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Review

Anti-neutrophil cytoplasmic autoantibodies: Methodological aspects and clinical significance in systemic vasculitis☆

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ABSTRACT

Antineutrophil cytoplasmic antibodies (ANCA) are the serological hallmark of some idiopathic systemic vasculitides, such as granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and, to a lesser extent, Churg–Strauss syndrome (CCS), the so-called ANCA-associated vasculitides (AAV).
ANCA were first detected by immunofluorescence (IIFT), subsequently the target antigens myeloperoxidase (MPO) and proteinase 3 (PR3) were identified, allowing the development of the quantitative, antigen-specific assays. According to the guidelines, combining IIFT and PR3-ANCA/MPO-ANCA assures the optimal diagnostic specificity. Antigen specificity does not effectively differentiate among the different AAV, however C-ANCA/PR3-ANCA are mainly found in GPA, while P-ANCA/MPO-ANCA are more prevalent in MPA and CSS.
Despite their diagnostic value, the performance of the widespread immunometric assays for ANCA testing is disappointing, particularly for the low sensitivity. In recent years, more "sensitive" assays have been developed, using the microplate as well as fully the automated technologies, with promising preliminary results.
ANCA, may be detected in a number of pathological conditions other than small vessel vasculitis. However, in most of these non-vasculitic patients ANCA do not recognize MPO or PR3 as target antigens, but other granulocyte components, often multiple or unknown specificities.
A positive ANCA result by itself is not diagnostic for AAV, clinical evidence and possibly histological confirmation are always required. On the other hand, a negative test result cannot completely rule out a diagnosis of AAV, as AAV without detectable ANCA exist.
The appropriate use of ANCA testing strongly improves the diagnostic accuracy and clinical usefulness of the results.

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Contents

1. Introduction . . . 0
2. Epitope-specific MPO- and PR3-ANCA . . . 0
3. ANCA-testing . . . 0
 3.1. Indirect immunofluorescence technique (IIFT) . . . 0
 3.2. Antigen-specific immunometric assays . . . 0
 3.3. Alternative assays for ANCA testing . . . 0
 3.4. Clinical significance of ANCA . . . 0
 3.5. ANCA testing in monitoring disease activity and predicting relapse . . . 0
4. Other antigenic specificities in AAV . . . 0
5. ANCA in non-vasculitic diseases . . . 0
6. ANCA problems in clinical practice . . . 0
 6.1. Lack of standardization of the current assays . . . 0
 6.2. The use of different diagnostic algorithms . . . 0
 6.3. Appropriateness of the requests . . . 0

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7. Different strategies for ANCA testing . . . 0
8. Conclusions . . . 0
Acknowledgments . . . 0
References . . . 0

1. Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are serological markers of some idiopathic systemic vasculitides, predominantly afflicting small (medium)-sized blood vessels, such as granulomatosis with polyangiitis (GPA, previously called Wegener granulomatosis), microscopic polyangiitis (MPA) with its renal limited form pauci-immune necrotizing crescentic glomerulonephritis (*pi*NCGN) and, to a lesser extent Churg–Strauss syndrome (CSS, proposed to be called eosinophilic granulomatosis with polyangiitis EGPA) [1–4].

The term "ANCA-associated vasculitis" (AAV) is used generically to include these primary vasculitic syndromes, in which circulating ANCA are commonly found. 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 possible pathogenetic potential [1,5,6].

ANCA were first detected by the immunofluorescence technique, using ethanol-fixed leukocytes or human purified neutrophils as cellular substrates [7,8].

The main fluoroscopic staining patterns are the diffuse, granular cytoplasmic (C-ANCA), and the perinuclear (P-ANCA) (Fig. 1a, b); the former largely due to the presence of autoantibodies targeting the serine protease proteinase-3 (PR3-ANCA), while the latter caused by antibodies directed against many antigens, among which the myeloperoxidase (MPO-ANCA) is the most frequent one in AAV [9,10].

