Antineutrophil cytoplasmic antibodies (ANCA) – their role in pathogenesis, diagnosis, and treatment monitoring of ANCA-associated vasculitis

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Abstract
Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) constitutes a group of rare diseases characterized by necrotizing inflammation of small blood vessels and the presence of ANCA. Increasing clinical and experimental evidences support their pathogenic role in AAV, but the exact mechanism is not fully understood. Recently, the important role of neutrophil extracellular traps (NETs) in pathogenesis of AAV is underlined. There is an indication that NETs can be a source for the formation of ANCA. The most common ANCA target antigens are myeloperoxidase (MPO) and proteinase 3 (PR3). Though the mechanism of action of ANCA is still under exploration, ANCA serology is being increasingly used for classification of AAV and revealed as key in defining various disease subsets associated with different genetic background, clinical features, treatment response, and prognosis. Controversy exists regarding the utility of serial measurements of ANCA in patients with AAV to monitor treatment and predict disease relapse.

Key words: ANCA, vasculitis, pathogenesis, diagnosis, treatment monitoring.

Introduction
The antineutrophil cytoplasmic autoantibodies (ANCA)-associated vasculitides (AAV) comprise a group of diseases characterized by necrotizing vasculitis of small vessels, with frequent involvement of the kidneys, lungs, lack or paucity of immune deposits in the vessel wall, and the presence – in the majority of cases – of autoantibodies to neutrophil constituents [1]. The consequence is ischemia and necrosis of organs supplied by these vessels. Clinically and pathologically, three forms are traditionally recognized such as granulomatosis with polyangiitis (GPA, formerly called Wegener’s granulomatosis), which is distinguished by necrotizing granulomas on biopsy and frequent upper respiratory tract involvement, microscopic polyangiitis (MPA) mainly with kidney involvement, and eosinophilic granulomatosis with polyangiitis (EGPA, formerly called Churg-Strauss syndrome), which is much less common and distinguished by a prominent eosinophilia and allergic features [1].

Characteristic for AAV is the presence of ANCA. They were first reported in 1982 by Davies et al. [2] in serum from a few patients with segmental necrotizing glomerulonephritis. The data was largely disregarded until Van der Woude et al. [3] described similar patterns of autoantibodies in patients with systemic vasculitis (GPA) in 1985. Then, myeloperoxidase (MPO) and proteinase (PR3) were recognized as the major autoantigens accounting for the perinuclear (p-ANCA) and cytoplasmic (c-ANCA) patterns, respectively [4, 5]. ANCA in serum were originally detected by indirect immunofluorescence (IIF), and a positive IIF staining of only neutrophils without lymphocytes distinguishes ANCA from other types of autoantibodies. Currently, they are widely considered as a laboratory test biomarker in the diagnosis of patients suspected with vasculitis.

Although ANCA are characteristic feature for AAV, they not always occur in AAV patients: c-ANCA are detected in 95% of cases of new onset GPA, and p-ANCA in 80% of patients of new onset MPA, and only in 40% of EGPA [4-6]. Additionally, PR3-AAV clearly differs in its clinical presentation from MPO-AAV. Franssen et al. [7] observed that patients with PR3-ANCA have, in comparison with patients positive for MPO-ANCA, more organs involved, resulting in faster decline in renal function, more frequent relapses of the disease, and in particular, granulomatous nec-
rotizing inflammation in the airways. Recently, an emerging concept in AAV found a relationship between disease serotype and phenotype [8]. Since the manifestations, clinical course and relapse rates correlate closely with target antigen, with some clinicians using the terms PR3- or MPO-AAV rather than GPA, MPA, and EGPA [9].

