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Epidemiology and pathogenesis of ANCA-associated vasculitis

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ABSTRACT

Antineutrophil cytoplasmic antibody **(ANCA)** associated vasculitis **(AAV)** represents a heterogeneous group of rare diseases characterized by necrotizing inflammation of small blood vessels and the presence of ANCA with specificity for proteinase-3 **(PR3)** or myeloperoxidase **(MPO)**. Genetic susceptibility along with environmental exposures to agents such as infections, silica or drugs is involved in the disease process. To date, growing evidence reveals that ANCA specificity defines better than clinical diagnosis homogeneous groups of patients, as PR3 ANCA and MPO-ANCA are linked with different genetic backgrounds and epidemiology. Also, although ANCAs were initially used to classify pauci-immune vasculitis, increasing clinical and experimental evidence now suggests their pathogenic role. ANCA-neutrophil interactions induce critical cellular responses that result in highly inflammatory necrotizing vascular damage. Activation of the complement system, mainly via the alternative complement pathway, and a no adequate T cell and B cell regulation of the ANCA autoimmune response also plays a crucial role in the pathogenesis of AAV. This review summarizes the central pathogenesis concepts of AAV and highlights the value of ANCA specificity for future classification criteria.

Keywords: Antineutrophil cytoplasmic autoantibodies **(ANCA)**, Antineutrophil cytoplasmic antibody-associated vasculitis **(AAV)**, Epidemiology, Genetics, Pathogenesis.

INTRODUCTION

The anti-neutrophil cytoplasmic antibody **(ANCA)** associated vasculitides **(AAVs)** are rare, potentially life-threatening multisystemic autoimmune diseases, identified by the nomenclature of the 2012 revised International Chapel Hill Consensus Conference **(CHCC)** as a specific group of small vessel vasculitides **(SVVs)** different from the immune complex-mediated vasculitides [1] **(**Figure 1**)**. According to their clinical and pathological features, AAVs have been traditionally subclassified into three clinicopathologic variants: i) granulomatosis with polyangiitis **(GPA)**, formerly Wegener's granulomatosis ii) microscopic polyangiitis **(MPA)** iii) eosinophilic granulomatosis with polyangiitis **(EGPA)**, formerly Churg-Strauss syndrome [1]. However, the traditional division of AAVs based on clinical phenotype has been recently challenged on epidemiological and genetic grounds. Accumulated evidence now suggests that ANCA specificity, against the two major target antigens: myeloperoxidase **(MPO)** and proteinase 3 **(PR3)**, could be better define homogeneous groups of patients than those identified by clinical diagnosis [2,3].

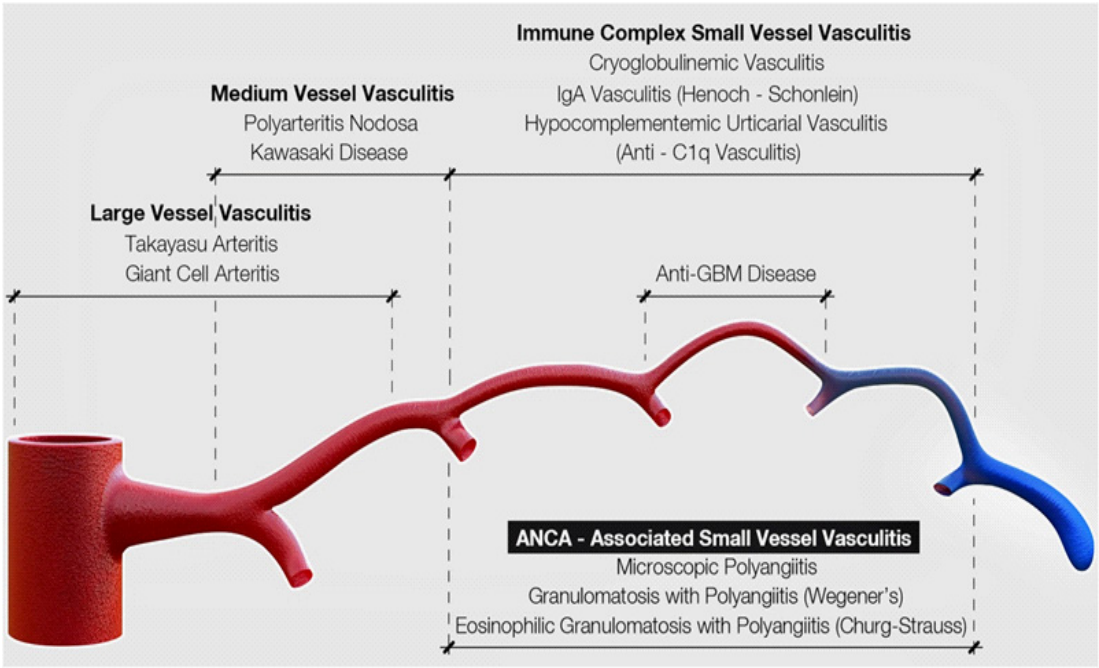

Figure 1: Distribution of vessel involvement by large vessel vasculitis, medium vessel vasculitis, and small vessel vasculitis. The diagram depicts (from left to right) aorta, large artery, medium artery, small artery/arteriole, capillary, venule, and vein. Anti-GBM: anti–glomerular basement membrane; ANCA: antineutrophil cytoplasmic antibody.

