



## CKJ REVIEW

## Current and novel biomarkers in anti-neutrophil cytoplasm-associated vasculitis

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### Abstract

Anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV) is characterized by a variable disease course, with up to 50% of patients having one relapse within 5 years and many progressing to end-stage organ damage despite modern treatment strategies. Moreover, complications arising from treatment dominate the causes of mortality and morbidity both early and late during disease, especially in the elderly and those with severe renal involvement, and there is additional uncertainty as to how long treatment should be continued. There is, therefore, an urgent clinical need to identify robust biomarkers to better predict treatment responses, risk of disease relapse and eventual complete clinical and immunological quiescence. To date, no such biomarkers exist, but better understanding of disease pathogenesis and the underlying immune dysfunction has provided some potential candidates linked to the discovery of new antibodies, different leukocyte activation states, the role of the alternative complement pathway and markers of vascular activation. With all promising new biomarkers, there is the need to rapidly replicate and validate early findings using large biobanks of samples that could be brought together by leaders in the field.

**Key words:** ANCA-associated vasculitis, biomarkers, glomerulonephritis

### Introduction

Anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV) comprises three different clinical entities: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA). They are a group of systemic diseases characterized by pauci-immune necrotizing inflammation of small- to medium-sized blood vessels and are differentiated by subtle differences in clinical phenotype. AAV is defined as a rare autoimmune disease with an incidence of 20 patients per million/year [1]; however, recent reports suggest this may be increasing [2].

In most patients with GPA and MPA, ANCAs are detected and are frequently directed against proteinase-3 (PR3) and myeloperoxidase

(MPO), respectively, while only half of those patients with EGPA have a detectable ANCA [3]. In addition, a significant minority of patients with limited GPA (often confined to the upper respiratory tract) are ANCA negative, as are 5–10% of patients with focal necrotizing, pauci-immune glomerulonephritis. Whether these represent variants of AAV or have different aetiologies with similar final pathologies is uncertain, but they appear to behave in similar fashion to ANCA-positive disease. AAV affects multiple organ systems, with renal (glomerulonephritis) and pulmonary (haemorrhage, nodules, fibrosis) involvement being the most frequent and severe manifestations.

AAV is characterized by a variable disease course, which can be limited and mild or systemic and life-threatening; with treatment, it can recover and resolve leaving no permanent organ

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dysfunction or progress and lead to scarring and end-stage organ damage. In conjunction with the short- and long-term toxicity of current therapies, this makes optimal management of AAV patients a highly complex field, which is often guided by physician-biased approaches rather than more objective approaches. Customization of treatment is therefore highly desirable but is limited by the lack of markers signalling the likely outcome of disease. Tissue sampling to identify ongoing disease and differentiate it from chronic damage may be helpful but is limited in its use as a longitudinal marker. Kidney biopsies, for example, may be biased by sampling error especially when considering focal disease, and are invasive, making repeated assessments less appealing. For this reason, there has been a longstanding search for non-invasive biomarkers that can predict treatment responses and disease relapses [4].

The aim of this review is to discuss some of the current and potential future biomarkers in AAV and understand how they are implicated in the pathogenesis of the disease (Table 1).

## Current biomarkers

Inflammatory markers, such as the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), are hampered by being non-specific and therefore unable to differentiate infection from disease, a common clinical dilemma. Similar to ANCA directed against PR3 or MPO, these markers are helpful for diagnosis in AAV, in conjunction with appropriate clinical and pathological findings; however, they are less useful when assessing disease activity in patients with an established diagnosis, or in predicting relapses.

ANCAs are important in the pathogenesis and diagnosis of AAV; however, data supporting their utility as biomarkers of disease activity are less clear. Since a proportion of patients remain ANCA positive despite clinical remission, the use of ANCA as markers of disease remission or immunological quiescence is limited. Early studies from single centres or small cohorts suggested that in those who become ANCA negative during disease remission, or in those with stable ANCA positivity, an increase in titre may be used as a marker of pending disease relapse, in some cases prompting change in management [5]. In 2006, a meta-analysis of 22 studies on the value of ANCA measurements to predict relapses concluded that no firm conclusions could be drawn because of considerable methodological heterogeneity in

the different studies, such as differences of follow-up time and time between serial ANCA measurements, variations in definition of remission and relapse, different techniques for ANCA measurement and different definitions of a rising titre [6]. In another meta-analysis [7], Tomasson *et al.* evaluated nine studies and found that both rising ANCA or persistently positive ANCA were poorly associated with flares (positive likelihood ratio 2.84 and 1.97, with a sensitivity of 0.56 and 0.38; specificity 0.82 and 0.78, respectively). In contrast, Kemna *et al.* [8] noted that longitudinal ANCA measurements may be useful in a subset of patients with renal involvement. They found that rising ANCA titres were significantly correlated with relapses in patients with renal disease (hazard ratio: 11.09), whereas this association was less clear-cut in those subjects without overt renal disease (hazard ratio: 2.79). However, less than half of the patients with a rising ANCA titre experienced a relapse within a year, making it difficult to use these data as a means of pre-emptively treating patients to prevent disease relapse.

