Current Rheumatology Reports (2019) 21: 76
https://doi.org/10.1007/s11926-019-0872-3

VASCULITIS (LR ESPINOZA, SECTION EDITOR)

Anti-neutrophil Cytoplasmic Antibodies (ANCA) as Disease Activity Biomarkers in a "Personalized Medicine Approach" in ANCA-Associated Vasculitis

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Published online: 26 December 2019

Abstract

Purpose of Review ANCA-associated vasculitides (AAV) are a group of rare diseases characterized by blood vessel inflammation and the presence of circulating anti-neutrophil cytoplasmic antibodies recognizing proteinase-3 (PR3) (PR3-ANCA) or myeloperoxidase (MPO), MPO-ANCA.

Recent Findings Historically, ANCAs have been used as biomarkers for disease associations and increases of ANCA levels as predictors of relapse in patients with AAV.

Summary In this review, we will summarize and highlight the most recent developments for using ANCA as predictive biomarkers and review some of the important disease-specific features in patients with AAV.

Keywords Vasculitis · Anti-neutrophil cytoplasmic antibodies · Relapse · Remission · Genetics · Glycosylation

Introduction

Over the past 35 years, our understanding of "pauci-immune" vasculitis has evolved from diseases associated with circulating anti-neutrophil cytoplasmic antibodies (ANCA), to diseases where the specificity of ANCA is predictive of specific clinical manifestations, patient prognosis, and probability of disease remission and/or relapse [1]. Granulomatosis with polyangiitis (GPA, formerly Wegener's) and microscopic polyangiitis (MPA) are systemic diseases that result in small-to-medium-sized blood vessel inflammation which may lead to organ and/or life-threatening complications [2]. Both diseases are characterized by circulating ANCA with specificity for either MPO or PR3 [3, 4]. Patients with these diseases are clustered within the spectrum of ANCA-associated vasculitis (AAV). Most patients with GPA have ANCAs that have a cytoplasmic pattern (c-ANCA) on IIF that recognize the serine protease proteinase-3 (PR3) as the dominant antigen, while patients with MPA more often have ANCAs with a perinuclear IIF pattern (p-ANCA) which recognize myeloperoxidase (MPO) [5]. Following the introduction of specific assays that can detect the presence of circulating PR3-ANCA or MPO-ANCA, it has become more feasible to accurately diagnose these conditions early and treat patients with immunosuppressive therapy to prevent life-threatening complications. Also, as relapses are common in a majority of patients, different strategies have been employed using ANCA-specific assays to predict relapses of disease. In other words, a simple and fundamental question that arises is whether ANCA levels or modifications of ANCA be used to predict disease relapse and possibly remission. In this review, we will discuss how ANCA testing can be used for the classification of AAV, predicting patient likelihood of relapse and remission, and how serial ANCA measurement can be useful in specific clinical settings. We will also review how post-translational modifications of ANCA may be utilized for predicting relapse and remission. The choice of therapies for achieving remission has been extensively reviewed in other articles and is beyond the scope of this article.

ANCA Serotype Are Useful for Classifying Patients and Predicting Overall Outcomes Although it is becoming more accepted that patients with PR3-ANCA-associated vasculitis (PR3-AAV) disease have a distinct clinical profile and

This article is part of the Topical Collection on *Vasculitis*

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different genetic risk factors when compared with patients with MPO-AAV, the use of ANCA serotype for the classification of these patients, as suggested more than 25 years ago, has only recently been widely accepted [6]. Historically, GPA was described as a histopathological triad comprised of necrotizing angiitis, granulomatous inflammation, and necrotizing crescentic glomerulonephritis [2]. This triad was implemented in the initial classification criteria for GPA without the use of ANCA serotypes. As it became apparent that many patients did not have histopathological evidence of AAV when diagnosed in an early stage, ANCA serotype was added to histological classification criteria to further aid clinicians in stratifying disease subsets.

Recently, it has been suggested that the presence of either PR3-ANCA or MPO-ANCA is more useful than a diagnosis of GPA or MPA in predicting disease relapse [7]. The authors of this study compared three different classification methods with biopsy-proven AAV: the histological method based on the Chapel Hill Consensus classification (CHCC), a combined histological and serological stratification based on the European Medicine Agency (EMA) classification criteria, or patient classification based solely on ANCA serotype. Interestingly, 78% of the patients that were diagnosed with MPA using the CHCC were re-classified as having GPA when the EMA system was used which may suggest that histopathological diagnoses may not be as specific as once thought. Also, PR3-ANCA was more predictive than the histopathological diagnosis in this study for clinically relevant outcomes even though there were no differences in the relapse risk between GPA and MPA patients.