As with most autoimmune diseases, the etiopathogenic process of AAV is likely to be multifactorial and varies among individuals and may involve a complex interplay between innate and acquired characteristics of the immune system, as well as environmental exposures,

infections, and genetic influences. Clinical and experimental data support that ANCA, in addition, to be an important serological biomarker for the diagnosis and classification of the disease, also plays a central role in the pathogenesis of AAV by promoting the recruitment of neutrophils and monocytes, and their adhesion to endothelial cells thereby causing extensive tissue damage [4,5]. Additionally, ANCAs are capable of inducing NETosis in neutrophils, as neutrophil extracellular traps **(NETs)** are visualized at the sites of vasculitic lesions. In turn, NETs might also be important for the generation of ANCAs [6]. Furthermore, the ANCA autoimmune response is facilitated by a defect of adaptive immunity with insufficient T-cell and B-cell regulation [7,8]. More recently, both in vitro and in vivo experimental data and histopathological studies from AAV patients highlighted the role of the alternative complement pathway, and particularly of C5a, in the pathophysiology of AAV [9].

In this review, we summarize the mechanisms and pathways of immunogenesis and pathogenetic mechanisms involved in AAVs and discuss how current epidemiological and genetic data could be the guide for an improved classification system based on ANCA specificity.

EPIDEMIOLOGY OF ANCA ASSOCIATED VASCULITIS

The annual incidence of AAVs ranging from 13 to~20 cases per million individuals and the prevalence from 46 to~184 cases per million individuals worldwide. EGPA is less common than GPA or MPA with an annual incidence in the range of 0.5-2.0/million and a prevalence of 10-45/million. The gender distribution is fairly similar, and the peak incidence occurs in the middle of the sixth decade of life [10,11]. Over the past few years, the increasingly well-documented epidemiology of AAVs highlighted geographical and ethnic differences according to clinical variants and their associations with ANCA subtypes. GPA is commoner in the north of Europe, while MPA is more common in the south of Europe [12]. The populations studied in the Western USA, Australia, and New Zealand were predominately white Caucasian of North European descent, and the incidence of GPA has been reported very similar to Europe [11]. A multiethnic population study conducted in Paris reported that the prevalence of AAVs in individuals of European descent **(104.7/million)** was twice that of non-Europeans **(52.5/million)**. In the same study, GPA was less frequent in people of non-European ancestry than MPA, and none of the GPA patients came from Africa [13]. In a multi-ethnic series from Chapel Hill in the USA, GPA was also relatively uncommon in African Americans compared to whites [14]. Two geographical regions with an apparently high incidence of MPA and much less GPA cases are Japan and Kuwait [15,16]. Large case series from China also reported that MPA is more common than GPA in that country [17].

In European studies, the ANCA antigen specificity of GPA is most frequently associated with PR3, and in MPA with MPO. However, the overlap between ANCA specificity and the clinical syndrome is only partial. PR3 ANCAs are present in about two-thirds of patients with GPA, but also in one-fourth of patients with MPA, whereas MPO-ANCAs are present in the majority of patients with MPA, but also in up to one-fourth of patients with GPA [18]. In China, 60% of GPA

patients have MPO-ANCA [19]. In Japan, MPO-ANCA is present in 83% of cases of AAV, including a significant proportion of those with GPA [15].

Recent and future developments in knowledge of the genetic background could explain these geographical and ethnic differences and help us to a better understanding of the disease pathogenesis.

PATHOGENESIS OF ANCA ASSOCIATED VASCULITIS

Cause and effect are still an elusive link in the AAV. Presumably, like in most autoimmune conditions could represent the interaction between predisposing genes and triggering environmental factors, leading to the loss of tolerance against self-antigens. Then, the presence of increased levels of autoantibodies causes a consequent immune-mediated destruction of autologous cells and tissues.

Genetic Associations in ANCA Associated Vasculitis

Over the years, several studies based on candidate gene approaches have been performed in the field of AAV genetics. However, many of these studies were small and not replicated. Our knowledge has dramatically increased after the publication of two genome-wide association studies **(GWASs)**. The two GWASs were conducted respectively by the European Vasculitis Genetic Consortium **(EVGC)** [20] and the US Vasculitis Clinical Research Consortium **(VCRC)** [21].

The EVGC GWAS was performed in a cohort of UK AAV patients and matched controls. This analysis reported both major-histocompatibility-complex **(MHC)** and non-MHC associations with AAV. Subsequently, 158 single nucleotide polymorphisms **(SNPs)** were genotyped in a replication cohort of Northern European AAV patients and controls, to confirm the GWAS data. Combined analysis of the two cohorts showed that the strongest genetic associations were with the antigenic specificity of ANCA **(PR3 or MPO)** rather than the clinical syndrome **(GPA or MPA)**. HLA-DP, Serpin peptidase inhibitor clade A member 1 **(SERPINA1)** which encodes alpha-1 antitrypsin **(AAT)**, a serine protease that represents the major inhibitor of PR3 activity, and Proteinase 3 **(PRTN3)** which encodes PR3, were in association with GPA and stronger association with PR3-AAV. Their specific loci and single nucleotide polymorphisms **(SNPs)** are given as follows:

- HLA-DP **(**6p21.32, SNP rs3117242**)**
- PRTN3 **(**19p13.3, SNP rs62132295**)**
- SERPINA1 **(**14q32.13, SNP rs7151526**)**.

A weaker association was found between MPO-AAV and HLA-DQ **(**6p21.32, SNP rs5000634**)**.

