

EliA™ JOURNAL



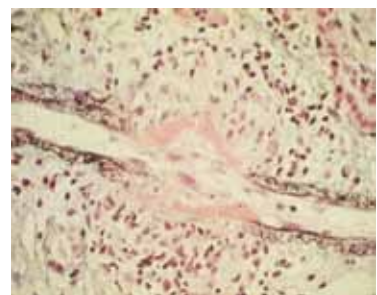
■ *Review*

ANCA and associated diseases



■ *The Assays*

EliA® MPO^S and EliA® PR3^S



■ *Experience*

ANCA testing by high sensitive methods

Editorial



Some months ago a Swiss colleague sent me a publication on ANCA and associated diseases which was printed in the Swiss Medical Forum, written by well-known rheumatologists from Switzerland such as Alan Tyndall, Thomas Vogt and Thomas Daikeler.

I was very pleased as the publication reviewed the topic of ANCA and ANCA-associated vasculitis in a very clear and comprehensive way, and it is a pleasure to be able to make this review available for you in this EliA Journal (page 3).

Specialists worldwide recommend the development of updated criteria and re-evaluation of current disease definitions of ANCA-associated vasculitides, as the criteria from 1990 are incomplete and misleading. One major point which has to be changed is the implementation of ANCA into the criteria, but which method or algorithm should be recommended? In an International Consensus Statement in 1999 it was agreed that indirect immunofluorescence (IIF) using ethanol-fixed neutrophils should be the basis for detection of ANCA. Accordingly, many clinical laboratories screen for ANCA in patients suspected for vasculitis by IIF and confirm positive results with ELISA. However, ELISA can be easily automated and, therefore, is an attractive alternative to IIF as a screening method. Vermeersch et al. (Clin Chim Acta 2008; 397:77-81) showed in their comparative study that screening with EliA and confirming with IIF has an expected clinical utility that is comparable to screening with IIF and confirming with EliA. Moreover, using only EliA has an expected clinical utility that almost equals that of screening with IIF and confirming with ELISA. The highest expected clinical utility was found when both IIF and ELISA were performed on all samples, thereby confirming previous studies. The lowest utility was found when only IIF was performed.

With the development of highly sensitive tests, such as the new EliA PR3^S or EliA MPO^S, the clinical utility is even more increased and, indeed, more and more studies call the current role of IIF as gold standard into question. Please find some information on the high sensitive EliA PR3^S and EliA MPO^S on page 6 and a comparative study of these assays with IIF in patients with ANCA-associated diseases on page 7 of this EliA Journal.

Enjoy reading!



3 Review

ANCA and associated diseases

6 The Assays

EliA[®] MPO^S and EliA[®] PR3^S –
Cutting edge automated ANCA testing

7 Experience

ANCA testing by high sensitive methods in
ANCA-associated systemic vasculitis (AASV)
diagnosis

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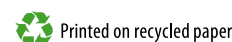
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Editor: Nina Olschowka

Contributors: Thomas Daikeler, Stephan Regenass,
Ingmar Heijnen, Alan Tyndall,
Thomas Vogt
Nina Olschowka
Antonella Radice, Laura Bianchi,
C. Farina, C. Zehnder,
Renato Alberto Sinico

Layout: Melanie Tritschler, Tom Bernhard

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ANCA and associated diseases

Thomas Daikeler^a, Stephan Regenass^b, Ingmar Heijnen^c, Alan Tyndall^a,
Thomas Vogt^a

^a Department of Rheumatology, University Hospital and Felix Platter-Spital, Basel, Switzerland

^b Diagnostik AKI, Department of Immunology, University Hospital, Zurich, Switzerland

^c Medical Immunology, Laboratory Medicine, University Hospital, Basel, Switzerland