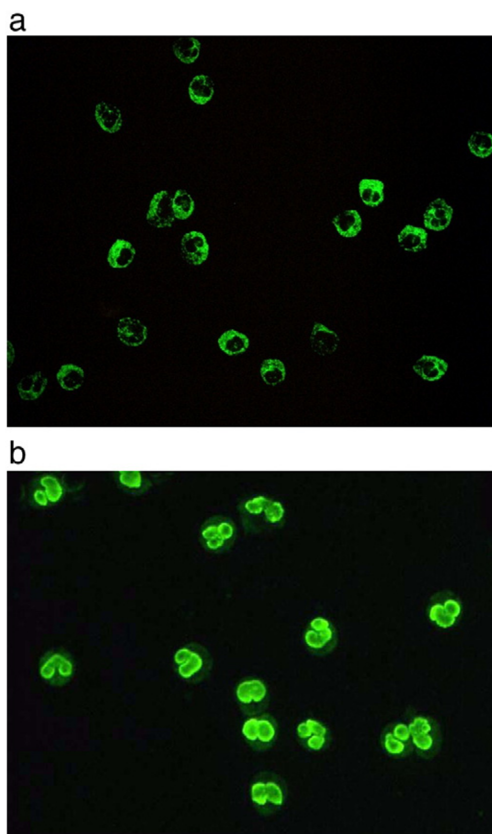

Fig. 1.

Less frequently, a cytoplasmic atypical and an atypical ANCA can be identified by indirect immunofluorescence (IIFT), however their target antigens usually differ from PR3 and MPO, multiple and unidentified specificities are common, and their presence is generally not related to AAV [11]. The target proteins PR3 and MPO [12,13] are localized in the azurophile (primary) granules of polymorphonuclear cells and monocytes, from where they can be exposed on the cellular surface, as well as excreted in the extracellular environment, as a result of various inflammatory stimula.

Ethanol treatment causes solubilization of the granule membranes, thus allowing mobilization 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. The use of the cross-linking fixative formaldehyde prevents the redistribution of the MPO (and other positive-charged proteins) and therefore the perinuclear staining [2].

The discovery and the purification of the antigens allowed the availability of a number of antigen-specific assays, the conventional enzyme-linked immunosorbent assay (ELISA) [14] and, more recently, methods based on different fully automated technologies i.e. fluoro-enzyme-immuno-assay (FEIA), chemiluminescent immuno-assay (CIA), the microsphere-based multiplex technology, image analysis, etc.

In AAV only IgG-ANCA are considered relevant. Even though IgA and IgM-ANCA have been described in patients with Henoch–Schonlein purpura and pulmonary-renal syndrome respectively, those results have not been confirmed [15–18].

The role and clinical significance of the different IgG-ANCA subclasses are still unclear. Increased IgG1 and IgG4, as well as a higher pathogenicity of the IgG3 subclass have been reported in GPA and in the other idiopathic systemic vasculitides, but also an unexpected activity of IgG4 fraction in some patients [19–21]. By the way, these data have not been confirmed afterwards [22].

After the standardization of the methods for ANCA detection and the evaluation of their clinical application, a document produced by an International Consensus of experts was published, with suggestions for the correct ANCA testing and reporting [14,6,23].

Because the specificity of the tests taken individually (C-ANCA, P-ANCA, PR3-ANCA and MPO-ANCA) is not satisfactory enough, the guideline suggests combining IIFT on ethanol-fixed leukocytes (or purified neutrophils) and PR3-ANCA/MPO-ANCA, which allows a 99% specificity versus the pathological controls, with only minor loss of sensitivity.

An Addendum to the International Consensus Statement on Testing and Reporting of ANCA has been published in 2003 with the Quality Control Guidelines, comments, and recommendations for ANCA testing in other autoimmune diseases [24].

The diagnostic work-up of a suspected autoimmune disease includes the assessment of organ involvement and the laboratory investigations in order to explore the systemic involvement and the etiopathogenetic mechanisms. ANCA detection plays a central role when a small vessel systemic vasculitis is suspected [25].

2. Epitope-specific MPO- and PR3-ANCA

ANCA recognizing "pro-forms" and diverse conformational epitopes on the target proteins are documented, and a number of mouse monoclonal antibodies targeting them are available [26–28].

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Because ANCA recognize conformational epitopes, epitope-mapping strategies using linear peptides have failed the identification of the fine specificities. Therefore, recombinant human-murine chimeric MPO and PR3, as well as peptide molecules synthesized after peptide library screening for antigen–antibody interactions, have been developed and used for the mapping of such conformational epitopes [29,30].

Both, MPO and PR3-ANCA, seem to recognize a restricted number of epitopes whose clinical significance is unknown. In particular, MPO-ANCA react against multiple epitopes on the heavy chain [31].

MPO-ANCA and PR3-ANCA are highly specific for the associated small vessel vasculitides, and this suggests that they are implicated in the pathogenesis of such diseases [32]. If ANCA exert pathological effects in AAV, the heterogeneous clinical manifestations and the contradictory results of *in vivo* animal models and laboratory tests might be explained by a different pathogenic potential of epitope-specific ANCA.