ANCA serotype has consistently been associated with different clinical outcomes in several studies. Patients with PR3-ANCA have increased risk of having severe inflammatory lung disease (e.g., alveolar hemorrhage) and systemic disease that involve many organs at the time of diagnosis [1, 6, 8], whereas patients with MPO-ANCA are more likely to have renal-limited disease and/or lung fibrosis [1, 6].

AAV increases the overall death from cardiovascular complications (ischemic stroke and coronary artery disease) compared with age and gender-matched controls [9–11]. It has been postulated that ongoing microvascular inflammation may result in endothelial cell dysfunction which in turn results accelerated atherogenesis and poor cardiovascular outcomes. This is supported by studies in AAV in which patients were assessed for cardiac involvement by different imaging modalities [12, 13]. Patients with AAV had a higher rate of cardiac involvement compared with age matched controls using echo (46%) and cardiac MRI (62%) independently of clinical symptoms or disease activity [12] which may suggest that subclinical endothelial cell dysfunction in AAV patients may drive cardiovascular progression especially when they have an increased risk of metabolic syndrome [14]. Future studies assessing cardiovascular outcomes in AAV patients treated with "glucocorticoid light" regimens may be instrumental in better defining this phenomenon as glucocorticoids are also known to promote cardiovascular disease in other rheumatic diseases such as systemic lupus erythematosus [15, 16]. Indeed, a recent pilot study using a reduced course of glucocorticoids suggested that using such a regimen is plausible in many patients with AAV treated with rituximab (RXB) [17, 18].

ANCA Relapse and Genetic Associations

Patients with increasing PR3-ANCA titers have an increased risk for relapse when they have renal or alveolar hemorrhage compared with patients that do not [8, 19]. This observation may reflect differences in the pathogenesis of systemic disease and limited disease, which is corroborated by animal studies where ANCA alone is sufficient to induce necrotizing vasculitis, but not granulomas [20]. Recent genetic studies support the notion that PR3-AAV patients have more systemic immune dysregulation involving antigen-presenting cells (e.g., dendritic cells, B and T lymphocytes) than MPO-AAV patients. For example, one of the strongest associations for relapse is the HLA-DPB1*0401 (HLA-DPB4), which varies in frequency in populations of different ethnicity [21]. Like other HLA alleles, it is also expressed in a co-dominant way, which may explain why patients that are homozygous in this allele carry the highest risk of relapse compared with patients that are heterozygous or non-carriers (Fig. 1). The presence of the *HLA-DPB4* allele was predictive of relapse regardless of ANCA specificity in Caucasian patients, suggesting that antigen-presenting cells expressing HLA-DPB4 may promote loss of immune homeostasis [21]. Other immunoregulatory alleles that are associated with an increased risk for PR3-ANCA and T cell dysfunction include cytotoxic T lymphocyte antigen 4 (CTLA) 4, which when lost promotes systemic immune dysregulation and dysregulation both in animal models and in patients genetically deficient in these proteins [22]. Interestingly, the single-nucleotide-linked polymorphism (SNP) for *CTLA4* allele associated with ANCA (rs3087243) resulting in increased expression was protective [23] (Fig. 1).

Likewise, an SNP associated with decreased expression in program death 1 (PD1), a key regulator of peripheral immune homeostasis, is associated with ANCA [22]. Of note, PD1 expressed on circulating T cells is functionally impaired in PR3-ANCA [24]. Clinically, decreased PD1 expression or impaired functionality may potentiate ANCA vasculitis particularly in patients exposed to chronic inflammatory signals such as those with exposure to silica [25]. This observation may also explain the diminished potency of regulatory T cells in patients with PR3-ANCA which express lower levels of PD1 [24]. It is also supported by the development of PR3-ANCA in patients treated with monoclonal antibodies