The discovery of ANCA have revolutionized the diagnostic process of AAV, but beyond a diagnostic serological marker, clinical and experimental data support a pathogenic role for ANCA, which promote activation of primed neutrophils and monocytes and their adhesion to the endothelium, leading to subsequent tissue damage [10, 11]. Although various hypotheses with different kinds of triggers have been suggested concerning ANCA formation, none has been confirmed to date. In contrast to an established, although not fully understood, role of ANCA in pathogenesis of vasculitis, their role for monitoring disease activity and treatment results of patients with AAV remains still unclear. Rising titers of c-ANCA were shown to predict a relapse of disease [12], and pre-emptive treatment with cyclophosphamide and steroids based on rising titers of c-ANCA was able to prevent the occurrence of relapses [13]. Other authors, however, could not confirm a strong correlation between rising titers and ensuing relapses [14, 15]. In this review summary of current knowledge about the role of ANCA in pathogenesis, diagnosis and monitoring of treatment of AAV was presented.

ANCA and their role in pathogenesis of AAV

How ANCA can lead to the development of AAV

A breakthrough regarding the pathogenicity of ANCA was based on the discovery by Falk et al. [10] who proved that ANCA can activate primed neutrophils to produce reactive oxygen species (ROS) and the release of lytic enzymes. Antigens such as microbial components or complement stimulate ANCA autoantigen expression on the membrane surface of neutrophils [16, 17]. ANCA are thereby developed and released from B cells and activate neutrophils by binding to the ANCA autoantigens. Once activated, neutrophils attach to the endothelium of blood vessels, and release ROS and inflammatory cytokines leading to systemic vasculitis and multiple organ injuries [18]. The signaling pathways involved in priming are not fully known, but the activation of the p38 mitogen-activated protein kinase, extra signal regulated kinase, and phosphoinositol-3 kinase appear to be important steps in the translocation of ANCA antigens to the cell membrane [19, 20]. Although neutrophils play a key role in the pathogenesis of AAV, it has been demonstrated that other immune cells (e.g., T cells and monocytes) are also involved [21].

The strong evidence that MPO-ANCA are involved in AAV pathogenesis comes from studies using MPO-deficient mice [22, 23]. Additionally, in a human case study, a newborn was reported who developed pulmonary-renal syndrome with placential transmission of MPA-ANCA [24]. In PR3-AAV, the pathogenic role of PR3-ANCA is not fully confirmed [25-28], but although no sufficient experimental model can be used, there are some clinical arguments confirming this relation. The first comes from some observations that the persistence of ANCA after induction of remission in patients with AAV is associated with relapse of the disease [29]. Another argument comes from two studies describing the efficacy of rituximab, a B-cell-depleting monoclonal antibody in patients with severe, active AAV [30, 31]. Both showed that rituximab was not inferior to cyclophosphamide treatment (regardless of the route of administration), and may be more effective in severe, with impending renal insufficiency, and relapsing disease [30, 31]. Although the results of B-cell depletion may be more extensive, its efficacy in AAV can be explained at least in part by its effect on ANCA production.

The mechanism of ANCA formation in AAV

There is considerable evidence that ANCA are involved in the pathogenesis of AAV, but the mechanism leading to their formation is unclear. Experimental [32] and epidemiological [33] studies have provided strong supportive evidence that infections may trigger exacerbations of AAV and may even be the main pathogenetic mechanism of ANCA formation. The presence of transient immunoglobulin M (IgM) PR3-ANCA with an acute respiratory manifestation of AAV also suggests a possible link between an infectious trigger and AAV disease activation [34].

One factor that has been linked to GPA and anti-PR3 antibodies is nasal carriage of *Staphylococcus aureus* (*S. aureus*). Stegeman et al. [29] first noted that 63% of patients with GPA were chronic nasal carriers of *S. aureus*, and that this carriage was associated with a higher relapse rate, with an adjusted relative risk of 7.16. Furthermore, it has been shown that a treatment with cotrimoxazole could prevent the occurrence of relapse [35]. The main influence of *S. aureus* on AAV activity is mainly attributed to its superantigens (SAgs), an extremely potent immunostimulatory molecules [36, 37], but so far, the results of clinical studies evaluating a critical role of SAgs for AAV activity have not been unequivocal [38, 39].