Moreover, epitope-specific ANCA subsets, some better associated with disease activity than others, may partially explain controversial results between clinical activity and laboratory findings during patient monitoring. Finally, ANCA subpopulations may change during the course of the disease, at least in some patients.

However, the currently available routine methods for ANCA detection do not distinguish these different ANCA subsets, and their clinical and functional significance still needs to be assessed [33].

3. ANCA-testing

3.1. Indirect immunofluorescence technique (IIFT)

According to the guidelines, the immunofluorescence technique using normal human ethanol-fixed neutrophils as cellular substrate is frequently performed as a first step of ANCA detection, followed by the confirmation of any positive or doubt sample, irrespective of the staining pattern, by the PR3 and MPO-specific immunometric assays.

Since a negative IIFT result does not completely rule out the diagnosis of small vessel vasculitis, also the negative samples should be tested for PR3 and MPO-ANCA if such disease is strongly suspected.

Because a P-ANCA (or a P-ANCA like) pattern may be caused by different autoantibodies, IIFT on formalin-fixed cells has been suggested to help distinguish between P-ANCA/MPO-ANCA and similar fluoroscopic stainings due to the presence of antinuclear antibodies (ANA) [2,34].

However, the usefulness of the latter test is controversial, and its inclusion in the algorithm for ANCA testing in AAV has not been largely adopted [35].

As well known, the sensitivity of the IIFT is high while the specificity is low due to the presence of P-ANCA not directed against MPO, for example in inflammatory bowel diseases (IBD), and to the interference of anti-nuclear antibodies [1,6,24,36,37].

3.2. Antigen-specific immunometric assays

The characterization and purification of the relevant target molecules of ANCA, PR3 and MPO, allowed the quantitative and antigen-specific detection of such autoantibodies to become easy to interpret and widely diffused. However, the purity of the antigens, as well as the presence and accessibility of all relevant epitopes, are required in order to get the expected high specificity and sensitivity.

Although the clinical decisions also depend on ANCA testing results, the basic standards are not always present in the largely diffused assays, as clearly demonstrated by Csernok and collaborators in repeated comparative evaluations of commercially available kits for PR3 and MPO-ANCA detection [38,39].

The classical and widespread immunometric systems are based on the direct coating of the purified antigens onto the solid-phase. As a result of the direct binding of PR3 and MPO to the support, conformational changes of the molecules can occur, as well as the loss and/or the hiding of some relevant epitopes, and this fact may be responsible, at least in part, for the low sensitivity and the wide variability of the results between different assays from different manufacturers.

Recently, newly designed assays for detecting and quantifying PR3 (MPO)-ANCA have been described, namely capture-ELISA and anchor(sensitive)-ELISA. The former (2nd generation) uses, as a capturing ligand, a mouse monoclonal antibody directed towards an epitope on the specific antigen rarely targeted by human ANCAs (56), while the latter (3rd generation) uses different little molecules to anchor the antigens to the solid-phase. In both cases the coating strategy has changed from direct to indirect binding through “bridging” spacers, potentially assuring the optimal exposition of the antigenic proteins and of their most relevant epitopes, with an expected increase in sensitivity for AAV [40,41].

A large number of studies have been realized 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 [42,43].

On the contrary, only few data are available on the performance of the MPO-ANCA detection with “sensitive” methods, as they have been proposed lately.

The 3rd generation assays for ANCA testing have also recently been applied on fully automated systems such as FEIA and CLIA.

Recently, the diagnostic effectiveness of the EliA MPO^S in detecting AAV patients has been retrospectively evaluated, in comparison with some of the most performant commercial kits (EliA-MPO Phadia, anti-MPO Orgentec, and Wieslab captureMPO-ANCA Eurodiagnostica) on a large cohort of AAV patients (MPA 52, EGPA 20), pathological (123: SLE, UCTD, MCTD, SSc, AR, IBD, cryoglobulinemia, other vasculitis, infectious) and normal controls (20). The EliA MPO^S exhibited a better sensitivity, while maintaining a high specificity, compared to the direct assay by the same manufacturer (employing the same purified antigen), and similar to those of the best competitors (A. Radice et al. 14th International Vasculitis & ANCA workshop Chapel Hill, USA — May, 2011 — [11]).

It is worth outlining that direct comparison of the absolute data from different published series is never possible, because of the different selection criteria for AAV patients and controls.

Finally, the first commercially available test for PR3-ANCA detection, 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, so as to be recognized by most autoantibodies in the patients' sera [44,45].

Because of the encouraging results of the preliminary tests, large and prospective studies are warranted to confirm the performance of this innovative format.