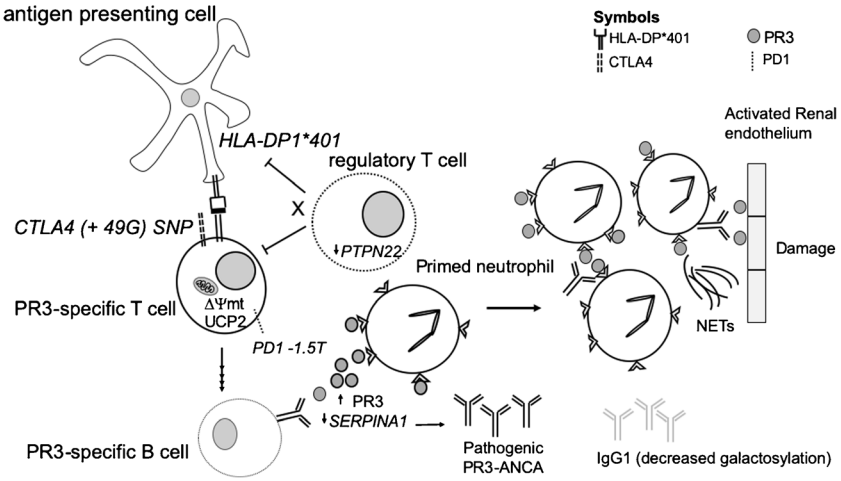

Fig. 1 Genetic factors regulating immune cell homeostasis and dysregulated immunoglobulin glycosylation are important mediators of disease relapse in PR3-ANCA vasculitis. Patients at risk of relapse more commonly express specific HLA polymorphisms (*HLA-DPB1*401*) and less commonly express functional immunoregulatory receptors (*CTLA4 + 49G* and *PD1 -1.5 T*) on antigen-presenting cells and T lymphocytes, respectively. Altered mitochondrial suppression in patients with PR3-ANCA may also increase their risk of relapse. Regulatory T cells in relapsing patients are less functional by virtue of decreased PTPN22 expression. Neutrophils in relapsing patients are more readily primed which stems from an imbalance in PR3 activity (from decreased SERPINA1 levels), higher PR3 expression, decreased immunoregulatory antibodies with lower levels of glycosylation, and increased levels of pathogenic conformational PR3-ANCA. Together, these factors culminate in increased risk of renal damage and clinical relapse in patients with PR3-ANCA. ANCA, anti-neutrophil cytoplasmic antibody; CTLA4, cytotoxic T lymphocyte activation protein 4; HLA, human leukocyte antigen; ΔΨmt, mitochondrial membrane potential; NETs, neutrophil extracellular traps; PD1, program death 1; PR3, proteinase 3; PTPN22, protein tyrosine phosphatase 22; UCP2, uncoupling protein 2

targeting PD1 [26]. Other important genetic contributors to the pathogenesis of PR3-ANCA include the protein phosphatase *PTPN22* where a loss of function SNP (rs2476601) is associated with the disease (Figs. 1 and 2) [23]. Decreased expression of PTPN22 may result in decreased T_{reg} differentiation as supported by in vivo animal studies [27]. Finally, patients with GPA carry a higher frequency of SNP polymorphisms in proteinase 3 (*PR3)*, the antigen targeted by PR3-ANCA and alpha-1-antitrypsin (*SERPINA1*), a protein that negatively regulates extracellular PR3 function, compared with MPO patients and healthy controls [28]. Thus, the pathogenesis of AAV, and possibly relapses associated with PR3-ANCA, may be in part determined genetically. With genetic tests being more accessible and more cost effective, future studies assessing multiple loci in a given patient may be used to identify patients at increased risk of relapse, and possibly those that warrant frequent serial assessments to predict severe systemic vasculitis relapses, and renal vasculitis.

The risk of relapse of in PR3-ANCA ANCA has recently been associated with metabolic syndrome [14] which may reflect abnormal metabolism at the cellular level which is governed by mitochondria (Figs. 1 and 2). Recently, mitochondrial dysfunction has been linked to the development of many inflammatory diseases [29]. Suppression of mitochondrial function is also one of the earliest steps in immune cell activation [29]. One of the important pathways regulating mitochondrial function is uncoupling protein 2 (UCP2) which is required for transporting calcium into mitochondria [30]. Loss of UCP2 results in decreased mitochondrial aerobic respiration as a result of decreased calcium in the mitochondrial matrix [30]. Interestingly, patients carrying a loss of function single nucleotide polymorphism (SNP) in the *UCP2* promoter resulting in decreased mRNA expression have an increased risk of metabolic syndrome and inflammatory diseases such as multiple sclerosis [31–33], whereas those carrying an SNP resulting in higher *UCP2* expression less frequently develop AAV [32], cardiovascular disease, and metabolic syndrome [31]—suggesting that mitochondrial dysfunction may be another factor important in the pathogenesis of ANCA (Fig. 1). Future studies assessing the risk of relapse with mitochondrial dysfunction may provide a novel perspective in this area as mitochondria are key regulators of epigenetic modifications (e.g., protein acetylation) and inflammation via the NLRP3-inflammasome [29], and many PR3-ANCA patients with a high risk of relapse are subjected to stimuli resulting in aberrant inflammatory signals such as exposure to silica [25] and nasal carriage of *Staphylococcus aureus* [34, 35].