Taken together, persistent ANCA or a rising titre seems insufficient to change treatment decisions. However, in patients with AAV and renal involvement, an increase in ANCA titre could be used as a rationale to more carefully follow these patients, monitoring for other earlier signs of disease flare.

Another important issue about ANCA as a biomarker was the presence of AAV patients with negative ANCA, presenting an important challenge regarding the pathogenic role of this antibody. To address this issue, Roth *et al.* developed an epitope mapping of murine and human MPO-ANCA and revealed that this antibody in ANCA-negative disease reacted against a sole linear sequence and was capable of *in vitro* neutrophil activation and induction nephritis in mice. This important discovery could provide a better understanding of the evolution of the disease and enhance the ability to identify at-risk individuals prior to disease development [9].

## New biomarkers in AAV

### Other antibodies

It is possible that additional humoral mediators, other than the classical ANCA, are involved in development of AAV in patients, and may serve as markers of disease activity. There are a number of other, recently described, autoantibodies, such as those directed at lysosome-associated membrane protein-2 (LAMP-2), moesin,

**Table 1.** Summary of biomarkers in AAV

Biomarker	Comment
Inflammatory markers	Non-specific. Unable to differentiate infection from disease.
ANCA	Persistent ANCA or rising titre insufficient to change treatment decisions. More useful in patients with renal disease.
LAM-2 antibodies (Ab)	Titres fall rapidly after treatment and recur in relapse. Need standardized techniques.
Moesin Ab	Associated with more renal damage. No data on disease relapse. Not replicated outside Japan.
Plasminogen Ab	Mostly found in active disease. Correlates with glomerular lesion severity. No data on disease relapse.
Pentraxin-3 Ab	Mostly found in active disease. No data on disease relapse.
Bregs	Contradictory data in active and remission. No data on disease relapse. Need a homogenous marker and functional assays.
T cells	CD8+ profile: able to identify patients more likely to relapse. Must do a prospective validation.
Granulocytes subsets	Treatment with RTX: better response in patients with higher GI, versus CYC: better response with a lower GI.
Complement	Higher levels in active compared with remission disease. Results need to be replicated in larger studies.
Calprotectin	Failure to decrease serum calprotectin by Month 2 or 6 compared with baseline: higher relapse risk in PR3-ANCA treated with RTX.
Urine MCP-1	Higher levels associated with relapse and poor prognosis.
Urine-soluble CD163	Higher levels in active renal disease compared with remission.

RTX, rituximab; CYC, cyclophosphamide; GI, granularity index.

plasminogen and pentraxin-3 that have been found in AAV patients.

Autoantibodies to LAMP-2 are co-expressed with ANCA directed against MPO or PR3 in patients with pauci-immune glomerulonephritis but have also been found in ANCA-negative cases. LAMP-2 is expressed in neutrophils and in endothelial cells. Autoantibodies to LAMP-2 have been described in 80–90% of patients presenting with AAV, become rapidly undetectable after the start of immunosuppressive treatment, are rare in patients during disease remission, but recur in those with clinical relapses [10, 11]. However, a lower frequency of antibodies has been reported by other investigators, who found anti-LAMP-2 antibodies in only 21% of AAV patients compared with 16% of controls. In addition, the assays for their detection have not been standardized or made widely available. These conflicting results make the value of currently using this antibody as a biomarker of disease activity unlikely [12].

Moesin is a member of the ezrin family of proteins that link actin to the plasma membrane. Anti-moesin antibodies were originally described in serum from SCG/Kj mice that spontaneously develop vasculitis. More recently, a cross-sectional study of 60 Japanese patients with MPO-AAV found that anti-moesin antibodies were present in the serum of half of the cohort. The anti-moesin antibodies were detected in both active disease and remission, but were associated with more renal damage (as assessed by levels of blood urea nitrogen, serum creatinine and proteinuria). Interestingly, the autoantibodies bound to moesin in neutrophils and monocytes result in a cytoplasmic ANCA pattern on indirect immunofluorescence. However, testing for the presence of the antibody in non-Japanese populations has not been carried out, nor has their presence been correlated with susceptibility for relapse [13].