We recently compared the diagnostic performances of nine different 1st, 2nd and 3rd generation PR3-specific ANCA assays in detecting GPA patients (Fig. 2). Our results (not published yet), while confirming the relevance of a better exposition and accessibility of the antigen epitopes, as it may be achieved using an indirect coating strategy, also show that it may be not sufficient to assure the optimal sensitivity.

Bead-based flow cytometry assays, capable of measuring analytes simultaneously (multiplex technology), are available for ANCA testing [46]. In our opinion, the application of such powerful technology for ANCA testing or differential diagnosis of patients with rapidly progressive glomerulonephritis, where only MPO, PR3 and anti-glomerular basement membrane (GBM) antibodies detection is relevant, is questionable.

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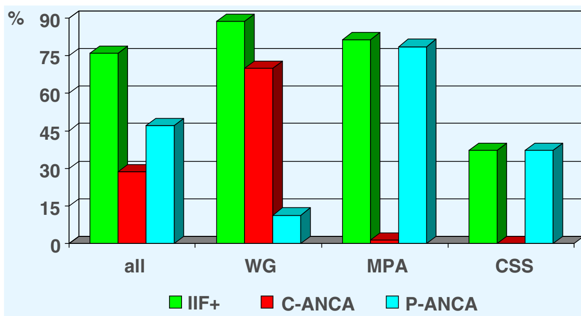

Q3 **Fig. 2.**

3.3. Alternative assays for ANCA testing

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. Instead, the ANCA biochip (Europlus™ Granulocyte mosaic) is based on a different principle, where the presence of cells (ethanol- and formalin-fixed) and antigen-specific spots enables the detection of the fluoroscopic pattern and the confirmation of antigenic specificity.

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.

Nevertheless, being aware of all these limitations, there are a few situations where their use could be considered: i) in small routine series, where ANCA testing often relies on the IIFT (or ELISAs) alone, ii) in "true" urgent ANCA requests.

By the way, we do not suggest the use of the biochips in the first setting, not only due to the costs, but also because the diagnosis and management of AAV patients needs laboratory and clinical expertise.

Instead, in selected cases, i.e. in patients strongly suspected for having a small vessel systemic vasculitis, with severe renal and/or pulmonary involvement, having ANCA results available in a short time could be really valuable for the specialist, helping in the exclusion or confirmation of the diagnosis.

In this critical clinical setting, the availability of an alternative method, in particular the "biochip" that allows to perform simultaneously, on the same support (slide), all tests recommended for the optimal ANCA testing, enables more confidence when interpreting and communicating the results.

Preliminarily, we evaluated the ANCA results on a large cohort of AAV patients and controls, achieved by using the biochips or the standard techniques. Briefly, the agreement for AAV diagnosis was 81.3–97.2% (depending on inclusion/exclusion of Churg–Strauss syndrome patients), while the agreement for IIFT and antigenic specificity (MPO, PR3) was about 90%.

Afterwards, due to satisfying results, we decided to perform the ANCA-biochip on consecutive patients with life-threatening disease, where ANCA testing immediately was mandatory. In this clinical setting (patients with high pre-test probability of having a small vessel vasculitis) we correctly classified 12 out of 13 patients (AAV vs. non-AAV), confirming its clinical utility in such critical situations. In the last case, a "true" positive analytical result (concordance between biochip and standard techniques) was found in a patient strongly suspected, but confirmed, having AAV.

3.4. Clinical significance of ANCA

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 [6,47–50].

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. P-ANCA, in disease controls, can be also caused by the presence of anti-nuclear antibodies, especially in systemic lupus erythematosus patients. C-ANCA have been reported in infectious diseases and are frequently found in cystic fibrosis (Table 1).

The use of immunometric assays with purified antigens improves the specificity up to 98% or more, with a slight loss in sensitivity [6,47–49].

Mc Laren et al. examined the role of routine ANCA testing in achieving a diagnosis of AASV in a clinical setting in five UK hospitals [51]. In all departments apart from Rheumatology, the majority of patients (88–100%) with a positive ANCA by IIF did not have confirmed AAV. This wide variation in diagnostic yield among departments was likely based on differences in clinical practice and experience. Only

Table 1
Immunofluorescence pattern, antigen specificity and clinical associations.