The mechanisms underlying the relationship between *S. aureus* and AAV remain unclear, but numerous theories have been postulated [16, 40]. Autoantigen complementarity has been suggested as one of the mechanisms that can break a tolerance to ANCA antigens. According to this hypothesis, the initial immune response in patients with AAV is not directed towards the autoantigen, but rather to a peptide that is complementary to the autoantigen epitope. The complementary peptide immunogen could arise from antisense transcription of autoantigen gene or exist as mi-
crobial exogenous peptide that mimics the complementary antisense peptide. Antibodies to a complementary protein may induce anti-idiotypic antibodies that cross-react with the original protein. Another hypothesis is based on molecular mimicry. It has been theorized that similarities between antibodies to pathogens and epitopes of self-antigens may lead to cross-reactivity and mounting an autoimmune response. Such molecular mimicry may also be the primary mechanism in the development of focal necrotizing glomerulonephritis in patients with ANCA directed against human lysosome membrane protein (hLAMP-2) [16].

Recently, the important role of neutrophil extracellular traps (NETs) in the pathogenesis of AAV is underlined. There are evidences supported the concept that NETs can be a source for the formation of ANCA. NETs were first described in 2004 and were initially associated with antibacterial role in bacteria trapping [41]. These formations characterize a special type of neutrophil-related cell death named NETosis, which in neutrophils leads to a production of a meshwork of chromatin fibers associated with citrullinated histone H3 and antimicrobial proteins, including MPO and PR3. This meshwork forms extracellular nets that trap and kill microbial pathogens [16]. In 2009, a study demonstrated that NETs released after in vitro ANCA-induced neutrophil activation contained MPO and PR3 [42]. Another study showed that MPO and PR3 transfer from NETing neutrophils into dendritic cells, with subsequent generation of anti-MPO-ANCA and development of autoimmune vasculitis in animals [44]. Additionally, PR3 and elastase containing NETs have been detected in affected human glomeruli [42]. Results of recent studies showed that simultaneous stimulation of neutrophils by different NETosis-induced agents result in diminished formation of NETs, as compared to a single stimulation. This indicates that cells may possess an internal regulatory mechanism that prevent overgeneration of NETs among healthy people [44].

On the other hand, the relation between ANCA and NETs in clinical studies is not evident. Kraaij et al. [45] investigated NET formation in 99 patients with AAV. They found a significant excess of ex vivo NET formation in both MPO-ANCA- and PR3-ANCA-positive patients with AAV, as compared to healthy individuals. Excessive NET formation did not correlate with serum ANCA levels. Likewise, immunoglobulin G depletion had no effect on excessive NET formation in patients with AAV, indicating an ANCA-independent process. Next, they demonstrated that excessive NET formation was predominantly found during active disease, more so than during remission. Excessive NET formation was found in patients with AAV hospitalized for disease relapse, but not during severe infection. Authors concluded that excessive NET formation in AAV was independent of ANCA, and an excess of ex vivo NET formation was related to active clinical disease in patients with AAV and a marker of autoimmunity rather than infection.

ANCA and genetics

Recently investigated by genome-wide association study (GWAS) methods showed that the pathogenesis of AAV has a genetic component, and that the association of these genetic polymorphism is related to the ANCA type. PR3-AAV is strongly associated with HLA-DP (human leucocyte antigens-DP) and the genes encoding for α-1-antitrypsin (SERPINA1 gene) and PR3 (PRTN3 gene), while MPO-AAV is significantly associated with HLA-DQ [8, 46]. Whereas EGPA is associated with HLA-DRB1*04 and HLA-DRB4 [47, 48], it suggests a strong CD4+T lymphocyte activation, possibly triggered by allergens or antigens. Patients with AAV are also more likely to carry polymorphism in CTLA4 and PTPN22, which are thought to be “general” susceptibility factors associated with autoimmune disease. Ultimately, genetic studies indicated AAV as a polygenic disease and supported the pathogenicity of ANCA, with HLA and non-HLA genes variants predisposing to this condition [47]. Genetic associations suggest that AAV could be divided into several subsets beyond the classical phenotypic diagnoses of GPA, MPA, and EGPA. Particularly, some findings indicated that classifying patients according to ANCA status and specificity could be more pertinent in practice than phenotypic MPA or GPA diagnosis, since ANCA status might correspond better to differences in clinical presentations and outcomes [9]. However, this leads to various problems when considering the significant minority of patients with ANCA-negative disease. It would be of great interest to examine genetic associations in the ANCA-negative patients; however, numbers needed are likely to limit the ability to perform such study [47].