Serial ANCA Measurements and Relapse

Many studies have assessed the utility of using ANCA levels as markers of disease remission.

Remission in AAV is defined when there is a complete absence of clinical disease activity and patients are using less than 7.5 mg equivalents of daily prednisone either without

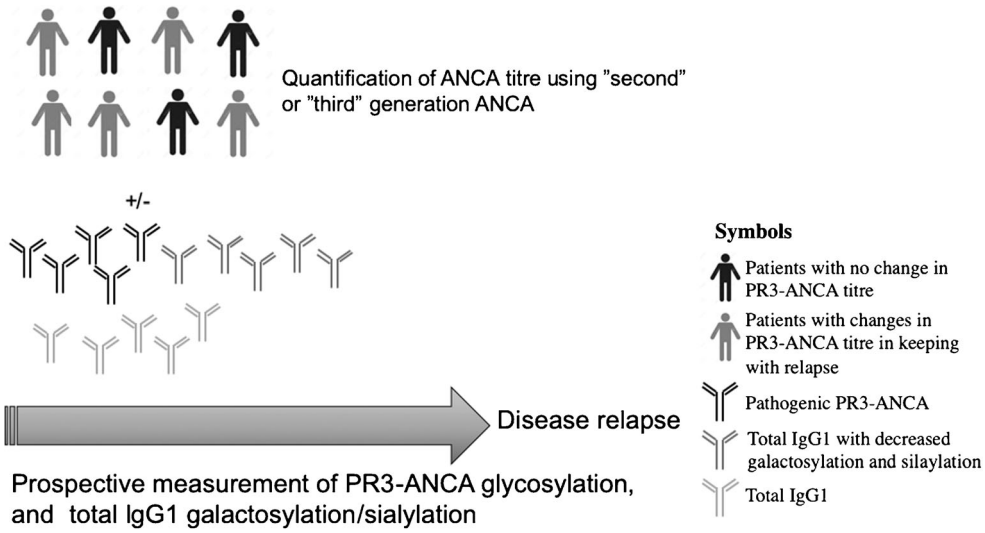

Fig. 2 Personalized medicine strategy for identifying patients at risk of relapse using PR3-ANCA. Patients at risk of renal relapse have increasing PR3-ANCA titers, decreased total IgG glycosylation, and increased conformation-specific "pathogenic ANCAs." A future prospective study incorporating both strategies may identify patients at highest risk of relapse

other immunomodulatory therapies or on a maintenance therapy (e.g., azathioprine, methotrexate, RXB) [36]. In contrast, relapses are defined as patients that are demonstrating new and/or relapsing organ manifestations and are requiring an escalation in immunosuppressive therapy and/or an increase in prednisone [36]. ANCA titers often decrease after the initiation of induction therapy (e.g., cyclophosphamide or rituximab) [37, 38]. Furthermore, in many patients, ANCA become undetectable during follow-up. The absence of ANCA is not a prerequisite for patients to be labelled as being in a complete remission, although recently the absence of detectable PR3-ANCA in patients with GPA treated with rituximab resulted in longer lasting remission [39]. Importantly, an increase in ANCA titer for an individual patient once remission is achieved is useful in predicting relapses in patients with ANCA, particularly in patients with renal disease [8, 19]. Patients with GPA complicated by a renal relapse are almost always positive for ANCA [5, 37], and many patients with renal disease are carriers for genetic susceptibility loci for relapse [21]. Approximately 45% of patients with PR3-ANCA and renal involvement become seronegative following the induction of remission using rituximab [5, 37]. As many of the genetic loci associated with PR3-ANCA are important regulators of immune homeostasis and T cell activation (e.g., *HLA-DRB1*, *CTLA4*, and *PD1*), increased PR3-ANCA titers may represent a higher state of immune dysregulation and loss of ANCA may similarly reflect restoration of immune homeostasis particularly in PR-3-AAV patients with renal disease.