IIFT pattern	Target antigen	Associated disease
C-ANCA	PR3	GPA (40–90%)
		MPA (15–20%)
		*pi*NCGN (10%)
		CSS (<10%)
		Infectious diseases (SBE)
C-ANCA Atypical	BPI	Cystic fibrosis (90%)
	BPI, MPO, catepsin G, …, often multiple specificities	IBD, PSC, RA
P-ANCA	MPO	MPA (65–90%),
		*pi*NCGN (70–90%)
	Multiple specificities including: elastase, catepsin G, lysozyme, BPI…(primary granules)	CSS (30–40%)
		IBD, AIH, RA, CTDs (SLE, SSc)
		DIV/DIL (also anti-MPO+)
		Parasitic infestation
	Lactoferrin, lysozyme, …(secondary granules) α-enolasi, actin,…(cytoplasm)	
	Catalase (peroxisomes)	
	HMG1/2, heterochromatin and nuclear membrane-associated proteins (nucleus)	
Atypical ANCA	MPO	CSS
	Multiple specificities (see above)	DIL/DIV, IBD, RA
	Elastase (anti-PR3 positive in about 30–40%)	Cocaine abuse

Abbreviations: PR3: proteinase 3; BPI: Bactericidal/permeability-increasing protein; MPO: myeloperoxidase; HMG1/2: high mobility group protein 1–2; GPA: granulomatosis with polyangiitis; MPA: microscopic polyangiitis; *pi*NCGN: pauciimmune-necrotizing crescentic glomerulonephritis; CSS: Churg–Strauss syndrome; SBE: subacute bacterial endocarditis; IBD: inflammatory bowel disease; PSC: primary sclerosing cholangitis; RA: rheumatoid arthritis; AIH: autoimmune hepatitis; CTDs: connective tissue diseases; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; DIV: Drug-induced vasculitis; DIL: Lupus-like syndrome.

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Table 2
Clinical indications for ANCA testing (Modified from [23]).

○ Glomerulonephritis, especially rapidly progressive glomerulonephritis
○ Pulmonary hemorrhage, especially pulmonary-renal syndrome
○ Cutaneous vasculitis, especially with systemic features
○ Multiple lung nodules
○ Chronic destructive disease of the upper airways
○ Long-standing sinusitis or otitis
○ Subglottic tracheal stenosis
○ Mononeuritis multiplex or peripheral neuropathy
○ Retro-orbital mass
Other possible indications for ANCA testing:
○ Pulmonary fibrosis, with systemic features
○ Episcleritis, uveitis, retinal vasculitis, with systemic features

The presence of any of these features in the absence of another obvious cause indicates that ANCA testing is warranted.

the Rheumatology department had significant sensitivity, specificity, PPV and NPV of ANCA by the combined IIF/ELISA test for AASV (68%, 75%, 79%, and 63% respectively). These data strongly suggest that ANCA testing should be used in selected cases, where AAV is highly suspected, with typical features as determined by initial clinical assessment (Table 2).

ANCA testing in patients with signs and symptoms suggestive for AAV is most useful to confirm the suspected diagnosis, whereas ANCA testing in patients with weak clinical evidence for AAV is most useful to rule out the diagnosis.

Antigen specificity (PR3 or MPO) does not effectively differentiate among the different AAV, however C-ANCA/PR3-ANCA are mainly found in GPA, while P-ANCA/MPO-ANCA are more prevalent in MPA, *pi*NCGN and CSS. ANCA are detected in 70–90% of active, generalized GPA, but only in about 40–50% of the loco-regional forms [27].

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-hemorrhagic lung involvement [3].

Such reports suggested the hypothesis of the existence of two disease subsets with different clinical manifestations and, possibly, pathogenetic mechanisms [52].

Fig. 3 shows the ANCA prevalence in AAV patients diagnosed and followed by the Authors' Institution.

3.5. ANCA testing in monitoring disease activity and predicting relapse

Whereas the diagnostic value of ANCA in AAV has been widely recognized [6,47–49,53], the role of ANCA in monitoring disease activity in patients with vasculitis is still controversial.

Boomsma et al. found that serial measurement of ANCA levels, as detected by ELISA or immunofluorescence, was valuable for early prediction of relapses in patients with GPA (22, while others found that changes in serial C-ANCA titers temporally correlated with changes in disease activity in only 24% of patients [50,54].