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Key messages

- ANCA testing with immunofluorescence microscopy yields characteristic staining patterns (C-ANCA, P-ANCA, ANCA with atypical pattern).
- The best-documented target antigens for ANCA are myeloperoxidase (MPO, target antigen of MPO-ANCA) and serine proteinase 3 (PR3, target antigen of PR3-ANCA). These can be measured specifically using enzyme immunoassays (EIA).
- The group of disorders known as ANCA-associated vasculitides (AAV) includes Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS). The ANCA involved in this association are PR3- and MPO-ANCA. Immunofluorescence has also revealed the presence of ANCA in a number of non-vasculitic diseases. In these cases the antibodies concerned are usually others and not PR3- or MPO-ANCA.
- ANCA testing should involve both immunofluorescence and EIA (to determine the MPO and PR3 specificity). Alternatively EIA can be performed as the primary procedure with specific testing for PR3- and MPO-ANCA. A negative ANCA test does not exclude vasculitis. A precise diagnosis always includes a histological examination of suitable tissue as well.

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) constitute a heterogeneous group of antibodies. They were first described in 1982. Subsequently, an association was revealed between ANCA and a group of vasculitides affecting small blood vessels. At first, immunofluorescence microscopy was used to detect ANCA. In ethanol-fixed neutrophil granulocytes the target antigens of ANCA are located mainly in the granules, and also partly in the cytosol. As a consequence, a characteristic pattern can be observed in the cytoplasm of these cells when antibodies are marked. The result of this method represents a merely visual description of the observed fluorescence pattern. Three major patterns can be distinguished: ANCA with the classic cytoplasmic granular fluorescence pattern (C-ANCA), ANCA with perinuclear fluorescence (P-ANCA), and ANCA with an atypical pattern.

Target antigens of the different types of ANCA were characterized at a later stage. ANCA targeting these antigens can nowadays be measured specifically by using enzyme immunoassays (e.g., ELISA, immunoblot). The best-documented ANCA target antigens are myeloperoxidase (MPO, target antigen of MPO-ANCA) and serine-proteinase 3 (PR3, target antigen of PR3-ANCA).

The different ANCA specificities identified by ELISA are associated with particular patterns of immunofluorescence. PR3-ANCA usually generate a C-ANCA pattern whereas MPO-ANCA usually result in a P-ANCA pattern. Besides MPO- and PR3-ANCA, other autoantibodies exist that may also generate an ANCA fluorescence pattern, most frequently a P-ANCA. In conjunction with clinical symptoms and other laboratory results, the detection of ANCA allows meaningful conclusions about the presence of diseases (Fig. 1).

Significance of ANCA

According to the ANCA cytokine sequence theory, ANCA (either PR3- or MPO-ANCA) can bind to granulocytes that have been activated, for example by infection. This triggers the release of lysosomal enzymes in small blood vessels which then lead to necrotizing changes in the vessels (Fig. 2). This theory is supported by clinical and experimental evidence.

ANCA targeting antigens other than PR3 and MPO have been reported to occur in various diseases, but the underlying mechanisms in these cases are less well clarified.

ANCA-associated vasculitides

ANCA play a major role in the differential diagnosis of vasculitides. Because of their association with ANCA (especially PR3- and MPO-ANCA), Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS) are collectively known as ANCA-associated vasculitides (AAV). Considering the size of the affected blood vessels, AAV are classified as small-vessel vasculitides. A typical characteristic of AAV is the general absence of detectable immune complexes in the inflamed blood vessels, hence the name "pauci-immune" vasculitis. The classification criteria that are currently still in use (*American College of Rheumatology* 1990, *Chapel Hill* 1994) do not consider ANCA, albeit ANCA have now become established in clinical practice. New criteria for classification and diagnosis are currently being developed by an EULAR/ACR committee and it can be expected that these will include ANCA.