The VCRC GWAS was effectuated in a population of only GPA patients and controls of European ancestry. The strongest association was with HLA-DPB1 **(**6p21.32, SNP rs9277554**)** and HLA-DPA1 **(**6p21.32, SNP rs9277341**)**, and even more significant among the PR3-cANCA

positive subgroup. An independent, single SNP **(**rs26595**)** near semaphorin 6A **(SEMA6A)** on chromosome 5, was also associated with GPA, reaching genome-wide significance [21], but this was not confirmed in an independent cohort of European patients [22]. The association between SERPINA1 and GPA was also confirmed in this GWAS [21].

Interestingly, the allele HLA-DRB1*15 has been associated with PR3-AAV only in African Americans [14], whereas HLA-DRB1*1202 has been associated with PR3-AAV in a population of Han Chinese [23]. Furthermore, it is now possible to suppose that the almost rare appearance of PR3-AAV in China and Japan is due the low frequency of the HLA-DPB1*0401 and the PI*Z allele of SERPINA1 genetic variants [11].

A recent meta-analysis performed by EVGC on 62 studies, including the 2 GWASs, identified 33 genetic variants, in or near 15 genes, associated with AAV [24].

CD226 antigen **(CD226)**, Cytotoxic T lymphocyte-associated antigen-4 **(CTLA-4)**, Fc fragment of IgG receptor IIa **(FCGR2A)**, HLA-B, HLA-DP, HLA-DQ, HLA-DR, Hydroxysteroid 17-beta dehydrogenase 8 **(HSD17B8)**, Interferon regulatory factor 5 **(IRF5)**, Protein tyrosine phosphatase non receptor type 22 **(PTPN22)**, Ring finger protein 1 **(RING1)**, Retinoid X receptor beta **(RXRB)**, Signal transducer and activator of transcription 4 **(STAT4)**, SERPINA1 and Toll-like receptor 9 **(TLR9)**. Moreover, genetic distinctions between GPA and MPO and between PR3-AAV and MPO-AAV were identified [24].

All these data provide clear evidence that GPA and MPA have distinct genetic associations and support the concept that PR3-AAV and MPO-AAV are distinct autoimmune syndromes.

Environmental Associations and Immunogenicity of ANCA

Several microbial and non-microbial environmental factors, like silica and drugs, have been linked to AAVs (Table 1).

Table 1: Environmental associations with ANCA formation and AAV.

Silica [49-54]
Infectious agents [30-37]
Bacterial species
Staphylococcus
Streptococcus
Enterococcus
Escherichia coli
Klebsiella pneumoniae
Virus species
Parvovirus B-19
Epstein-Barr virus
Ross River Virus
Protozoa
Entamoeba histolytica
Drugs [55-58]
Antibiotics
Cefotaxime
Minocycline
Antithyroid drugs
Benzylthiouracil
Carbimazole
Methimazole
Propylthiouracil
Anti-tumor necrosis factor agents
Adalimumab
Etanercept
Infliximab
Psychoactive agents
Clozapine
Thioridazine
Miscellaneous drugs
Allopurinol
D-Penicillamine
Hydralazine
Levamisole
Phenytoin
Sulfasalazine

Infections

The seasonal or temporal oscillations of disease incidence, reported in various epidemiological studies [25-29], could be suggestive of an infectious agent. Particular attention has been given to Staphylococcus aureus **(S. aureus)** since, in patients with GPA, chronic nasal carriage of S. aureus correlates with relapsing disease [30,31]. Conversely, antimicrobial therapy with co-trimoxazole leads to substantial reduction in the relapse incidence [32]. These findings are supported by the fact that the immunization of rats with bacterial proteins derived from S.aureus has been shown to cause AAV [33]. Other infectious agents have also been associated with the onset of AAV, especially in patients with subacute bacterial endocarditis. In an analysis of 50 AAV case reports, three species of bacteria: Streptococcus, Staphylococcus, and Enterococcus, were associated with

63% of the reported episodes of infectious endocarditis and 42% of all infections reported [34]. Among viruses suspected to play a pathogenic role in AAV, parvovirus B19 [35], Epstein-Barr virus [36], Ross River Virus are reported [37].

The involvement of infectious agents in the pathogenesis of AAV could be explained through a process known as "neutrophil priming ." Infections induce the production of proinflammatory cytokines as Tumor necrosis factor alpha **(TNF-a)**, Interferon gamma **(IFN-γ)** and Interleukin **(IL-1)**, leading neutrophils and monocytes in a pre-activation ("primed") state by the migration of autoantigen targets **(PR3 and MPO)** to the cell surface in order to get activated by ANCA [38,39].

Infectious agents can also be involved in the immunogenicity of ANCA. The so-called "autoantigen complementarity theory" argues that the initial immune reaction is developed versus a complementary peptide **(c-peptide)** to the self-antigen (original autoantigen). The c-peptide could be produced from the transcription of an endogenous

antisense strand of DNA or could be a mimic of an antisense peptide that is produced by a pathogen. Therefore, the initial immune reaction versus the c-peptide results in the formation of anti-c-peptide antibodies which in turn produce anti-idiotypic antibodies that identify not only the idiotope on the anti-c-peptide antibodies but also the epitope on the sense peptide (original autoantigen) [40]. Numerous microbial homologs of the complementary PR3 **(c-PR3)**, including antigens from microbes established to be correlated with GPA and PR3-ANCA, such as Staphylococcus aureus, Ross River virus and Entamoeba histolytica have been described [41].