Plasminogen is a key protein in the fibrinolysis system, and antibodies directed to it were found in 24 and 26% of AAV patients from UK and Dutch cohorts (compared with 2% of healthy controls) [14]. The presence of antibody correlated with *in vitro* prolongation of fibrinolysis in a proportion of patients, and with glomerular lesion severity. Similar findings were reported in a Chinese cohort of 104 AAV patients, in whom anti-plasminogen antibodies were present in 18% of patients with active disease and only 2% of those in remission. Weak correlations were found between antibody titre and ESR, renal function and certain renal biopsy features [15].

Pentraxin-3 [16] is stored in neutrophil granules and is expressed on the surface of apoptotic neutrophils. Antibodies to pentraxin-3 were measured in 150 AAV patients and various healthy or disease controls. They were found in the sera of 37% of AAV patients (compared with 5.3% of healthy controls and 7.5% of scleroderma patients), and in up to 50% of ANCA-negative AAV. Levels were higher in patients with active disease compared with those in remission, but considerable overlap was found. No data on disease relapse were provided. These antibodies may be useful in making a diagnosis of disease in a subset of patients, but their significance and correlation with disease activity have yet to be validated and may be no better than ANCA itself.

Taken together, these novel autoantibodies appear in only a proportion of patients and may serve to identify a subset with more severe disease features, which may be useful in customizing therapy for those with a worse phenotype. None of the studies has investigated the impact of these antibodies for predicting relapse or compared them with change in ANCA titre. Few of them have been validated in larger independent cohorts, which is obviously the next critical step in understanding their significance and utility.

## Leukocyte subsets

The importance of B lymphocytes in the pathogenesis of AAV was confirmed through the clinical efficacy of B-cell-target therapy. In the RAVE and RITUXIVAS studies, rituximab (RTX) was demonstrated to be non-inferior to cyclophosphamide (CYC) for induction of remission in severe AAV [17, 18].

B lymphocytes can be further classified according to function and expression of certain markers, dividing them into memory cells, regulatory cells, mature cells and plasmablasts. B subsets, particularly regulatory B-cells (Bregs), have been investigated as potential biomarkers in AAV since they are implicated in inhibiting autoimmune disease. One critical issue is that different groups use different markers to define Bregs, with few using functional assays of suppression to confirm their regulatory phenotype. Eriksson *et al.* [19] found CD25+ B cells to be increased in clinical remission relative to acute disease. Bunch *et al.* [20] described a numerical deficiency in CD5+ B cells in AAV patients compared with controls, which was most profound during active disease. In contrast, the RAVE group measured the absolute and relative number of CD5+ B cells longitudinally in their trial participants [21]. After an initial decline, absolute CD5+ B cell numbers progressively increased in patients in the RTX arm, but remained low in patients in the CYC arm. Interestingly, during relapse the percentage of CD5+ B cells correlated inversely with disease activity only in RTX-treated patients, but the dynamics of CD5+ cells did not predict disease relapse. Todd *et al.* [22] found a numerical, but not functional, deficiency in Breg defined as CD24<sup>hi</sup>CD38<sup>hi</sup> B cells during disease remission in PR3- and MPO-AAV patients, while the frequency of memory cells (CD24<sup>hi</sup>Cd38<sup>lo</sup>) was reduced in active disease and restored during remission. However, no correlation with disease relapse was investigated. In summary, Bregs do not, at present, seem a good biomarker for relapse, but standardization of staining and gating strategies with validation in different cohorts may allow more consistent findings and conclusions to be made.

T cells are also involved in the pathogenesis of AAV, being abundantly present in inflammatory lesions, and have the potential to destroy tissue integrity by driving granuloma formation (in GPA and EGPA), by cytokine production or by direct effect on endothelial cells [23], being the predominant cells in inflammatory tissue infiltrates. The most promising study related to T cells in AAV was published in 2010. McKinney *et al.* demonstrated that a particular gene expression signature seen in CD8+ T cells, markers related to T cell survival and memory T cell, showed a strong association with subsequent disease relapse [24]. If this finding is validated in prospective studies and if it can be adapted into a clinically viable test, then it could help identify a group of patients with greater propensity for relapse, allowing customization of therapy.

Recently, Nasrallah *et al.* identified distinct subsets of granulocytes in patients that participated in the RAVE study, based on granularity index (GI), which measures the difference between the percentage of hypergranular and hypogranular granulocytes. They found that RTX-treated patients who achieved remission had a significantly higher GI at baseline than those who did not ( $P = 0.0085$ ) and that this pattern was reversed in CYC-treated patients ( $P = 0.037$ ). These different granulocyte profiles could be interesting for identifying the patients who best respond to these different therapeutic strategies in AAV [25].