Clinical features of ANCA-associated vasculitides

The underlying mechanism in AAV is an inflammatory reaction that primarily affects the small vessels. It is possible that not just several, but also single organs are affected by the disease, depending on the extent of vascular involvement. The symptoms of the vasculitides depend on the organ system affected, and may therefore be uncharacteristic. Symptoms such as fatigue, fever and weight loss may dominate. Vasculitic skin changes frequently occur, such as palpable purpura, ulcerations and leucocytoclastic vasculitis. All AAV can cause pulmonary-renal syndrome. AAV should always be considered a possibility in patients with systemic inflammatory disorders lacking a definite focus. ANCA can aid in the differential diagnosis in these cases. Laboratory testing usually

shows elevated parameters of inflammation (e.g., Erythrocyte sedimentation rate and CRP). A blood count can reveal both anemia and reactive thrombocytosis. The following sections address the individual disease entities and their association with specific types of ANCA.

Wegener's granulomatosis

Wegener's granulomatosis is a granulomatous inflammation of small- and, to some extent, medium-sized blood vessels. The disease usually presents with symptoms involving the upper airways, lungs and kid-

neys, but other organs can also be affected. The annual incidence is approximately ten new cases per million population, and the prevalence estimate is one hundred cases per million, although marked regional differences have been reported. In the case of localized disease, Wegener's granulomatosis can initially present as chronic sinusitis and should, thus, also be considered in cases with bloody nasal discharge mixed with crusts. In more disseminated disease, a wide range of pulmonary symptoms can occur. In this context, Wegener's granulomatosis can mimic tuberculosis on

imaging, and vice versa. Therefore, if examination of the lungs gives no clear result, Wegener's should be considered as a possible diagnosis. Over 70% of patients with Wegener's granulomatosis have kidney involvement in the form of rapidly progressing glomerulonephritis. This can lead to irreversible loss of kidney function. Mononeuritis, with attendant paralysis and sensory disturbances, can result from involvement of the blood vessels supplying peripheral nerves. Inflammation of the eye, in most cases episcleritis, is also not unusual and may threaten visual acuity.

Histologically, Wegener's granulomatosis is characterized by granulomatous inflammation with evidence of giant cells and necrotizing vasculitis of the small arterial vessels.

On examination with immunofluorescence microscopy, C-ANCA are found in 90% of patients with Wegener's granulomatosis, but P-ANCA are occasionally present. The target antigen is PR3 in 80-95% of cases and MPO in a smaller proportion. In the case of limited disease (e.g., only affecting the upper airways or kidneys), ANCA may be negative.

If ANCA are measured by using ELISA, changes in the concentration of PR3- or MPO-ANCA may be identified. In Wegener's granulomatosis these results may provide an indication of the disease activity and the risk of relapse. However, ANCA are not the only factor involved in causing disease symptoms. In individual patients a rise in an ANCA level can indicate a recurrence of vasculitis, but this observation cannot be generalized. The treatment strategy therefore still depends on the clinical picture.

Microscopic polyangiitis

Microscopic polyangiitis (MPA) used to be considered a particular form of polyarteritis nodosa. It is characterized by the development of rapidly progressing glomerulonephritis and pulmonary hemorrhage. Clinically it also includes general symptoms and skin involvement in the form of palpable purpura.

Inflammation of the small arterial vessels underlies AAV

With immunofluorescence, ANCA (usually P-ANCA) are found in 70% of patients. ELISA shows this to consist almost exclusively of MPO-ANCA. When PR3-ANCA are present the disease is difficult to distinguish from Wegener's granulomatosis.


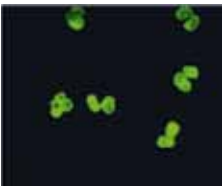
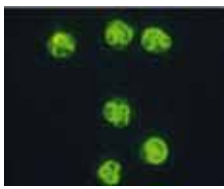
Occurrence	AAV (ANCA-associated vasculitides)		Other disorders
Immuno-fluorescence pattern (ethanol-fixed granulocytes)	C-ANCA 	P-ANCA 	P-ANCA or atypical 
Most frequent antigen (ELISA)	PR3	MPO	Various, many unknown
Described in	Wegener's granulomatosis (usually PR3-ANCA) microscopic polyangiitis (usually MPO-ANCA) Churg-Strauss syndrome (usually MPO-ANCA)		Rheumatoid arthritis, chronic inflammatory bowel disease (ulcerative colitis>Crohn's disease), autoimmune hepatitis, cystic fibrosis and others.