An alternative theory based on molecular mimicry between bacterial and self-antigens have been proposed as a mechanism for the immunogenesis of ANCA. Kain et al [42] reported the presence of ANCA against lysosomal membrane protein-2 **(LAMP-2)** in about 90% of patients with active ANCA associated with necrotizing crescentic glomerulonephritis **(NCGN)**. More importantly, the researchers showed that human LAMP-2 has 100% homology with the bacterial adhesin FimH, which is found in Gram-negative bacteria such as Escherichia coli and Klebsiella pneumonia. Accordingly, rats immunized with FimH developed pauci-immune NCGN and antibodies to rat and human LAMP2 [43]. However, this finding was not confirmed by Roth et al in another animal model using Wistar-Kyoto **(WKY)** rats [44]. Therefore, the pathogenicity of LAMP2 antibodies remains uncertain. Recently, in another experimental model of molecular mimicry, Kim et al. [45] showed that immunization of C57BL/6 mice with a recombinant serine protease of Saccharomonospora Viridis, which has >30% homology with human PR3, produced high levels of ANCA against mouse PR3.

Also, as stimulation of Toll-like receptor 9 **(TLR-9)** by bacterial DNA, is known to promote B cell proliferation and antibody production [46]. Hurtado et al [47], investigated and confirmed the possibility that unmethylated CpG oligodeoxynucleotides, found in bacterial and viral DNA, stimulate circulating autoreactive B cells to produce ANCA in patients with AAV.

Silica

Silica exposure has been associated with several autoimmune disorders [48], and various case-control studies have documented that 22-60% of patients with AAV, principally those with MPO positivity, were previously exposed to silica [49-51]. Hogan et al showed that there is an enhanced risk for the disease with long lifetime silica exposure, but there is no evidence of a correlation between the length of time since last exposure and disease onset [51]. It has been suggested that silica exposure could evoke an accelerated apoptosis of polymorphonuclear leukocytes and macrophages. The release of MPO could then be taken up by alveolar macrophages and presented to immunocompetent cells, resulting in the development of anti-MPO antibodies [52]. Furthermore, in vitro studies showed that silica could activate apoptosis in human peripheral blood lymphocytes, with an increase in Fas-mediated cell death [53]. Fas expression was significantly higher in regulatory T cells **(Treg)** for silicosis patients compared with healthy donors, and when cultivated with an agonistic anti-Fas antibody, Treg from silicosis patients also expressed a higher degree of apoptosis [54]. Consequently, the reduction of Treg function makes these patients more sensitive to a disruption of self-tolerance.

Drugs

Drug-induced AAV has also been described (Table 1). The most common drug implicated with AAV is propylthiouracil **(PTU)**, described in 80-90% cases of AAV induced by antithyroid drugs [55]. Evidence for an association with the development of drug-induced AAV has also been shown for the following drugs: hydralazine, minocycline, levamisole-adulterated cocaine, anti-tumor necrosis factor-a **(TNF-a)** agents (Adalimumab, Etanercept, and Infliximab), sulfasalazine, D-penicillamine, allopurinol, psychoactive agents (clozapine, thioridazine), cefotaxime and phenytoin [56-58]. However, in most cases, the evidence is restricted only to case reports and the causative link is much less convincing.

PTU is used to treat hyperthyroidism due to its ability to inactivate thyroid peroxidase **(TPO)**. Human MPO and TPO nucleotide sequences display an average similarity of 46%, which becomes 58% within the sequence encoding the functional MPO protein, while the similarities of the amino acid sequences are 44% [59]. Then, PTU treatment could reduce or competitively inactivate the oxidation activity of MPO in a dose-dependent manner, also altering its configuration [60-62]. The altered MPO might, therefore, serve as a neoantigen. Furthermore, Jiang et al [63] proposed that the offending drugs such as PTU and hydralazine, in the presence of extracellular hydrogen peroxide, could be transformed into cytotoxic metabolites by neutrophil-derived MPO. In such conditions, the drugs and their metabolites are immunogenic for T cells, which in turn activate B cells to produce ANCA [64].

Hydralazine, a DNA methylation inhibitor with suspected reverse epigenetic silencing properties, has been shown to increase also the expression of MPO and PR3 autoantigens in neutrophils [56]. While some drugs such as sulfasalazine could activate neutrophil apoptosis,

which in the absence of priming is related to translocation of ANCA antigens to the cell surface and a subsequent production of ANCA [65].

ANCAs with specificity to human neutrophil elastase **(HNE)** are rare and infrequently detected in patients with AAV, but importantly might be useful for the diagnosis of cocaine-induced midline destructive disease and other drugs associated with AAV as PTU and hydralazine [56,66].

Pathogenicity of ANCA

Evidence for the pathogenicity of ANCA derives from clinical observations, in vitro experiments and animal models.

Clinical Observations Supporting the Pathogenicity of ANCA

After successful induction of remission, ANCA titers decrease or disappear in many patients. The efficacies of anti-B cell therapy (Rituximab) and of plasma exchange, which reduce circulating ANCA titers, are consistent with an essential role for ANCA in the pathogenesis of the disease [67-69]. However, clinical studies have failed to show a definitive correlation between ANCA levels and disease activity or disease relapse after remission [70-73]. The most convincing clinical evidence that ANCAs are pathogenic derives from two reports of MPO-induced pulmonary-renal disease following a passive transplacental transfer of maternal MPO-ANCA IgG to human neonates [74,75]. In contrast, no clear reports exist of similar disease induced by transfer of PR3-ANCA to a neonate from a mother with AAV [76].