Since initially proposed [38, 40], using serial ANCA measurements for predicting disease relapse has been highly debated. This may stem from several issues which are underscored by the heterogeneity in the definition of relapses in many of these studies and the limited number of studies that prospectively assess the utility of ANCA in predicting relapse.

In the 1980s, van der Woude et al. suggested that ANCA might be a useful biomarker in assessing disease relapse [38, 41]. As a result, a double-blinded prospective study was conducted whereby ANCA titers were measured every month in patients with PR3-ANCA (most of which had renal involvement) [42]. During the 16-month follow-up period, 18 of 35 patients demonstrated a rise in ANCA levels and in 17 of 18 patients, relapse followed. Subsequently, in a randomized controlled study, patients with an increase in ANCA titers were randomized to receive pre-emptive cyclophosphamide and corticosteroid therapy or no therapy until clinical relapse occurred [40]. During the 24-month study period, 20 of 58 patients had ANCA rise, and the 9 patients randomized for pre-emptive treatment did not relapse, whereas nine out of 11 patients that were not treated relapsed [40]. This observation was confirmed by a different group in a subsequent unblinded study where 8 patients not receiving pre-emptive therapy all relapsed during the study follow-up period, while only two out of 11 patients relapsed in the pre-emptive study arm [43] suggesting that changes in ANCA predict changes in disease activity.

In a study by Kemna et al., 72 consecutive patients with renal involvement and PR3-ANCA or 36 patients with only PR3-ANCA and no renal involvement were prospectively followed over approximately 50 months and had serial ANCA measurements [19]. ANCA rises correlated with relapses in patients with both PR3-ANCA and renal involvement (HR 11.09), but not patients with PR3-ANCA (HR 2.79) [19]. The predictive value of PR3-ANCA titers for patients with pulmonary and/or renal involvement was confirmed in the RXB vs. cyclophosphamide in ANCA-associated vasculitis (RAVE) [8]. In this study, 55 of 93 patients relapsed of which an increase in ANCA titer as measured by direct ELISA or capture ELISA corresponded to a severe relapse within 1 year in patients with renal involvement (HR 7.94, $P < 0.001$) or alveolar hemorrhage (HR 5.8; $P = 0.002$). In the recently published MAINRITSAN2 study, a fixed RXB infusion regimen (500 mg every 6 months after induction, 81 patients) was compared with patients that only

received rituximab if their B cell numbers were detectable post induction and/or the ANCA titers increased from baseline (i.e., it was positive when previously negative, or the ELISA titer for PR3-ANCA or MPO-ANCA doubled, 81 patients (tailored regimen)) [44]. Only a fraction of the patients were positive for PR3-ANCA at inclusion (26 and 22 % in the fixed vs. tailored regimens, respectively). Ten out of thirteen patients relapsed in the tailored arm, while three out of 8 patients relapsed in the fixed treatment regimen (a non-statistically significant difference). This study supported the notion of using biological disease-specific markers, i.e., circulating B cell re-emergence post-depletion or an increase in ANCA titre, as tools for deciding when to administer RXB as this approach was non-inferior to a standard approach, i.e., "a fixed regimen." It also highlights the importance of B cells and ANCA as important pathogenic mediators of the disease (Fig. 1). Future studies implementing biological disease-specific outcomes are currently underway. For example, COMBIVAS is a randomized, double-blinded, placebo-controlled trial where patients with active severe (newly diagnosed or relapsing AAV) and PR3-ANCA will be treated with both belimumab and RXB (compared with placebo). Interestingly, the study's primary outcome is the time to PR3-ANCA negativity which may highlight the importance of PR3-ANCA titer in predicting a sustained remission [39].

A rise in the type of ANCA (e.g., PR3 or MPO) may not be as important as patients that become positive after a period of previous negativity for predicting relapse [19]. This may stem from the role of genetic factors on the type of ANCA. For example, Japanese patients do not commonly carry the HLA-DRB SNP associated with PR3-AAV in Caucasians [45], which may explain why they rarely develop PR3-AAV. In contrast, Japanese patients more commonly develop MPO-AAV [46]. An increase in MPO-ANCA is also associated with relapse [19, 47, 48]. For instance, in a Japanese study, nearly half of the patients (48%, 46/118) became positive for MPO-ANCA prior to relapse during follow-up [47].

