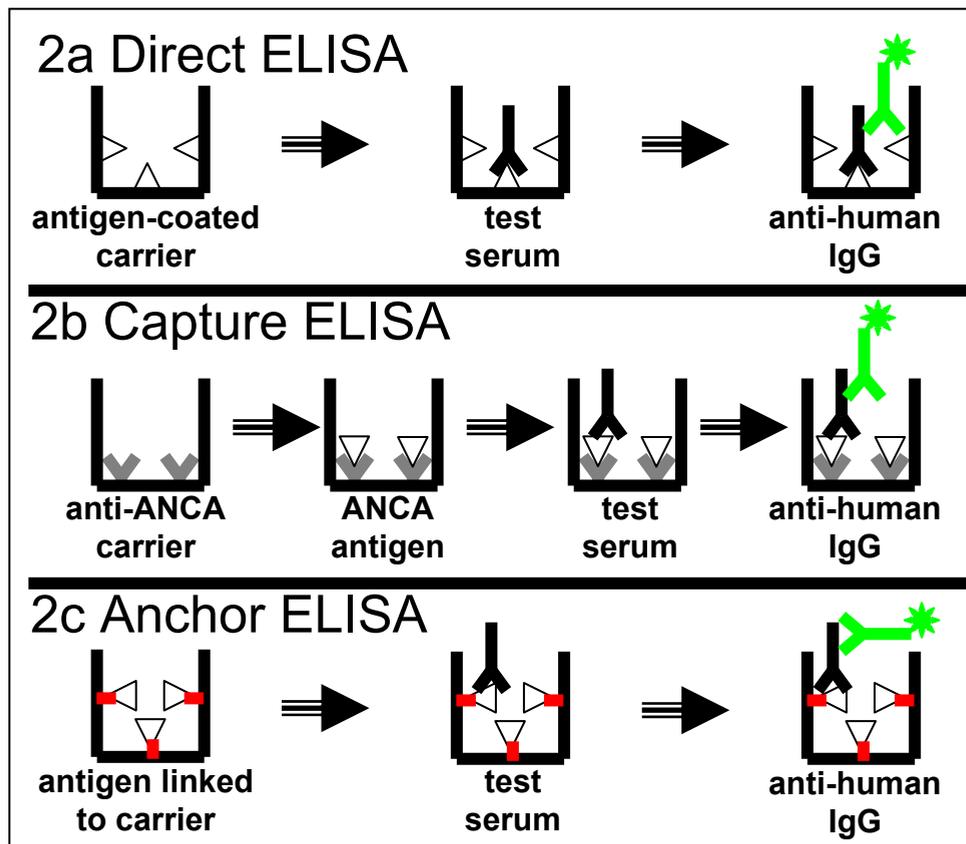
Fig. 1. ANCA detection by IIF.

Antigen-specificity of ANCA is generally determined by enzyme-linked immunosorbent assays (ELISA). In most cases a direct, non-competitive ELISA is the method of choice for detection of antigen-specific antibodies (Fig. 2a) (Damoiseaux & Cohen Tervaert, 2005). In this assay the antigen is solid-phase bound to the microtiter plates and unoccupied protein-binding sites on the carrier are blocked to prevent non-specific binding of antibodies. Since the antigen is directly coated to the solid-phase, this type of ELISA is referred to as direct ELISA. After coating of the antigen, patient serum is diluted and incubated to enable