In Vitro Evidence Supporting the Pathogenicity of ANCA

In vitro studies showed that incubation of MPO-ANCA or PR3-ANCA with neutrophils "primed" by proinflammatory stimuli such as TNF-α, results in their full activation with a subsequent oxidative burst that generates reactive oxygen species **(ROS)** and degranulation of numerous lytic granule enzymes [4,77].

The interaction between ANCAs and "primed" neutrophils occurs via binding of their F**(ab')**2 fragments to PR3 or MPO, translocated on the neutrophil surface, as well as by binding of their Fc fragments to FcγRIIA **(CD32B)** and FcγRIIIB **(CD16B)** IgG receptors on the neutrophil membrane [78].

Furthermore, in vitro studies showed that human and mouse neutrophils activated by ANCA release complement activators (e.g., properdin) that activates the alternative complement pathway [79-82]. Subsequently, the complement activation generates C5a and the recruitment of further neutrophils at the site of inflammation. Similarly, incubation of monocytes with ANCA IgG induces the release of ROS, monocyte chemoattractant protein-1 **(MCP-1)**, and interleukin-8 **(IL-8)** [83-85]. IL-8 can attract and activate neutrophils and thus could amplify neutrophil-mediated injury, while MCP-1 attracts monocytes and macrophages at the site of inflammation. ANCA activation of TNF-primed neutrophils in vitro also leads to accelerated and dysregulated

apoptosis and secondary necrosis of these neutrophils by a mechanism dependent on NADPH oxidase and generation of ROS [86]. Then, the opsonization of apoptotic neutrophils by ANCA induces an increased uptake by macrophages and a further release of proinflammatory cytokines. More recently, activation of neutrophils by ANCA was shown to result in the formation of NETs [87] that contain chromatin fibers and neutrophil proteins, including MPO and PR3, and can be identified at the sites of injury in ANCA NCGN kidney biopsy specimens [88]. ANCA antigens in NETs could serve as targets for localized in situ immune-complex formation and might trigger adaptive autoimmunity through their exposure to antigen presenting cells **(**APC**)** as well as lead to a further development of inflammation in situ [87,89]. Furthermore, other studies have documented that neutrophil activation by ANCA occurs only when neutrophils are attached to a surface, and not in solution [90]. ANCAs promote migration and stable adhesion of neutrophils to the endothelium via a β2-integrin/intercellular adhesion molecule-1 **(ICAM-1)** interaction [91]. Incubation of ANCA IgG and neutrophils with endothelial monolayers causes neutrophil activation and the death of endothelial cells [92]. This cellular injury can be inhibited by antibodies against β2 integrins [93]. In summary, these findings in vitro support a pathogenic role for ANCAs and also suggest potential novel therapeutic targets for AAVs.

Animal models supporting the pathogenicity of ANCA

Animal models supporting the pathogenicity of ANCA-MPO: The first animal model was described in 2002 by Xiao et al [94]. MPO knockout mice **(MPO-/-)** were immunized with murine MPO to produce high titers of anti-murine MPO antibodies. Then, to test the pathogenic potential of antibodies alone, purified anti-MPO IgG from MPO-/- mice were injected intravenously into recombinase-activating gene-2-deficient **(Rag2-/-)** mice which lack the function of B and T lymphocytes, and in wild-type C57BL/6J mice. Both wild-type and immune-deficient mice developed pauci-immune NCGN and systemic AAV, documenting a pathogenic role for MPO-ANCA. A more severe disease was induced by the transfer of splenocytes from MPO-/- mice into Rag2-/- mice, but the presence of significant immune deposits in glomeruli of these mice do not match with the pauci-immune nature of the human disease and represents a possible bias of this model.

In an alternative mouse model, the immunization of MPO-/- mice with murine MPO to produce high titers of anti-MPO antibodies, was followed by irradiation and transplantation of bone marrow from MPO-positive wild-type mice. Because antibody-producing plasma cells are almost radioresistant, circulating anti-MPO antibody levels were preserved. Bone marrow-derived cells were sufficient targets to induce anti-MPO disease in the absence of MPO in other cell types after irradiation [95].

Little et al [96,97] immunized WKY rats with human MPO **(**hMPO**)** to develop anti-hMPO antibodies. Subsequently, anti-hMPO antibodies were shown to cross-react with rat MPO. Over time, these rats developed NCGN and focal pulmonary capillaritis. Interestingly, only WKY rats

were affected after immunization with hMPO, while Lewis, Wistar Furth and Brown Norway strains did not, despite having similar anti-hMPO antibody titers. The latter finding suggests an important genetic susceptibility of this MPO-AAV animal model.

Animal models supporting the pathogenicity of ANCA-PR3: Pfister et al [98], in a similar way to the MPO -/- approach, described the generation of anti-PR3 antibodies by immunizing PR3/elastase-/- mice with recombinant murine PR3 **(r-mPR3)**. The injection of these PR3 autoantibodies into lipopolysaccharide **(LPS)** -primed wild-type mice failed to induce NCGN or AAV.

Van der Geld et al [99] investigated the differences between human and murine PR3 by generating a series of chimeric human/murine PR3 molecules and immunizing both mice and WKY/Brown Norway rats. A robust antibody response was observed without the development of the pathological features of vasculitis.