Figure 1 ANCA patterns in immunofluorescence microscopy: ANCA with the classic cytoplasmic granular fluorescence pattern (C-ANCA), ANCA with perinuclear fluorescence (P-ANCA) and ANCA with atypical pattern.

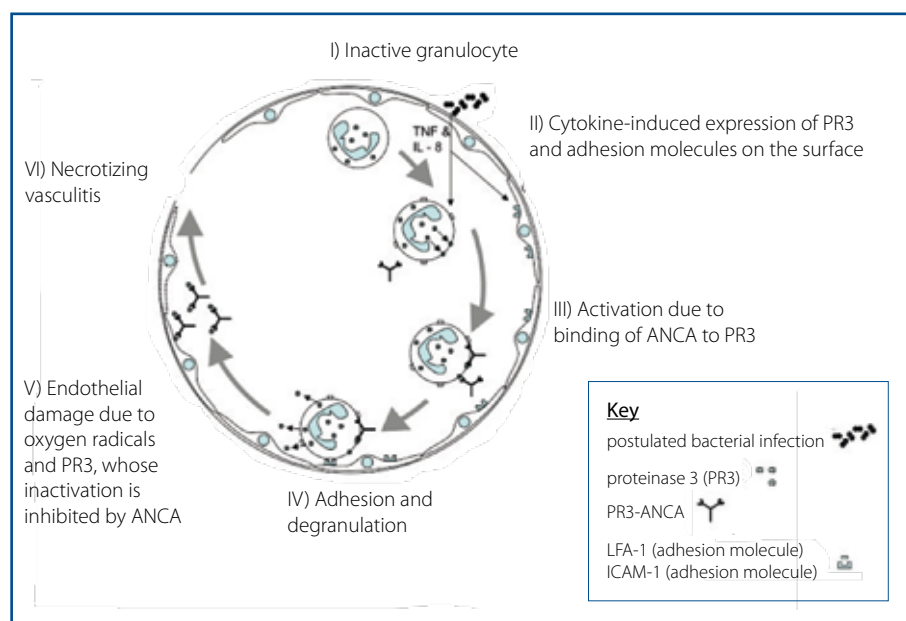


Figure 2 ANCA (in this case PR3-ANCA) bind to granulocytes that have been activated, for example by an infection, and trigger the release of lysosomal enzymes in small blood vessels. This results in the necrotizing changes in the vessels.

Churg Strauss syndrome

Besides general symptoms of disease, Churg-Strauss syndrome typically includes blood eosinophilia and is almost always preceded by a history of allergic asthma. A diagnosis of Churg-Strauss may be indicated if rhinitis occurs early in the illness as the prominent symptom with polyp formation, which may be pronounced. Systemic manifestations such as sinusitis and mononeuritis multiplex are also seen.

In blood smears, eosinophilia is typically, but not always present. Radiology reveals transient migratory pulmonary infiltrates. Histological examination reveals granulomatous necrotizing vasculitic lesions with high concentrations of eosinophils.

Overall Churg-Strauss syndrome is rare with an incidence of approximately three per million population with regional variations existing.

Immunofluorescence shows ANCA to be present in about 50% of patients. These are frequently MPO-ANCA while PR3-ANCA are less common.

Renal-limited vasculitis

Renal-limited vasculitis is a pauci-immune vasculitis of the kidney without systemic involvement. ANCA, primarily MPO-ANCA, are almost always present. If other systemic manifestations occur in the course of the disease, reclassification as Wegener's granulomatosis or MPA may be appropriate.

Drug-induced ANCA-associated vasculitis

Drugs, particularly propylthiouracil, hydralazine and minocycline, but also cocaine may cause localized vasculitis.

Occasionally the disease course can be severe and may require intensive immunosuppression. P-ANCA are often present, sometimes corresponding to MPO-ANCA in ELISA. However, other ANCA types can also occur, particularly atypical ANCA with different target antigens (e.g., lactoferrin, elastase).