antigen-specific binding of autoantibodies as present in the serum sample. After washing to remove non-specific antibodies, the anti-human IgG reagent, conjugated with an enzyme like horseradish peroxidase or alkaline phosphatase, is added. Binding of the anti-human IgG conjugate with the antigen-antibody complex, leads to the formation of an enzyme-labelled three-part complex that converts the finally added substrate to form a coloured solution. The rate of colour formation is a function of the amount of autoantibody present in the serum sample. Therefore, the ELISA is considered a true quantitative assay when a reference standard is available, which happens to be the case for both PR3-ANCA as well as MPO-ANCA (IUIS-CDC reference preparations). These standards enable to report the results in international units, an important step forward in standardisation of ANCA diagnostics. During the last decade, multiple variants of the direct ELISA have become available. These assays may differ in terms of the carrier, for instance a membrane in dot-blot assays (Rutgers et al, 2004) and line-immuno-assays (LIA) or beads in multiplex flow cytometry (Nifli et al, 2006; Damoiseaux et al, 2007; Kaul et al, 2009), or by replacement of the enzyme by a fluorochrome, like in fluorescent-enzyme immuno-assays (FEIA) (Damoiseaux et al, 2005) as well as the Luminex-based technology (Nifli et al, 2006; Damoiseaux et al, 2007; Kaul et al, 2009). More recently, also assays based on chemiluminesence technology have become available. Some of the novel assays have the advantage that they simultaneously measure PR3- and MPO-ANCA, and often include anti-GBM antibodies as well. On the other hand, the dot-blot assays and LIA only reveal qualitative results and may be subjective in their read-out. Optimally, the dot-blot assays and LIA are scanned and analysed by appropriate software that takes into account the signal of a cut-off control integrated in the system.

The second generation antigen-specific assays for ANCA detection is based on the so-called capture technology (Fig. 2b). In these ELISA-based tests a monoclonal antibody, specific for either PR3 or MPO, is coated to the carrier and, next, the antigen preparation, either as a crude neutrophil extract (Cohen Tervaert et al, 1990) or as purified antigens (Westman et al, 1998; Boomsma et al, 2001) is added. The antigen is now captured by the monoclonal antibody, hence the name, and the assay is continued with subsequent incubations with patient serum, anti-human IgG conjugate, and, finally, the substrate, similar as in the direct ELISA described above. Theoretically, the advantage of the capture technology is twofold: 1. because of the better consolidation of the three dimensional structure of the antigen the sensitivity will be increased, and 2. because of the additional purification of the antigen by the use of the capturing monoclonal antibody the specificity will be increased. For detection of PR3-ANCA, indeed, both the sensitivity and the specificity are increased in the capture ELISA as compared to the direct ELISA (Csernok et al, 2004). However, capture ELISA's for MPO-ANCA only have increased specificity, but not sensitivity (Boomsma et al, 2001).

Quite recently, third generation ANCA assays were introduced which are referred to as anchor ELISA (Fig. 2c). In these assays the antigen is conjugated to a peptide linker which is used for coating to the carrier. Like in the capture ELISA the three dimensional configuration of the antigen is better maintained and this may result in improved sensitivity. Indeed, an increase in sensitivity for PR3-ANCA was observed in particular in patients with limited disease manifestations (Hellmich et al, 2007; Roggenbuck et al, 2009). These assays were first developed for PR3-ANCA only, but are now also available for MPO-ANCA, although for the latter assay no data are available yet.

2a Direct ELISA

**antigen-coated carrier** → **test serum** → **anti-human IgG**

2b Capture ELISA

**anti-ANCA carrier** → **ANCA antigen** → **test serum** → **anti-human IgG**

2c Anchor ELISA

**antigen linked to carrier** → **test serum** → **anti-human IgG**

*The antigen is either coated directly to the carrier (2a), or via a capturing monoclonal antibody (2b), or via a peptide linker (2c). Incubation with the patient serum and the final detection reagent is similar in these assays.*

Fig. 2. PR3- and MPO-ANCA detection by antigen-specific assays.

Interestingly, in almost all antigen-specific assays native antigen, purified from human neutrophils, is used. Assays based on recombinant antigen have appeared difficult to develop (Jenne & Kuhl, 2006). There is, however, one direct ELISA based on a mixture of native and recombinant PR3 on the market. The performance of this assay is very similar to the anchor ELISA's (Damoiseaux et al, 2009a). It is speculated that the HIS-tag, which is normally genetically attached to a recombinant protein for purification purposes, functions as a linker peptide for anchoring the antigen to the carrier.