Serial ANCA Measurement, Methodologies, and Relapse

The method of ANCA testing may influence the performance of serial ANCA testing for defining an increased risk for a severe vasculitic relapse. IIF is inferior for quantifying ANCA levels [19, 48]. Most studies have used first-generation ANCA assays, i.e., direct enzyme-linked immunoassay (ELISA) methods, which are less reliable in predicting relapses compared with second-generation "capture ELISA"–based methods and third-generation "anchor"–based methods [5]. There are no additional benefits for monitoring ANCA of the IgG subclass and for ANCAs that recognize the zymogenic form of PR3 [5].

The increased sensitivity for detecting severe vasculitis relapses in PR3-ANCA patients using increases in ANCA titers also relies on two additional parameters: frequency of serial measurements and definitions of cut-offs of an ANCA rise [19]. A higher association between disease relapse and serial measurements was observed when at least four measurements per year were obtained (HR 6.40 [95% CI 3.45–11.88]) compared with patients who were tested less frequently (HR 2.13 [95% CI 0.67–6.77]) [19]. However, more frequent sampling may not always be feasible. Also, improved association with relapse and increased titers was achieved using a receiver operating characteristic curve (ROC) to define the most optimal cut-off value [19, 48, 49]. In this method, the cut-off value closest to the upper left corner is chosen (the cut-off value closest to 100% sensitivity and 100% specificity).

ANCA Conformation, Avidity, Glycosylation, and Relapse

Post-translational modification is important for the recognition of PR3 by pathogenic antibodies derived from patient sera. These antibodies recognize the mature form of PR3 which is formed after the protein is cleaved at two sites then forms 4 disulfide bonds which hold the protein in its native conformation [50–53]. One critical modification is N-linked glycosylation of PR3 at two sites as suggested by in vitro generation of recombinant PR3 in an insect expression system (lacking mammalian glycosylation) results in decreased enzymatic activity of PR3 but retains ANCA binding [54]. Also, patients with relapsing renal vasculitis had PR3-ANCA with a higher avidity than patients with limited disease and patients that are non-relapsing [55]. In other words, pathogenic ANCAs are likely conformational and are only able to bind to the mature form of PR3, and this increased binding reflects an important aspect of their pathogenicity. This observation may provide an explanation for why direct ELISA or IIF methods for measuring ANCA are less reliable than capture or anchor ELISA for measuring disease relapse (Fig. 1). Like PR3, PR3-ANCAs are also glycosylated. In fact, patients with AAV with decreased levels of total IgG1 antibody galactosylation, but not ANCA IgG1, had an increased likelihood of a disease flare [56] and neutrophil oxidative burst [57] suggesting that ANCA may be modified in patients at risk for a disease flare, although more prospective studies are needed to further validate this. Specifically, antibodies from patients with active vasculitis more frequently had decreased 2,6-linked sialylated modifications within the Fc and the variable regions of the antibodies [56–59], while those with less active disease had more heavily glycosylated antibodies. Interestingly, antibodies that are more sialylated promote less activating leukocyte functions suggesting that they have anti-inflammatory properties [60]. Future studies exploiting

specific PR3 conformational epitopes or glycoforms may prove to be important in detecting pathogenic ANCA that are directly associated with disease activity. Alternatively, specific peptibodies or peptides targeting pathogenic ANCA that are linked to an antibody backbone may be a novel therapeutic approach that can help reduce self-reactive B cells in patients with AAV among other personalized medicine approaches (Fig. 2).

Conclusions

AAV continues to be a life-threatening disease characterized by a high amount of patient-related morbidity and mortality. The development of novel diagnostic and prognostic biomarkers in AAV has been an active area of research. ANCA remains the most important biomarker that is used in disease classification and predicting relapses in patients with more severe vasculitic manifestations such as alveolar hemorrhage and renal vasculitis. When combined with post-translational modifications and possibly genetic backgrounds in a personalized medicine approach, ANCA titers may even be more informative in predicting relapses (Fig. 2). Future proof-of-principle studies incorporating these components may provide more insight into the role of this combined strategy in patient care and follow-up, which can then be validated in large clinical trials.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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