Non-vasculitic disorders with presence of ANCA

Immunofluorescence testing shows ANCA to be present in a variety of non-vasculitic disorders in addition to AAV. Examples among the rheumatic diseases are rheumatoid arthritis, systemic lupus erythematosus, and myositides. ANCA can also be found in chronic inflammatory bowel disease, primary sclerosing cholangitis, autoimmune hepatitis, granulomatous inflammations such as tuberculosis and sarcoidosis, cystic fibrosis and subacute endocarditis. These diseases should therefore be taken into consideration for differential diagnosis when interpreting positive immunofluorescence findings for ANCA.

The description usually corresponds to an atypical ANCA pattern. Direct examination with ELISA shows that antibodies against MPO or PR3 are only rarely involved. Target antigens can be lactoferrin, cathepsin G, elastase, lysozyme, bactericidal/permeability-increasing protein and others. They are usually not determined because they lack significance.

P-ANCA are found more frequently in patients with ulcerative colitis (50-70%) than in Crohn's disease (10-30%). These P-ANCA patterns can therefore be helpful in distinguishing the two disorders in cases with no clear positive evidence of PR3- or MPO-ANCA.

ANCA testing: Indications and interpretation of results

As a rule the diagnostic significance of a test result depends on the likelihood of the disease being present. AAV are rare disorders. If immunofluorescence testing for ANCA is used as a screening method, it is likely that a large number of results will be false positive for AAV. For example, antinuclear antibodies (ANA) can sometimes generate a result mimicking the ANCA pattern. International consensus therefore requires immunofluorescence examinations to be accompanied by ELISA testing to determine MPO and PR3 specificity. The primary specific determination of PR3- and MPO-ANCA by means of ELISA can be an alternative strategy when AAV are specifically suspected. Autoimmune serology is just one part of the diagnostic process in identifying AAV and must be interpreted in combination with all other findings. It is essential that diagnostics be precise, as treatment is usually intensive and long-term with potentially dangerous side-effects. The diagnostic process should always include histological examination of appropriate tissue as well as clinical and laboratory testing.

Autoimmune serology is just one part of the diagnostic process in identifying AAV and must be interpreted in combination with all other findings

ANCA testing should be considered in cases with unexplained general symptoms if there is reason to suspect vasculitis. Not all systemic vasculitides are associated with the presence of ANCA and therefore a negative ANCA test does not exclude vasculitis. The indication for ANCA testing becomes more substantiated if additional features of AAV are found (Table 1).

If immunofluorescence testing yields a positive result for C- (or P-) ANCA, and ELISA identifies PR3-ANCA, Wegener's granulomatosis is likely when clinical features are also present. Positive P- (or C-) ANCA and evidence of MPO-ANCA in ELISA testing suggest MPA or CSS (provided the relevant clinical evidence is also present). A specialist should be consulted in either case and also if the suspicion remains despite negative results.

Suspicion of systemic vasculitis e.g. in cases with: – non-specific symptoms (e.g. fever, weight loss, myalgia, arthralgia) – arthritis – systemic inflammation
Glomerulonephritis, pulmonary bleeding or both (pulmonary-renal syndrome)
Vasculitic skin changes/ Livedo reticularis
Pulmonary nodule(s)/ infiltrates shown in thoracic X-ray
Pneumonia resistant to antibiotics
History of allergic asthma
Erosion of nasal mucous membrane, epistaxis
Long-lasting sinusitis / otitis, chronic rhinitis with polyp formation
Tracheal / subglottic stenosis
Retro-orbital tumor
Mononeuritis

Table 1 Evidence of ANCA-associated vasculitides indicating ANCA testing.

Specialist support is always essential for the next stage which involves histological confirmation and subsequent treatment.

Summary

Antineutrophil cytoplasmic antibodies (ANCA) constitute a heterogeneous group of antibodies with great importance in the differential diagnosis of vasculitides.