Finally, there is a novel technology that combines IIF and antigen specificity within a single assay. This biochip technology is a composite substrate of ethanol- and formalin-fixed granulocytes, Hep-2 cells and monkey liver (both for detecting potential interference of ANA), and microdots consisting of the PR3 and MPO antigens. This method has a very high concordance with a multi-test reference method based on IIF, direct ELISA, and capture ELISA (Damoiseaux et al, 2009b). More recently the biochip has even been extended by the inclusion of microdots consisting of the Goodpasture antigen, i.e. the non-collagenous part of the alpha 3 chain of type IV collagen. Although the biochip technology is qualitative in

nature, it is very useful in screening new patients suspected of AAV and, in the extended version, of the Goodpasture's syndrome.

## 3. Guidelines for ANCA detection

In 1999 an international consensus statement on testing and reporting of ANCA has been published (Savige et al, 1999). This is a consensus based on expert opinion. Importantly, this consensus has defined clinical indications for ANCA testing (Table 1). These indications are relevant as far as there is no other obvious cause for the clinical manifestations and they are restricted to the diseases that are associated with small vessel vasculitis. Other diseases that may be associated with ANCA (not MPO- or PR3-ANCA) are included in an addendum (Savige et al, 2003). The clinical indications as defined in table 1 are validated in a retrospective study (Mandl et al, 2002). When the guidelines for requesting ANCA would have been followed, there would have been a decrease of 23% in ANCA requests, while none of the vasculitis patients would have been missed. Due to the retrospective approach, there might have been a bias in potential misclassification. However, none of the ANCA-negative patients developed AAV within 8 to 12 months follow-up. This is somewhat surprising since AAV patients may also present with (epi)scleritis and/or other symptoms that are not included in table 1 (Cohen Tervaert et al, 1987). More recently, an extended gating policy for requesting ANCA has been evaluated (Arnold et al, 2010). Besides clinical manifestations that have a significant pre-test probability of small vessel vasculitis (grossly similar as indicated in table 1), ANCA testing was also granted for the following specialties: rheumatology, nephrology,

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| - 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 |

Table 1. Clinical indications for antineutrophil cytoplasmic antibody testing (Savige et al, 1999).

chest medicine, medical ophthalmology and ENT surgery. Additionally, a close liaison was ensured between the laboratory and referring clinician in order to offer requesters the option of further discussion. This approach resulted in a 50% decrease in ANCA requests and obviously in a concomitant reduction in false-positive results. Importantly, the gating policy did not result in either a missed or delayed diagnosis of small vessel vasculitis belonging to the spectrum of AAV.

According to the international consensus statement ANCA testing should start with screening the samples by IIF on ethanol-fixed neutrophils; titration may be useful but is optional. At least the IIF positive samples, irrespective of the pattern, should be tested in a quantitative way by ELISA for both PR3- and MPO-ANCA. Although the consensus states that antigen specificity should be determined by ELISA, it is obvious that other novel assays, nowadays available,

may also be applied for detection of PR3- and MPO-ANCA. The consensus is somewhat diffuse with respect to the interpretation of the results. Basically, one expects that only the combination C-ANCA/PR3-ANCA or P-ANCA/MPO-ANCA is relevant for the diagnosis of AAV (atypical ANCA or interfering ANA may replace the respective fluorescence patterns). However, the consensus allows the interpretation of results that reveal positivity only by IIF as being relevant for the diagnosis of AAV. This was merely based on the finding that ~10% of ANCA positive serum samples in patients with GPA or MPA were positive by IIF but negative by direct ELISA. As mentioned, by using capture and/or anchor ELISA's only few samples of AAV patients that are positive by IIF are negative by antigen specific assays. Furthermore, it is obvious that the specificity of this approach (IIF positive only) is strongly reduced. If it concerns a C-ANCA, the IIF result may only be relevant when there is a strong clinical suspicion of AAV, i.e., a high pre-test probability. However, the gap between P-ANCA reactivity and MPO-ANCA will not be closed, because autoantibodies to many other antigens, that are probably irrelevant for AAV give rise to this pattern.