## Markers of vascular activation

**Complement.** In animal models, alternative complement activation has been implicated in pathogenesis of AAV [26], and in

recent work, Xiao et al. proved that blockade of C5a receptor (C5aR) in transgenic mice expressing human C5aR protected against disease activity [27].

Following these discoveries, Gou et al. showed that plasma levels of C3a, C5a, soluble C5b-9 and Bb were increased in 66 patients with active AAV compared with 54 patients in remission [28]. The same group also found a positive correlation between urinary levels of Bb with serum creatinine in patients with active disease and an inverse correlation with the percentage of normal glomeruli in the renal biopsy [29]. These are small studies and the use of complement components as biomarkers in AAV must be evaluated among larger cohorts and in those with non-renal disease. In particular, prospective studies looking at these markers in renal biopsies or in serum need to be carried out to validate these markers.

*Toll-like receptor-4 and receptor for advanced glycation end-products.* The link between infection and AAV is well established. In animal models, toll-like receptor-4 (TLR4) ligation is involved in disease induction [30]. A recent publication [31] demonstrated that high-mobility group box-1 (HMGB1), a typical damage-associated molecular pattern (DAMP) protein associated with inflammatory conditions and tissue damage, is able to prime neutrophils by increasing ANCA antigen translocation, and the primed neutrophils could be further activated by ANCA, resulting in the respiratory burst and degranulation. This process is dependent on TLR4 and receptor for advanced glycation end-products (RAGE) through the MyD88/NF- $\kappa$ B pathway. Along similar lines, calprotectin (S100A8/S100A9), a calcium-binding protein found abundantly in neutrophils, monocytes and early-differentiated macrophages, and an endogenous ligand of TLR4 and RAGE showed interesting results [32–34]. Calprotectin has been shown to be upregulated in many inflammatory disorders, including AAV [35–38]. During remission in patients with limited systemic AAV, Pepper et al. [39] showed that calprotectin levels increased in those who developed a future relapse compared with those who did not, making calprotectin a possible useful biomarker to predict relapses. More recently, analysing 144 patients enrolled in the RAVE trial, it was shown that failure to decrease serum calprotectin by Month 2 or 6 compared with baseline identified a subgroup of PR3-ANCA patients treated with RTX at higher risk of subsequent relapse.

### Panel of biomarkers

Recent studies have tested numerous biomarkers to identify promising tools to identify predictors of treatment response and relapse in patients with AAV.

The most robust study [40] tested 28 markers of inflammation, angiogenesis, tissue damage and repair in 186 patients enrolled in the RAVE study, before and 6 months after treatment in order to distinguish active disease from remission. They identified three promising biomarkers (MMP-3, TIMP-1 and CXCL13) that best discriminated these two conditions. Follow-up studies on clinical relevant endpoints for the prediction of relapse rates, are needed.

### Urinary biomarkers

Urine sediment analysis and proteinuria could be helpful in detecting early relapse in AAV with renal involvement. However, this biomarker is non-specific; proteinuria may also indicate renal fibrosis and haematuria can persist for months or years despite clinical remission [41, 42]. However, two urinary biomarkers showed promising results in AAV as predictors of disease activity.

Monocyte-chemoattractant protein-1 (MCP-1), a chemokine that promotes monocyte recruitment to areas of inflammation, has been shown to be increased in AAV patients in active disease compared with other forms of glomerulonephritis with a specificity of 94% and a sensitivity of 89% (AUC 0.93, positive likelihood ratio: 8.5 and negative likelihood ratio: 0.07), and elevated levels were associated with poor prognosis and relapse [43, 44].

Recently, O'Reilly et al. demonstrated that urinary-soluble CD163, shed by monocytes and macrophages, was markedly higher in patients with active disease compared with patients in remission in a cohort of 177 patients with AAV. With a cut-off of 0.3 ng/mmol creatinine, they found a sensitivity of 83% and a specificity of 96% (positive likelihood ratio of 20.8) for detection of active renal vasculitis [45].

These two biomarkers could have an interesting use for diagnosing renal flare in AAV and to triage patients with undifferentiated acute kidney injury, when serologic markers or histologic samples are delayed.

### Conclusions

Numerous studies of potential biomarkers in AAV comparing active disease with disease remission have been reported. However, clinically useful biomarkers for prediction of relapse and response are still required for better management of patients with AAV. Many of the recent discoveries described in this review require validation in separate cohorts. As new insights into the pathogenesis of AAV emerge, so too new markers and therapies will help guide disease therapy.

### Conflict of interest statement

None declared.

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