ANCA produce characteristic patterns when examined using immunofluorescence microscopy. Three main patterns are distinguished: ANCA with the classic cytoplasmic granular fluorescence pattern (C-ANCA), ANCA with perinuclear fluorescence (P-ANCA) and ANCA with atypical pattern. Target antigens have been characterized, and ANCA targeting these antigens can be measured by enzyme immunoassay (EIA). PR3-ANCA usually generate a C-ANCA pattern, whereas MPO-ANCA usually give a P-ANCA pattern.

Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS) are grouped together under the heading of ANCA-associated vasculitides (AAV) because of their association with ANCA (especially PR3- and MPO-ANCA).

AAV should always be considered a possibility in patients with systemic inflammatory disorders without a definite focus. ANCA can aid in the differential diagnosis in these cases.

ANCA testing should always involve immunofluorescence supported by EIA to determine MPO and PR3 specificity. The diagnosis should be confirmed by histological examination in all cases. If necrotizing vasculitis is suspected the patient should be referred to a specialist.

Recommended literature:

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Correspondence:

Dr. med. Thomas Vogt
Rheumatologische Universitätsklinik
Felix Platter-Spital
Postfach
CH-4012 Basel
Switzerland
Thomas.vogt@fps-basel.ch

EliA[®] MPO^S and EliA[®] PR3^S – Cutting Edge Automated ANCA Testing

Nina Olschowka

Global Marketing, Phadia GmbH, Munzinger Straße 7, D-79111 Freiburg

Phadia offers new highly sensitive EliA[®] Tests for the detection of ANCA-associated vasculitis: EliA[®] MPO^S and EliA[®] PR3^S. Both tests work with the so-called anchor technology (see picture below). The advantage compared to the conventional EliA[®] Tests: a substantial increase in sensitivity, while keeping the high specificity. In other words: more patients with Wegener's granulomatosis or microscopic polyangiitis are detected without running the risk of getting more of false-positive results.

Therefore, the performance of these new, fully automated EliA[®] ANCA tests is one of the best performances of all commercial ANCA ELISA kits.

In an International Consensus Statement of 1999 [1] it was agreed that the screening for ANCA should be done with indirect immunofluorescence (IIF) on ethanol-fixed granulocytes and positive results should be confirmed with ELISA. However, an alternative strategy that uses a sensitive ELISA or EliA at the initial stage and IIF for confirmation has also been reported to reach high diagnostic accuracy [2,3]. Although proven clearly and statistically evident, this alternative was regarded with suspicion as IIF is the classical screening method. With the new generation of highly sensitive EliA tests, it is time to question old traditions.

The principle of the new technology is shown in the figure: the antigens (proteinase 3 and myeloperoxidase, respectively) are coated indirectly via so-called capture antibodies or anchor molecules, as in the case of the new EliA[®] tests. Thereby, even those patient-specific antibodies can be detected that are

bound to epitopes which might be not available when the antigens are coated directly (red triangles).

Advantages of EliA[®] PR3^S and EliA[®] MPO^S

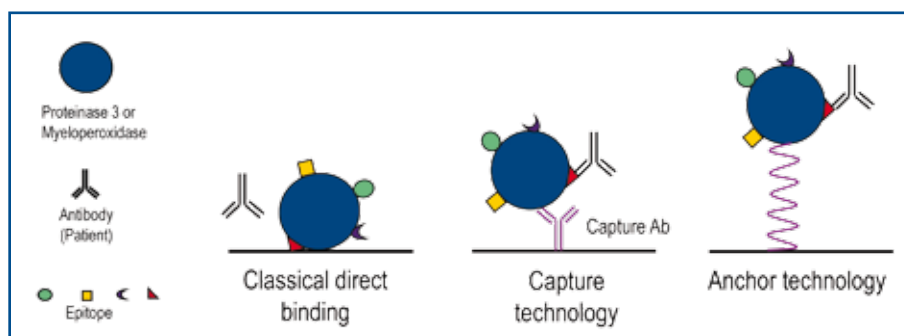
- A new dimension of sensitivity.
- Highest specificity, only in very rare cases positive in other autoimmune diseases.
- Reliable and easy detection of vasculitis-associated ANCA with the fully automated EliA method.
- Standardization against the new international CDC references for PR3-ANCA and MPO-ANCA improves the harmonization of test results. Results are given in international units (IU/ml).