As mentioned above, the international consensus is based on expert opinion. A systematic review of the literature on the role of ANCA testing for AAV is hampered by several issues:

- Standardization of assays is poor: only recently an international standard preparation (IUIS-CDC reference preparations) has become available for PR3- and MPO-ANCA, and the cut-off value in different assays is often not optimal (Holle et al, 2006),
- Serum dilutions for IIF are variable: the consensus does not decide for a 1:20 or a 1:40 dilution and correctly states that the cut-off for IIF not only depends on the serum dilution, but also on the substrate, the conjugate and the microscope,
- Many studies do not use diagnostic samples for testing the utility of novel assays. This is mandatory because, due to epitope spreading and affinity maturation, the autoantibody composite may change during ongoing disease,
- Characteristics of ANCA tests are often expressed in the context of the total AAV population. However, when including the total AAV population, test characteristics will depend on the mixture of the distinct diseases since they differ in the prevalence of PR3- and MPO-ANCA (Wiik, 2002). Also the geographical area may influence test characteristics because the prevalence of MPO-ANCA decreases, while the prevalence of PR3-ANCA increases, when more remote from the equator (Watts et al, 2005).

Altogether, there is one meta-analysis available that evaluates the value of ANCA testing, i.e., the combination C-ANCA/PR3-ANCA or P-ANCA/MPO-ANCA, in the whole spectrum of AAV (Choi et al, 2001). This meta-analysis is based on only 5 studies that fulfilled the stringent inclusion criteria. The study showed that the consensus approach for testing in combination with the strict interpretation revealed a sensitivity of 84.7% and a specificity of 98,6%. Although the specificity is high, one should be aware that the prevalence of AAV is only 15/100.000 (Mohammad et al, 2007 and references herein). The positive predictive value, therefore, will be very low if the selection of patients to be tested for ANCA is not based on appropriate clinical manifestations (Table 1).

Although the current consensus states that screening should be performed with IIF because 10% of the AAV patients may be only positive by IIF, they also advise to assess all samples for both PR3- and MPO-ANCA because 5% of the AAV patients may be positive by ELISA but negative by IIF. When strict criteria are used for interpretation of ANCA results, i.e., the combination C-ANCA/PR3-ANCA or P-ANCA/MPO-ANCA, it does not matter which test is being used as screening assay. There are, however, several arguments that plead for IIF as the best choice. First, depending on the amount of ANCA requests in a laboratory and the

degree of automation, it may be more cost effective to screen with a single assay (IIF) versus two assays (PR3- and MPO-ANCA). The advent of multiplex testing, and in particular the biochip method, neutralizes this argument. Second, ANCA IIF results, but not antigen specific assays, may also be important for other chronic inflammatory diseases like inflammatory bowel disease, primary biliary sclerosis, primary sclerosing cholangitis, and chronic, active autoimmune hepatitis (Kallenberg et al, 1992). The way of testing and reporting ANCA in these chronic inflammatory diseases is described in the addendum to the international consensus (Savige et al, 2003). Third, it is often argued that the utility of the IIF technology is hampered by the requirement of expertise of reading the results. This is a relevant point, but if only PR3- and MPO-ANCA positive samples are tested by IIF, the expertise will be more difficult to maintain because the number of samples tested by IIF will be strongly reduced. On the other hand, the continuous improvement of the antigen specific assays, in particular the capture- and anchor-assays, may have resulted in the IIF being redundant. Indeed, decision tree analysis of different testing strategies revealed that a strategy based on screening for ANCA with ELISA or FEIA and confirmation by IIF is a valuable alternative to screening with IIF and confirming with ELISA or FEIA (Vermeersch et al, 2008).