Another model involving autoimmunity prone non-obese diabetic **(NOD)** mice immunized with r-mPR3, resulted in the development of high titers of anti-PR3 antibodies but failed to develop disease [100]. Conversely, the transfer of splenocytes from these mice to immunodeficient NOD/severe combined immunodeficiency **(SCID)** mice developed severe NCGN and AAV. However, as previously documented by Xiao et al [94], the splenocyte transfer technique induced marked immune complex deposition in the kidney also in this model, thereby making it non-representative of the pauci-immune human condition.

To overcome the species differences concerning the expression and physicochemical properties of PR3, Little et al [101] studied the induction of vasculitis by human PR3-ANCA in mice with a human immune system. The humanized mice were generated through the injection of human hematopoietic stem cells into irradiated NOD/SCID/IL-2rγ-/- mice. After combining infusion of a low dose of LPS with human PR3-ANCA purified from patients with an acute pulmonary-renal syndrome, the chimeric mice developed mild proliferative glomerulonephritis and pulmonary hemorrhage. This model supports a role for the pathogenicity of PR3 ANCA in AAV and has the major advantage that the disease was induced using PR3 ANCA from patients with AAV in an in-vivo system in which there were circulating human neutrophils.

Are all ANCAs pathogenic?

The aforementioned clinical, in vitro and in vivo experimental findings are evocative for a pathogenic role of ANCA in the AAV. However, the existence of natural MPO and PR3 autoantibodies in healthy individuals [102], the absence of a robust correlation between ANCA titers and disease activity [72,73], and the occurrence of ANCA-negative vasculitis [103,104] suggest that not all ANCAs are pathogenic. Indeed, recent studies have highlighted the conception that epitope specificity determines the pathogenicity of ANCA. Therefore, epitope mapping of MPO and PR3 has been performed widely over the years. Erdbrügger et al [105] showed that

MPO-ANCA target a confined number of MPO epitopes, mainly in the carboxy terminus of the heavy chain. In a subsequent study, Bruner et al [106] identified seven major epitopes. 12 AAV patients with significant levels of MPO-ANCA exhibited reactivity toward these seven epitopes. Six of these epitopes were located on the heavy chain of the MPO protein, while the latter one was identified within the pro-peptide region of MPO. More recently, using a mass spectrometry-based approach; Roth et al [107] identified a total of 25 different epitopes bound by MPO-ANCA. Twelve of these were restricted to active disease, and 8 were also exposed in healthy individuals. One linear MPO epitope (aa447-459) exclusive to active disease showed a declined reactivity with the remission. The pathogenicity of MPO-ANCAs specific for the aa447-459 epitope was supported by their ability to activate neutrophils in vitro and by the consideration that DR2 transgenic mice immunized with the homologous murine epitope developed a pauci-immune NCGN. In the same study purified immunoglobulin fractions from serum samples of ANCA-negative patients were able to bind the pathogenic MPO aa447-459 epitope. This finding was explained by masking of the pathogenic epitope by a fragment of ceruloplasmin, an MPO inhibiting serum protein which interferes with standard assays. Further and more extensive studies are required to confirm this finding and determine the rate of ANCA-negative patients, which are reactive to masked or currently unrevealed epitopes.

Additionally, the close contiguity of the epitopes identified by natural ANCAs with the epitopes specific to ANCA disease suggested the authors advance the hypothesis that the development of pathogenic ANCA could be due to dysregulation of natural ANCA instead of a newly developed autoimmune reaction [107]. Therefore, the reaction may first develop against MPO's natural epitopes and then spread to pathogenic epitopes.

Another study investigated the correlation between linear MPO epitopes and disease activity in 77 AAV patients [108]. Six recombinant linear MPO epitopes produced from E.coli were recognized by MPO-ANCA from at least one patient. At the time of disease remission, the reactivity for all epitopes was definitely lower. Interestingly, half of the patients recognized the aa399-519 region, which also includes the aa447-459 epitope. Although previous studies found only reactivity for the heavy chain, reactivity to the light chain of the MPO was also detected in this study.

Fewer studies have been performed for PR3 with the attempt to map the pathogenic epitopes [109-113]. The vast majority of the pathogenic epitopes recognized in these studies was shown to be strictly linked to the catalytic site of PR3. Bruner et al [113] identified seven pathogenic antigen regions, three of which were contiguous to amino acids that shape the catalytic triad of the protein. The contiguity of these epitopes to the catalytic triad could support a potential mechanism by which anti-PR3 antibodies might change the normal proteolytic properties of PR3, thus perpetuating the disease process through epitope spreading, a common feature of other autoimmune diseases.

Cells and Pathways Involved in AAV Pathogenesis and Regulation of the Immune Response

Neutrophils in AAV

Neutrophils play a central role in the pathogenesis of AAV since they represent both target cells of the autoimmune response, and effector cells responsible for endothelial damage **(**Figure 2**)**. We have already shown in this review how ANCA interact with neutrophils to trigger and provoke vascular damage discussing the evidence in support of the pathogenicity of ANCA coming from in vitro experimental models. Besides, we reported the in vivo experimental model performed by Schreiber et al [95] which demonstrates that induction of NCGN requires both anti-MPO antibodies and MPO-positive neutrophils in the circulation.