ANCA and their utility in the diagnostic process of AAV

ANCA as a preliminary diagnostic tool

ANCA have been recognized as an important diagnostic marker in patients with AAV. Therefore, patients presenting clinical features of systemic small-vessel vasculitis suspected of AAV are routinely tested for ANCA [49]. Moreover, target antigens of ANCA (PR3 and MPO) should be identified as they are helpful for reaching a diagnosis [50, 51]. On the other hand, ANCA are not a specific marker for AAV. Interestingly, detectable MPO- and PR3-ANCA but also ANCA directed against other autoantigens (such as lactoferin) have been described in different kinds of non-vasculitis disorders with a controversial clinical relevance [52]. Furthermore, circulating autoantibodies against MPO and PR3 have been reported in healthy individuals and designated as “natural” autoantibodies [53]. Lower titre, lower avidity, and lack of IgG3 subclass of these autoantibodies suggest their non-pathogenic co-existence in serum, but whether ANCA are targeting these
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Evident that ANCA specificity is associated with different GPA, MPA, and EGPA [64]. In the last years, it became distinguished based on clinical and pathological features: patients with AAV are suspected, the detection of ANCA required only tests for PR3- and MPO-ANCA, without categorical need for IIF. If both are negative, and there is still a strong suspicion of small vessel vasculitis, the use of other immunoassays and/or IIF, or a referral to an experienced laboratory is recommended [63]. The guidelines underline that a positive PR3- and/or MPO-ANCA result contributes only to the diagnostic work-up for AAV, but is not diagnostic as such, and also negative ANCA tests (PR3 and MPO) cannot be the basis for AAV exclusion [63].

ANCA specificity as a basis of new phenotyping patients with AAV

Traditionally, three distinct AAV diseases have been distinguished based on clinical and pathological features: GPA, MPA, and EGPA [64]. In the last years, it became evident that ANCA specificity is associated with different genetic background, clinical features, treatment response, and prognosis, and it could be keener in defining different disease subsets than clinical diagnosis of GPA or MPA [65]. The basis of these findings are genetic data from GWAS and African American population pointing to genetic differences between PR3- and MPO-ANCA patients. This study showed that autoantigen specificities of PR3 and MPO correlate better with different HLA risk genes (PR3-ANCA with HLA-DP, MPO-ANCA with HLA-DQ) than with clinical and pathological phenotypes of GPA and MPA. Moreover, the association with genes encoding PR3 (PRTN3) and its inhibitor α-1-antitrypsin (SERPINA1) with PR3-ANCA disease and/or GPA additionally supports central pathogenetic role of autoantigens and their neutralizing counterparts [8].

Although few studies have indicated that ANCA subtype does not impact disease outcome [66, 67], accumulating evidences indicate that ANCA type better identifies AAV patients that the GPA/MPA classification [9, 68-73]. PR3-ANCA-positivity vasculitis is associated with destructive granulomatous inflammation, more upper airway involvement, more extensive extrarenal organ manifestation (on average four organs are involved), and a higher relapse risk. In contrast, MPO-ANCA-positivity vasculitis is frequent in kidney-limited disease with glomerular sclerosis and a worse renal prognosis. Patients with MPO-ANCA are more likely than patients with PR3-ANCA to have a profibrotic phenotype such as strong association with lung fibrosis. Among patients with clinical diagnosis of GPA, those positive for MPO-ANCA more frequently have limited disease without severe organ involvement, higher prevalence of subglottic stenosis (SGS), less frequent need for cyclophosphamide or rituximab therapy, and fewer relapses than patients who are PR3-ANCA-positive [9, 68-73]. Furthermore, PR3-ANCA patients respond better to rituximab for induction and maintenance of remission than cyclophosphamide/azathioprine, whereas these treatments were equally effective in MPO-ANCA patients [73, 74]. Finally, MPO-ANCA vasculitis is characterized by fewer relapses and is associated with poorer survival, as compared with relapsing PR3-ANCA vasculitis [9, 75, 76]. Detailed list of study results shows Table 1.