Finally, the current international consensus does not discuss the need for rapid ANCA testing being available. Especially renal and pulmonary manifestations may have a rapidly progressive onset which requires early diagnosis and treatment in order to prevent organ failure or even death of the patient. In these patients it is important to have results available within 24 hours. Because rapidly progressive glomerulonephritis and alveolar haemorrhage have a high pre-test probability for the diagnosis AAV, it may be sufficient to test only with antigen specific assays, i.e., PR3- and MPO-ANCA, and not IIF. Since the Goodpasture syndrome is also within the differential diagnoses of the renal-pulmonary syndrome, it is advised to include additional testing for anti-GBM antibodies. Although nowadays most automates for autoantibody testing have random access and thereby enable rapid testing in the routine setting, there are also several rapid test assays available for laboratories that have not yet automated their ANCA diagnostics (Westman et al, 1997; Rutgers et al, 2004). Interestingly, capture technology has recently been introduced in these rapid tests. Results obtained in the rapid tests are often qualitative only and should eventually be confirmed and quantified by the assays being in use for routine testing.

## 4. Interpretation of ANCA results

According to the international consensus positive ANCA IIF results should be reported by the pattern (C-ANCA, atypical C-ANCA, P-ANCA, atypical ANCA, or not to be determined because of interfering ANA). Results of the antigen specific assays should be reported in arbitrary units in combination with the cut-off value. Assays that are calibrated on the new international standard can be expressed in international units. Preferentially, results of IIF and antigen specific assays should be released simultaneously. It is also advised that results are reported together with information regarding the interpretation. This information may include sensitivity and specificity values. It should be realized, however, that such information is of limited value for a clinician who has to decide on the diagnosis of a single patient.

An alternative way of presenting data, which is more valuable for an individual patient, is by likelihood ratios (Bossuyt, 2009). The likelihood ratio provides an estimation of whether there will be significant change in pre-test to post-test probability of a disease given the test result (American college of rheumatology, 2002). A likelihood ratio of 1 implies that the test, either positive or negative, will add nothing to the diagnosis. The higher the likelihood ratio

(>1) for a positive test result, the higher the probability that the patient will have the respective disease, while the lower the likelihood ratio (<1) for a negative test result, the lower the probability that the patient will have the respective disease. Importantly, likelihood ratios can also be determined for combinations of assays, like IIF and antigen-specific assays (Choi et al, 2001). The likelihood ratios depend on multiple independent parameters. First, the positive likelihood ratio corresponds to sensitivity / (1 – specificity) and the negative likelihood ratio to specificity / (1 – sensitivity). In clinical practice specificity is defined by the clinicians that request for ANCA: when the clinicians do not adhere strictly to the clinical manifestations defined in table 1, this may result in an increase in the number of false positive results and thus a reduced specificity. Similarly, if the final diagnosis of AAV is incorrect, this will affect the sensitivity. Altogether, this implies that the likelihood ratios of a certain test depend on the local settings, i.e., the clinicians involved. Second, the test system used for ANCA detection will influence the likelihood ratios. It is obvious that, as compared to antigen-specific assays, the likelihood ratio's for ANCA detection by IIF are less useful for the diagnosis of AAV, but also between distinct antigen-specific assays there may exist huge differences, especially when the cut-offs are not optimal. Finally, the level of the positive test result is associated with distinct likelihood ratios. The likelihood ratios have been defined as a function of ANCA concentration for different assays and from this study it is concluded that likelihood ratios can be best presented for different test-result intervals (Vermeersch et al, 2009). Since the patient population that requires ANCA testing and test system used for ANCA testing will be different between hospitals, this might imply that the likelihood ratios have to be determined locally based on consecutive, diagnostic samples that are sent to the laboratory for ANCA testing. Obviously, standardization of the clinical manifestations that warrant ANCA testing, together with standardization of the assays, for instance by the introduction of the international standards, will add to the generic use of likelihood ratios.
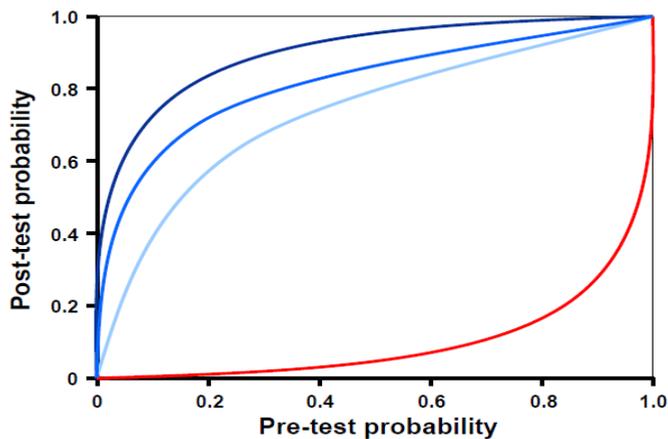