Which changes can you expect when changing to the new tests?

Because of the different coating and the new standardization the new test results will differ clearly from your former test with new values, measuring ranges and cut-offs. Particularly for follow-up sera this may lead to the need for double testing of some samples. However, due to the lower number of false negatives and the high degree of automation, this additional workload will be paid off after a short time.

Literature

1. Savage J et al. *Am J Clin Pathol* 1999; 111:507-13
2. Vermeersch P et al. *Clin Chim Acta* 2008; 397:77-81
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ANCA TESTING BY HIGH SENSITIVE METHODS IN ANCA-ASSOCIATED SYSTEMIC VASCULITIS (AASV) DIAGNOSIS

*A. Radice, °L. Bianchi, *C. Farina, ^C. Zehnder, °RA Sinico

*Microbiology Institute & °Nephrology and Clinical Immunology Unit, Osp S. Carlo Borromeo, Milano, ITALY; ^Future-Lab, Sementina, CH.

BACKGROUND

PR3 and MPO-ANCA are important serological markers for small vessel vasculitis such as WG, MPA and CSS (AASV). In the clinical suspicion of AASV, ANCA-testing is very useful to confirm or exclude the diagnosis, assuming that the available methods possess high sensitivity, specificity and diagnostic accuracy.

Although ANCA-testing is widely used to support diagnosis of AASV, until recently the diagnostic performances of the commercially available "direct" PR3 and MPO-ANCA assays were extremely variable, often lacking the basic standards. Subsequently, a significant improvement in PR3-ANCA performance was achieved by means of the novel and high performant assays (*capture, anchor*); unfortunately, the same progress was not achieved in MPO-ANCA assays. In this setting the novel EliA-MPO^S could allow to get over the gap.

AIM OF THE STUDY

To evaluate the diagnostic performance of the novel, high sensitive EliA-MPO^S and EliA-PR3^S in AASV patients.

RETROSPECTIVE STUDY: PATIENTS

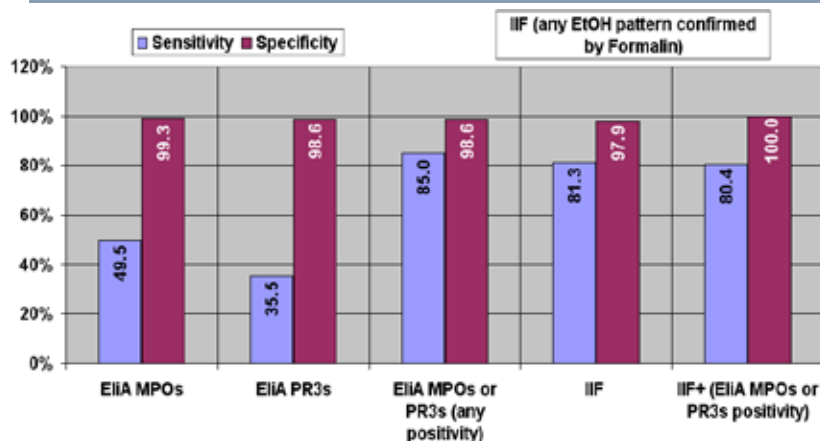
Sera from **107 AASV** (WG 55, MPA 52), **123 pathological** (SLE, CTDs, RA, IBD, Cryoglobulinemia, non-AASV vasculitis, infectious) and **20 healthy** controls, were retrospectively tested. **AASV were selected on the basis of the clinical diagnosis**, according to the available criteria & definitions (ACR, EUVAS, CHCC), irrespective of the disease activity / inactivity / extension, and ANCA status.