More recently, the predictive value for ANCA testing was evaluated in 55 patients with GPA: although relapses were more frequent when ANCA remained positive or reappeared, discordance between ANCA and disease activity was not unusual [55]. But, in a retrospective study published in 2003, the conclusions were completely different, and the authors stated that serial measurement of PR3 and MPO-ANCA titers in patients with AAV during remission can help predict relapses, and pre-emptive increases in immunosuppression following fourfold titer rises reduced the risk of relapses [56]. Sinico et al., using the fully automated enzyme fluoroimmunoassay (EliA™) for PR3 and MPO-ANCA testing, found a good discrimination between the active and inactive disease in PR3-ANCA positive but not in MPO-ANCA positive vasculitis, when patients were arbitrarily subdivided into two groups according to the Birmingham vasculitis activity score (BVAS ≤ 4 or > 4) [57]. In most of the 16 sequentially studied patients a rather good association between titer fluctuations and BVAS score was seen, however a few exceptions were present, with increasing ANCA level without change in disease activity.

In a recent evaluation, the BVAS score in the GPA group correlated with PR3-ANCA titers detected by a novel chemiluminescence immunoassay, but not by ELISA [58]. In the same study no correlation was found between the BVAS score in the MPA patients and the MPO results.

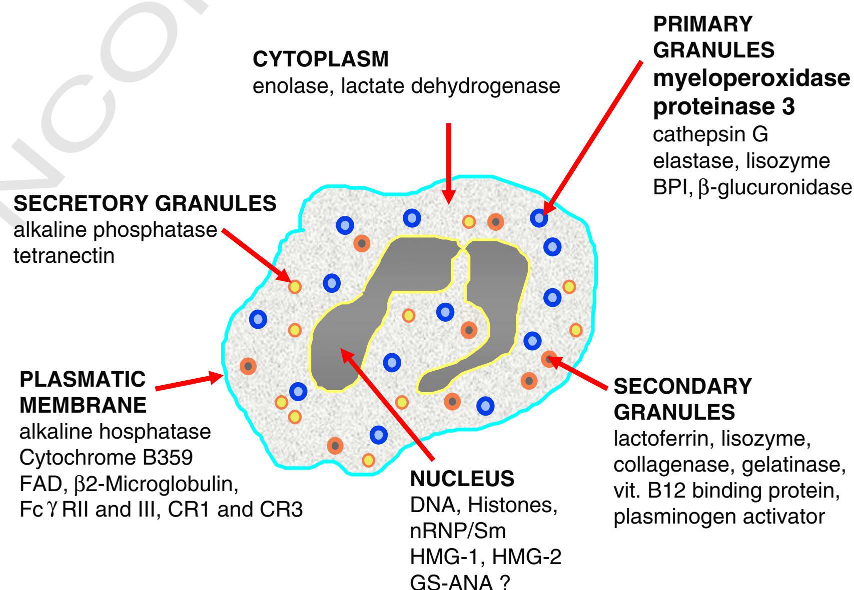

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Fig. 3.

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It seems to be reasonable, until unequivocal data will be available, to consider increasing of ANCA titer alone, in the absence of clinical manifestations, as a warning signal suggesting a more strict observation, but not a tool for treatment decisions.

4. Other antigenic specificities in AAV

Besides MPO and PR3, all other nuclear, cytosolic, and granular proteins could potentially be targeted by autoantibodies (Table 1, Fig. 3). So far, the clinical and diagnostic significance of ANCA recognizing "minor antigens" is unclear; so looking for other antigen specificities for routine purposes is not recommended. ANCA "profile" testing increases the costs without adding any useful information. Finally, reporting ANCA positivity for these specificities may be considered clinically relevant, which is not true, causing dangerous interpretation.

5. ANCA in non-vasculitic diseases

ANCA, especially P-ANCA may be detected by IIFT in a number of pathological conditions other than small vessel vasculitis. In most of these non-vasculitic patients ANCA do not recognize MPO or PR3 as target antigens, but other granulocyte components, often multiple or unknown specificities (Table 1).

P-ANCA positive/MPO-ANCA negative are frequently detected in sera from patients with ulcerative colitis (UC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH), connective tissue diseases (CTDs) and rheumatoid arthritis (RA).

Neutrophil-specific autoantibodies (also called NSA) in inflammatory bowel diseases and hepatobiliary disorder, which cause a P-ANCA-like pattern, can be hardly distinguished from ANCA found in AAV by the IIFT alone. Because most of the autoantigens targeted by neutrophil-specific autoantibodies in IBD are constituents of neutrophil nuclei, such as the heterochromatin and nuclear membrane, these antibodies should be better classified as antinuclear antibodies [59].

C-ANCA/PR3-ANCA negative are present in a large majority of patients suffering from cystic fibrosis, mostly targeting the bactericidal/permeability increasing protein (BPI) [60].

Atypical-ANCA, at times PR3 positive, are present in sera from cocaine abusers, where the relevant ANCA antigen is the serine protease elastase; the extensive homology of elastase with PR3 may cause cross-reactivity [61].