ANCA antigen specificity revealed to be more strongly associated with AAV clinical presentation and prognosis than the former diagnosis of the GPA or MPA. Therefore, there is a new proposal to use ANCA serotypes to classify disease and provide diagnosis based on the presence of PR3- and/or MPO-ANCA. That ANCA serotyping distinguishes distinct classes of ANCA disease: PR3-ANCA AAV, MPO-ANCA AAV, and ANCA-negative AAV [9, 65]. The identification of these distinct patients’ subsets is the big step forward in AAV research and may be a first step towards development of precision medicine for AAV, which may help to better individualize the treatment in future.
ANCA and their clinical utility in EGPA

The role of ANCA in differentiation of two phenotypes of EGPA was described already in 2005. Two large studies [77, 78] demonstrated that ANCA were positive in 38% of EGPA patients, and ANCA-positive patients more frequently had peripheral neuropathy, glomerulonephritis, and purpura (so-called “vasculitic phenotype”), whereas endo-myocardial involvement and lung infiltration dominated in the ANCA-negative subset (so-called “tissular phenotype”). Apart from the distinct clinical manifestation, these phenotypes are characterized by different clinical course of disease and prognosis: vasculitic phenotype is characterized by frequent relapses and better outcomes, while in tissular phenotype, the relapses are less frequent, but the outcomes are worse. Further studies confirmed the differences in clinical disease presentation based on ANCA status and indicated that ANCA-positive patients should be treated more aggressively [79].

Based on these observations, members of the European Respiratory Society CSS-Task Force recently prepared recommendations for the diagnosis of EGPA, including a definition of detailed criteria of vasculitis (or surrogates of vasculitis) in patients with asthma and blood eosinophils greater than 1.5 G/l [80]. They proposed that patients with asthma and blood eosinophils greater than 1.5 G/l without ANCA, vasculitis, or surrogates of vasculitis should be named as hypereosinophilic asthma with systemic manifestations (HASM), non-vasculitis. Supporting these proposals, Cottin et al. [81] published in 2017 their results of retrospective study of 157 patients’ population with EGPA and found that ANCA alone were an insufficient element to categorize patients with vasculitis features (47% of patients with systemic vasculitis features had no ANCA, while 29% of patients with MPO-ANCA had no vasculitis features) and in their opinion, the terminology of polyangiitis appears to be a misnomer for 41% of patients in this series. They fully approved the suggested criteria to separate patients with a prominent vasculitis phenotype (named as “EGPA”) from patients with predominant hypereosinophilic manifestations including asthma (named as “HASM”). In this regard, the proposed definition could serve as a basis for individualized future therapeutic intervention in EGPA.

ANCA and their utility in monitoring of AAV activity

Advances in therapy have transformed AAV from a frequently fatal disease into a chronic illness. Current treatments caused a remission in more than 80% of AAV patients, with a 5-year overall mortality rate of 10-15%...
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[82]. Although it is possible for most patients to achieve a remission with therapy, 38% of cases experience a disease relapse within 5 years despite an expert care [83]. There are few serologic predictors of relapse in AAV [84-86], but so far, no predictor has been finally shown to reliably guide therapeutic decision-making.