To further support the fact that neutrophils are key effector cells, Xiao et al [114] showed that mice depleted of circulating neutrophils were completely protected from anti-MPO IgG-induced NCGN, while all control mice developed NCGN. However, must be pointed out that not all neutrophils have the same characteristics and not all of them interact with the same intensity with the ANCAs. The expression of membrane PR3 **(mPR3)** is bimodal, as this target antigen is presented on the surface of a portion of the neutrophils [115]. The membrane-bound form is the only one able to interact with the circulating ANCAs. Neutrophil priming is a key mechanism to raise the surface exposition of PR3 in the subgroup of mPR3+ neutrophils, but the ratio of mPR3+ and mPR3− cells is stable within an individual even after priming and seems to be genetically determined [116,117]. Furthermore, a phenotype with a higher percentage of mPR3+ neutrophils is more common in patients with GPA when compared with controls (85 versus 55%) [118], and is associated with an increased frequency of relapse [119].

Interestingly, some observations suggest that ANCA-activated neutrophils could increase the production of ANCAs by stimulating B cells. Activated neutrophils represent an important source of TNF ligands superfamily members, including B-cell activating factor **(BAFF)** /B lymphocyte stimulator **(BLyS)**, known to play essential roles in B-cell differentiation, proliferation, and immunoglobulin production [120]. To date, studies report that serum levels of BAFF/BLyS are elevated in patients with active AAV and decline during remission [121,122].

All these findings raise the possibility for novel therapeutic strategies targeting ANCA-activated neutrophils.

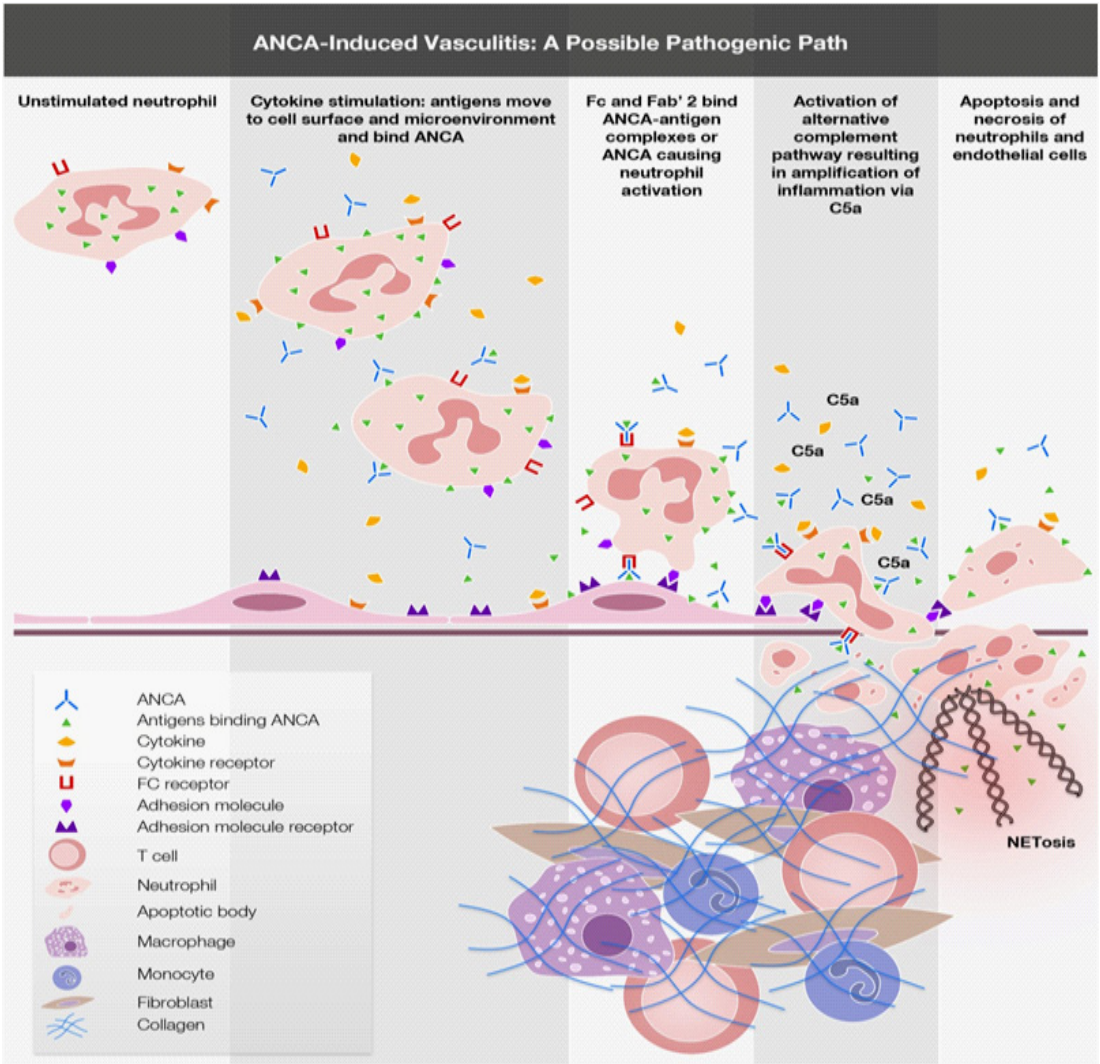

Figure 2: Putative sequence of pathogenic events in AAV. Neutrophils that have been primed (e.g., with cytokines) release ANCA antigens at the cell surface and into the adjacent microenvironment, where they bind ANCA. Fc receptor engagement by ANCA bound to ANCA antigens as well as $F(ab')_2$ binding to ANCA antigens on neutrophil surfaces cause neutrophil activation. Activated neutrophils release factors that activate the alternative complement pathway, generating C5a. C5a in turn recruits and primes more neutrophils for activation by ANCA and further activation of complement, resulting in an amplification loop. ANCA-activated neutrophils adhere to and penetrate vessel walls and undergo respiratory burst, degranulation and NETosis that cause vascular necrosis. The innate inflammatory response orchestrates the conversion of the initial acute neutrophil-rich inflammation into mononuclear leukocyte-rich inflammation and subsequent collagen deposition and fibrosis.