In addition, some infectious agents are able to induce ANCA production. Recently, a multicentric study reported several novel associations between GPA and past bacterial, viral and parasitic infections [62].

ANCA have been anecdotally reported in many infectious diseases by using the IIFT alone, or by positive specific ELISAs lacking of a suggestive fluoroscopic confirmation, but also detected combining IIFT and antigen-specific immunometric techniques in invasive amebiasis, acute streptococcal glomerulonephritis, tuberculosis, leprosys, acquired immune deficiency, etc. C/ANCA/PR3-ANCA are often described in patients with subacute bacterial endocarditis (SBE), a disease with clinical manifestations mimicking small vessel vasculitides, especially at presentation [63].

In a recent Italian multicenter survey organized by the "Forum Interdisciplinare per la ricerca nelle Malattie Autoimmuni" (FIRMA group, the Italian branch of European Autoimmune Standardization Initiative project) involving 25 reference centers, a sample from SBE was reported IIFT positive/doubt in 54.5% (75% C-ANCA, 25% P-ANCA) and PR3-ANCA positive in 80% of the participant labs, by using commercial assays of different brands.

ANCA may be induced by drugs such as penicillin, aminopenicillins, sulfonamides, allopurinol, thiazides, quinolones, hydantoins, propylthiouracil and hydralazine. The autoantibodies appearance can be the solely autoimmune manifestation or can be associated with the clinical picture of vasculitic damage [64,65]. P-ANCA/MPO-ANCA may be caused by antithyroid medications in genetically predisposed individuals; because the mere withdrawal of the offending drug is usually sufficient to attain complete remission of clinical symptoms (and ANCA disappearing), recognizing this condition is very important to avoid inappropriate treatment [66].

As a consequence, diagnosis of ANCA-associated vasculitis, is far from being sorted out by a positive ANCA testing. The differential diagnosis of AAV is a complex process that needs careful investigation and exclusion of the numerous pathological conditions mimicking vasculitides, or able to cause secondary forms of vasculitis, with a particular attention to infectious diseases that are common in the general population [67].

6. ANCA problems in clinical practice

Many problems with the extensive use of ANCA testing in the daily clinical practice still need to be addressed.

6.1. Lack of standardization of the current assays

Years ago, repeated surveys clearly demonstrated that the performances of the widely utilized direct assay for MPO and PR3-ANCA detection were not satisfying, with poor reliability in terms of sensitivity, specificity and diagnostic accuracy. Because ANCA recognize conformational epitopes on the target molecules, the loss of some antigenic epitopes, as well as their masking due to the direct coating of antigens to the solid phase, has been advocated to explain, at least in part, the discrepancies.

Lately, new methods have been developed in order to partially overcome these pitfalls and improve the performance of the traditional ANCA assays.

In the 2nd (capture) and 3rd (anchor, sensitive) generation assays the coating strategy has changed from direct to indirect, by means of "bridging" molecules: mouse monoclonal antibodies directed towards MPO/PR3 epitopes (rarely recognized by ANCA in vasculitis patients) in the "capture" or different little spacers molecules in the "anchor/sensitive" tests.

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 2nd and 3rd 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 generalized. The sensitivity of ANCA in AAV affected patients, detected by using the best performant kits and considering a specificity $\geq$95%, ranges between 70 and 90%, but the prevalence is lower (40–60%) in case of limited GPA.

The recent availability of MPO and PR3 reference sera should help in the standardization efforts. As far as we know, all except two commercial tests use different binding units, and antibody levels for individual serum samples varied significantly. However, quantitative PR3-ANCA measured by using the cPR3 Wieslab and the EliA PR3^S, whose results were expressed in International Units referred to the CDC PR3-ANCA Human Reference Serum $\neq$ 16, varied to the same degree (personal experience). Then, 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 standardization. It is important being wise to that and understand how it is difficult for clinicians to monitor response to treatment and to predict relapse, when consecutive serum samples are tested in laboratories using different assays.

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6.2. The use of different diagnostic algorithms

Even with these methodological improvements, the problem of inconsistent results still remains, since not all routine laboratories follow consensus recommendations.

A recent surveys on 25 laboratories joining the FIRMA group showed that only 14/25 (56%) and 6/25 (24%) of them followed the recommendations for the optimal ANCA testing, performing IIFT and MPO-ANCA/PR3-ANCA on all samples or on the IIFT positive ones, respectively. Two centers relied on the IIFT, and three on the MPO/PR3-ANCA assays alone (5/25, 20%). IIFT on formalin fixed cells was done on all samples in 6 laboratories (data not shown).