The utility of ANCA status for monitoring AAV activity and guidance of treatment decisions is widely studied, but results are still controversial. Some studies demonstrated a relationship between an increase in levels of PR3-ANCA and the occurrence of relapses. Boomsma et al. [87] in a study on 100 GPA patients with PR3-ANCA observed that 26 out of 33 relapses were preceded by a rise in PR3-ANCA measured by ELISA (sensitivity, 79%), whereas 12 of 38 increases were not followed by a relapse (specificity, 68%). In turn, Stegeman et al. [29] showed that persistence of ANCA presents a relative risk of 9.0 for a relapse. In their retrospective study, Han et al. [88] reported similar results, and they stated that serial measurement of ANCA titers in patients with GPA during remission can help to predict relapses and pre-emptive increase in immunosuppression following fourfold titer reduced the risk of relapses. On the other hand, there are studies reporting that changes in serial PR3-ANCA titers not always correlated with disease activity. Kerr et al. [14] demonstrated that changes in serial ANCA titer temporally correlated with a change in disease status in only 64% of patients with AAV. Furthermore, an increase in the c-ANCA titer preceded clinical exacerbation of disease in only 24% of patients with remission or low-grade, smoldering disease. In turn, Thai et al. [89] observed that although ANCA were positive during most systemic relapses or residual disease, no strict clinical-immunological correspondence was observed for 25% of their patients. Authors concluded that GPA management cannot be based on ANCA levels alone. Whereas, Girard et al. [90] reported in their small prospective study that relapses were more frequent when ANCA remained positive or reappeared, the mean time between inclusion and relapse did not differ between the patients who became ANCA-negative and those who were persistently ANCA-positive, and the mean time to ANCA disappearance was similar for the patients who relapsed and those who did not. They underlined that although usually the progression of AAV was more frequent when ANCA remained positive or reappeared, discordance between ANCA and disease activity was not unusual. Finally, a meta-analysis of nine studies concluded that the value of serial measurements of ANCA in patients in remission is limited and neither the increase nor the persistence of ANCA in patients who achieved remission is highly predictive of relapse [91], therefore targeted determination of ANCA levels may be useful only if a relapse is clinically suspected [92]. Fussner et al. [93] confirmed these results, showing that an increase in PR3-ANCA levels is not an overly sensitive and specific predictor of a subsequent relapse in patients with GPA.

Although there is a relevance for ANCA monitoring as a marker of disease activity, there is only 50% ANCA elevation, which ended in relapses, and around half of relapses occur even in the absence of rises in ANCA titer [94]. Therefore, physicians monitoring ANCA are sometimes confused by the results and they do not know what they should do. The outcomes of prospective studies are helpful in these situations. According to them, PR3-ANCA are associated with higher risk of relapse, while patients with MPO-ANCA relapse less frequently and might benefit in the future from shorter term drug administration with less ANCA monitoring. The PR3-ANCA at diagnosis and 12 months persistence after starting induction were considered as parameters significantly influencing relapse occurrence [83, 89, 95].

Typically, high circulating titres of ANCA are seen during active AAV, with subsequent falls during therapy. These observations may support a pathogenic role of ANCA in AAV but on the other hand, ANCA are not detectable in approximately 20% of cases [96]. Moreover, clinical benefit following rituximab therapy is often seen before a significant reduction in ANCA titre [97] and has also been reported in ANCA-negative cases [98]. Additionally, although rituximab efficacy against AAV activity appeared to significantly correspond with the ANCA profile (and CD19 levels) of treated patients [99], the results of a prospective trial (MAINRISTAN2) showed that the attempt to adjust the infusion schedule to ANCA titers (and/or the presence of circulating CD19) was unreliable for relapse prevention [100]. These facts suggest the possibility that ANCA can be some phenomenon accompanying but not necessarily driving the disease. Thus, although a significant improvement has been made in our understanding about the role of ANCA in AAV, their final role in treatment monitoring and therapeutic decision-making needs further investigations.

Conclusions

In conclusion, ANCA have been recognized as an important biomarker to aid diagnosis in patients with AAV, a life-threatening small vessel vasculitis characterized by the presence of ANCA, and renal, lung and upper respiratory tract involvement. Most probably, they are involved in the pathogenesis of AAV, but the exact mechanism is not clear. The etiology and pathogenesis of this complex diseases appear to be multifactorial and involves the interplay of initiating and predisposing environmental and genetic factors, loss of immune tolerance, and mediation of acute injury. The big step forward in AAV research was undoubtedly the sub-classification and phenotyping of these complex diseases, associated with different genetic background, clinical features, treatment response, and
prognosis in terms of relapse rate and survival rate. Identification of these distinct patients’ subsets may be a first step towards development of precision medicine for AAV, which may be helping to better individualize the treatment in the future. Though ANCA can be used to assess prognosis in patients with AAV, the utility of their serial measurement to predict disease relapse remains controversial. Thus, therapeutic decision to increase immunosuppression should not be based solely on the levels of ANCA titers.

The authors declare no conflict of interest.

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