*The red curve represents a negative test-result, the light-blue curve a low-positive test result, the medium-blue curve an intermediate-positive test result, and the dark-blue curve a high-positive test result. This is an imaginary example; the curves depend on multiple parameters, like the assay of choice and the patient population that is tested.*

Fig. 3. The function between pre-test probability and post-test probability representing test-result intervals.

Although in the diagnostic process of AAV likelihood ratios are of increased value, as compared to sensitivity and specificity, they still require transformation of pre-test probability to post-test probability. Likelihood ratios can be used to calculate post-test probabilities by application of Bayes' rule (Bossuyt, 2009). As depicted in figure 3, this will reveal the function between pre-test probability and post-test probability for both a positive, as well as a negative test result. Most optimal would be the report of results in relation to such pre-test versus post-test diagrams, ideally representing test-result intervals.

## 5. Discussion

ANCA are associated with distinct forms of primary small vessel vasculitides, collectively referred to as ANCA-associated vasculitis (AAV) (Jennette & Falk, 1997). ANCA were originally identified by IIF. Importantly, however, only ANCA directed to PR3 (PR3-ANCA) or MPO (MPO-ANCA) are relevant for the diagnosis AAV. Therefore there is international consensus that ANCA should be detected by a combination of IIF and antigen-specific assays, i.e. detection of PR3- and MPO-ANCA (Savige et al, 1999). In recent years multiple new developments have influenced detection and interpretation of ANCA. Several novel technologies have become available for antigen-specific ANCA detection. Most of these technologies apply direct binding of the antigen to a carrier and these developments have enabled automation as well as multiplex testing of antigen-specificities. New generations of ANCA tests are based on indirect binding of the antigen to the carrier, either via a monoclonal antibody (second generation capture technology) or via a peptide linker (third generation anchor technology). Although data on the third generation ANCA tests is still scarce, second generation tests have proven to be superior to traditional ELISA(-like) technologies. These second and, possibly, third generation technologies even challenge the dogma of the need for performing IIF as screening assay. The wish to abandon IIF is primarily instigated by the fact that this technology has several disadvantages. Especially the subjective reading of the slides is a serious drawback of this technique. The advent of microscope devices that have integrated software for pattern recognition might overcome this problem (Hiemann et al, 2009). Also for this development there is insufficient data if this approach is going to work in the near future for ANCA IIF. In the end, decision tree analyses have to give the ultimate answer on the role of IIF in ANCA diagnostics (Vermeersch et al, 2008). Finally, with respect to interpretation of ANCA results it is suggested that the data should be interpreted in the light of likelihood ratios for test result intervals (Bossuyt, 2009). This implies that the likelihood ratio increases with increasing ANCA levels. It is evident that these likelihood ratios are different for distinct assays, but it remains to be determined whether such likelihood ratios can be generalized and are applicable in different centres. Ideally, results are reported together with graphics for pre- versus post-test probabilities because this greatly facilitates interpretation by the clinicians. However, this requires adaptation of most hospital information systems to enable a link to the relevant graphics instead of providing only reference values.