6.3. Appropriateness of the requests

The widespread use of ANCA screening in the recent past, as the detection methods became available for most laboratories, made the occurrence of false positive results an emergent question.

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).

The laboratory contribution consists in the confirming or excluding the diagnostic hypothesis, by using the best assays and technologies available. On this topic it is worth keeping in mind that the predictive values of any laboratory test depend not only on the sensitivity and specificity of the given test, but also on the prevalence of the disease in the studied population. It is worth underlining that 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. 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 [68,69]. 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 [70].

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 which makes ANCA detection more clinically relevant.

7. Different strategies for ANCA testing

The most common reason for ordering an ANCA test is to diagnose or rule out GPA, MPA (also in its renal limited form) and CSS, and to monitor immunological activity in these diseases.

Table 2 shows the clinical manifestations that suggest a diagnosis of AASV.

In other conditions (e.g. IBD, PSC, AIH-1, Felty syndrome, scleroderma, SLE…) ANCA determination has sometimes been suggested to help in diagnosis, or to give some additional information.

Ulcerative colitis and Crohn's disease (CD) are diagnosed and differentiated more readily on clinical, radiological, endoscopic and histological criteria, but 10–15% of patients have features that initially do not fit either diagnosis. ANCA testing by IIFT in combination with anti-*Saccharomyces cerevisiae* antibody (ASCA) testing by ELISA may help distinguishing the two. Patients with UC are more likely to have P-ANCA positive but ASCA negative results, and those with CD are more likely to have P-ANCA negative but ASCA positive results. However, ANCA in IBD does not seem to correlate with disease activity, extent, treatment, or any other feature.

According to the international guidelines, Fig. 4a shows the suggested screening algorithm for ANCA detection.

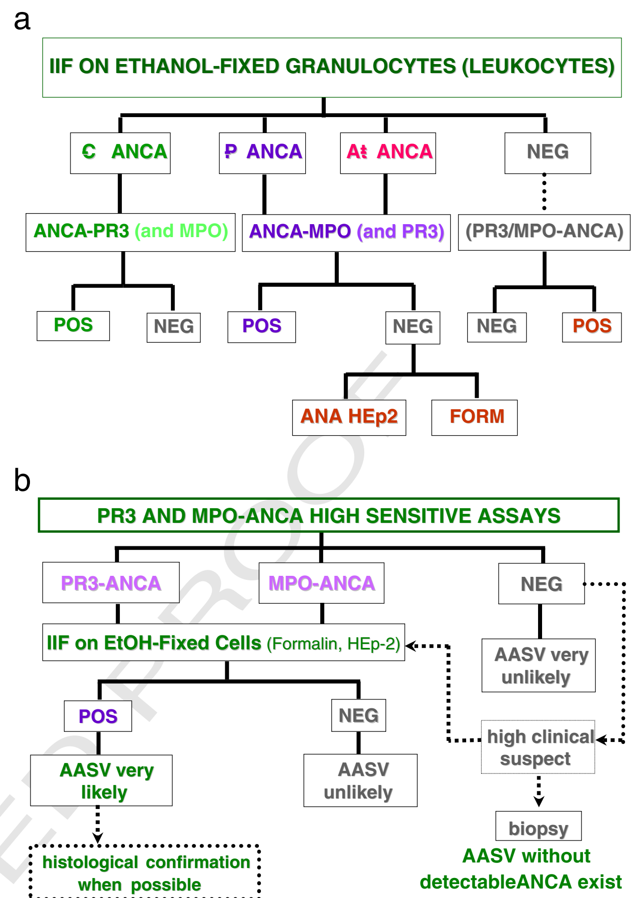

Fig. 4.

Because of the recent availability of novel high-sensitive assays for MPO and PR3-ANCA testing, whose preliminary performances seem very good, an alternative ANCA multitesting algorithm in suspected AAV has been suggested, with both the immunometric PR3 and MPO-ANCA assays used as first step (Fig. 4b).

Clearly, a screening algorithm using first the MPO and PR3-specific assays will exclude to detect ANCA in nonvasculitic conditions (as they are mostly seen by IIFT). This choice may determine an increase in direct costs (two tests instead of one), but probably can reduce indirect expenses because immunometric assays are easily automated.

8. Conclusions

- ANCA are a very sensitive and specific marker for some idiopathic necrotizing vasculitides (GPA, MPA and CSS), the so-called AAV.
- The best diagnostic performance is achieved combining indirect IF and PR3- and MPO-specific assays.
- 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 should not be used, by themselves, to guide treatment.

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