METHODS

- EliA MPO^S (Phadia)
- EliA PR3^S (Phadia)
- ANCA-IIF (EUROPLUS Granulocyte Mosaic 2, Euroimmun)

All assays were performed following the suppliers' instructions, and results calculated using the cut-off values suggested by the manufacturers

RESULTS



ANY TEST ALONE OR IN COMBINATION	LH+	LH-
EliA MPO ^S	70.8	0.51
EliA PR3 ^S	25.5	0.65
EliA MPO ^S or PR3 ^S (any positivity)	86.2	0.15
IIF	38.8	0.19
IIF+ (EliA MPO^S or PR3^S positivity)	→ ∞	→ 0

LH+ > 10	=	test result very useful
LH- < 0.2	=	test result very useful
0.2 < LH- < 0.5	=	test result of limited impact
LH- > 0.5	=	test result of poor impact

DISCUSSION AND CONCLUSIONS

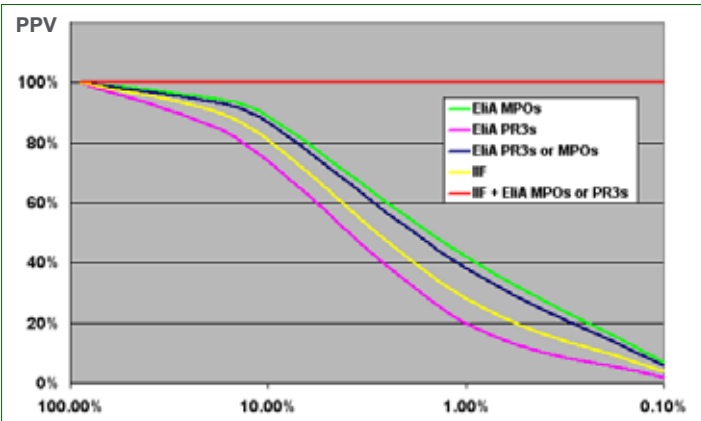
The sensitivity and specificity of both, EliA-MPO^S and EliA-PR3^S, in AASV diagnosis, was satisfactory; LH+/LH- and the diagnostic accuracy (data not shown) confirmed their ability in differentiating between patients with AASV and those who do not have the disease; the sensitivity of the combined tests for AASV diagnosis was as good (or something better) as the standard IIF technique. EliA-MPO^S & PR3^S are calibrated against the CDC Human Ref. Sera #15 & #16, then the results are given in International Units.

Such good performance makes the use of the combined EliA-MPO^S & EliA-PR3^S a possible first step in the algorithm of ANCA testing, followed by the confirmation of the positive results with the standard IIF technique. This alternative algorithm shows the same diagnostic sensitivity of the usually used, with the IIF assay as screening test.

Indeed, the combination of the two methods (IIF+EliA^S), as stated in the International Consensus, allows the best required specificity.

PPV/NPV were not reported because they depend, other than the intrinsic features of the tests, on the prevalence of the target disease in the evaluated population (below).

Then the PPV/NPV obtained in our high-prevalence cohort of patients (42.8%) cannot be extrapolated to other clinical settings.



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Phadia

Phadia GmbH, Munzinger Str. 7, D-79111 Freiburg Germany, Tel: +49 761 47-805-0, Fax: +49 761 47-805-120, autoimmunity@phadia.com, www.phadia.com

Head office Sweden +46 18 16 50 00 Austria +43 1 270 20 20 Belgium +32 2 749 55 15 Brazil +55 11 3345 5050 Czech Republic +420 220 518 743 Denmark +45 70 23 33 06 Finland +358 9 8520 2560 France +33 1 6137 3430 Germany +49 761 47 805 0 Italy +39 02 64 163 411 Japan +81 3 5365 8332 Korea +82 2 2027 5400 Norway +47 21 67 32 80 Portugal +351 21 423 53 50 Spain +34 935 765 800 South Africa +27 11 792 6790 Switzerland +41 43 343 40 50 Taiwan +886 2 2516 0925 The Netherlands +31 30 602 37 00 United Kingdom / Ireland +44 1908 76 91 10 Other Countries +46 18 16 50 00