Altogether, the new developments in ANCA diagnostics of the last decade are such that revision of the current international consensus on ANCA is required. Importantly, the

most recent developments, like third generation assays and automated pattern recognition software, need further evaluation to obtain objective data to be included. This might enable the development of evidence based guidelines instead of a consensus based on expert opinion. Issues that should be considered to be included in a novel consensus are summarized in Table 2. Besides the issues dealt with in this chapter, it is also relevant to define other issues, like the role of formalin-fixed neutrophils as a substrate in IIF and the usefulness of testing for other ANCA specificities. Formalin fixation of neutrophils destroys the antigenicity of multiple (irrelevant) antigens, including interfering ANA as well as multiple antigens other than PR3 or MPO, and reveals the shift in staining pattern from P-ANCA on ethanol-fixed neutrophils to C-ANCA on formalin-fixed neutrophils. As mentioned, the biochip technology enables to combine both substrates in a single incubation (Damoiseaux et al, 2009b). The usefulness of formalin-fixation is already a longstanding discussion (Bird, 1999). If eventually the role of IIF is considered to be redundant, the role of formalin-fixed neutrophils will be finished as well. With respect to other antigen-specificities of ANCA, there are at least three interesting candidates. First, anti-human elastase (HLE)-ANCA seems to be specific for midline destructive disease and/or vasculitis (Cohen Tervaert et al, 1993). Importantly, HLE-ANCA is relevant to identify drug-induced vasculitis (Merkel, 2001). In case of necrotizing lesions of the midline, i.e., nasal septum or palatum, detection of HLE-ANCA may discriminate between GPA and cocaine abuse (Trimarchi et al, 2001). However, thus far the assay is not standardized and data are limited. A second antigen of use might be the bactericidal-permeability increasing protein (BPI). BPI-ANCA have been associated with cystic fibrosis, but may also occur in other chronic inflammatory diseases. In terms of AAV detection, BPI-ANCA may be relevant to explain a C-ANCA result by IIF that can not be confirmed by specific assays for PR3-ANCA. In such a situation the likelihood for AAV is strongly decreased. Finally, human LAMP-2 has been described as a valuable ANCA subtype (Kain et al, 2008). In neutrophils, LAMP-2 is integrated into the membranes of MPO- and PR3-containing vesicles, and thus autoantibodies to LAMP-2 give positive results in ANCA IIF. These antibodies are typically associated with pauci-immune focal necrotizing glomerulonephritis and often co-occur with MPO- or PR3-ANCA. Altogether, more data is required before these different ANCA specificities can be included in routine ANCA diagnostics.

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1. Is it possible to screen by antigen-specific assays instead of IIF?
2. Are second (and perhaps third) generation antigen-specific assays mandatory for the diagnosis of ANCA-associated vasculitis?
3. Are antigen-specific assays to be calibrated on the international standard preparations for MPO- and PR3-ANCA?
4. Is there a place for antigen-specific assays other than MPO and PR3?
5. Is there still a role for indirect immunofluorescence (IIF)?
6. Are formalin-fixed (next to ethanol-fixed) neutrophils to be used in IIF?
7. Should rapid-testing be available in every routine laboratory that offers ANCA tests?
8. Is it manageable to present results as likelihood ratios for test result intervals?

Table 2. Issues to be incorporated in a novel international consensus on ANCA diagnostics.

## 6. Conclusion

Laboratory diagnostics of ANCA has evolved in fifty years with respect to assay technology and automation. The number of ANCA requests has increased simultaneously and this inevitably results in an increase in false-positive results. Altogether, these changes ask for a novel international consensus statement as well as a change in the way of reporting results.

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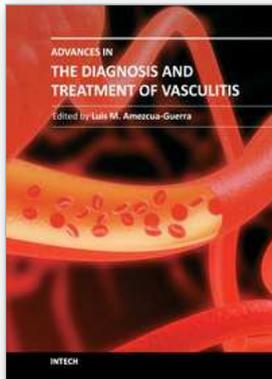
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Edited by Dr. Luis M Amezcua-Guerra

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