Lymphocytes in AAV

T cells in AAV: T cells are commonly localized into granulomas and other inflammatory lesions present in AAV, indicating a pathogenic contribution of the T-cell-mediated immune response [123-126]. According to these findings, elevated levels of soluble T cell activation markers [soluble interleukin-2 receptor **(sIL-2R)** and soluble CD30] are detected in GPA patients and have been shown to be associated with disease activity [127]. Interestingly, T cells are in a persistent state of activation both during the active phase of the disease as well as during the remission phase [128], suggesting a defect in the adaptive immunity with a no adequate T-cell regulation.

Wilde et al [129] reported a specific subset of effector memory T cells **(Tems)** expressing CD134 and glucocorticoid-induced TNF-receptor-related protein **(GITR)**. These specific CD134+ Tems were particularly expanded and easily detected in active lesions from patients with GPA. The ligand for CD134 **(CD134L)** is expressed on endothelial cells, and ligation of CD134 contributes to T cell migration and tissue infiltration through its interaction with CD134L on vascular endothelial cells [130,131]. Tems have also been revealed in a urine analysis of patients with an active renal disease, remarking their role in determining kidney damage and their potential utility as a biomarker of renal disease activity in AAV [132].

IL-17-producing T helper 17 **(Th17)** effector T cells have also shown to be involved in the pathogenesis of AAV. An in vitro study, Hoshino et al [133], showed that MPO-ANCA-activated neutrophils produce IL-17 and IL-23 developing the milieu to promote Th17-mediated autoimmunity. The secretion of IL-17 by Th17 cells stimulate the release of the pro-inflammatory cytokines IL-1b and TNF-a from macrophages, which are crucial for priming and activation of neutrophils [134]. This finding has been confirmed in an anti-MPO-induced GN murine model, where IL-17 played a role in mobilizing neutrophils and promoting the recruitment of macrophages into the kidney, supporting a pivotal pathophysiological role of the Th17 cells in the AAV [135].

The actions of Th cells can be counterbalanced by regulatory T cells **(Tregs)**, which are CD4+ cells expressing the interleukin **(IL)** -2 receptor α-chain **(CD25)** and high levels of the transcription factor forkhead box P3 **(FoxP3)**. Several autoimmune and chronic inflammatory disorders associated with vascular inflammation exhibited defects in Tregs function or a reduced number of Treg cells [136]. In patients with AAV different studies reported conflicting results regarding the frequency of Tregs, but all of these studies revealed impaired functionality of circulating Tregs [137-139]. Recently, Free et al [140] reported a defective Treg suppressive function which is associated with an increased usage of an exon 2-deficient FOXP3 splice variant as well as an increased frequency of a distinct proinflammatory effector T cell subset which is resistant to Treg suppression. The combination of these events could permit the formation of ANCAs, thereby perpetuating the disease.

B cells in AAV: Beyond the production of pathogenic ANCAs, B cells are also crucial in the regulation of the immune response in AAV. B cells exhibit different functional phenotypes. An increasing body of evidence indicates that regulatory B-cells **(Bregs)**, characterized by high CD5 surface expression and the production of IL-10, ameliorate immune responses in autoimmunity [141]. Bregs inhibit the differentiation of naive T-cells into Th-1 or Th-17 and promote the development of regulatory T-cells **(Tregs)** by cell-to-cell contact and TGF-β secretion [142,143]. Additionally, Bregs has been associated with induction of apoptosis of activated T cells through different signaling pathways involving programming death domain-1 **(PD-1)**, Fas ligand **(FasL)**, and tumor necrosis factor-related apoptosis-inducing ligand **(TRAIL)** [144]. Several studies reported lower percentages of circulating CD5+ B cells, without functional deficiencies, in active AAV patients, when compared with patients in remission and healthy controls [145-148]. Furthermore, Bunch et al [145] showed that the degree of normalization of circulating CD5+ B cells after therapy with Rituximab correlates with more persistent remission.

These findings suggest that the future direction for therapy in AAV could be the restoring of balance between B cell regulators and effectors, instead of the actual total depletion of B cells.

Complement in AAV

The histopathological feature of a pauci-immune NCGN and the absence of hypocomplementemia have initially motivated the supposition that the complement system was not implicated in the pathogenesis of AAV. However, current evidence from animal models, in vitro studies, and renal biopsy studies of patients with NCGN, suggest that activation of the complement system, and the alternative pathway **(AP)** in particular, is crucial for the development of AAV [149].

In a mouse model of MPO-AAV [79], C5 deficient and factor-B deficient mice were entirely protected from the disease, whereas AAV developed both in wild-type and C4 deficient mice, indicating activation of the complement system via the AP. Furthermore, treatment with an anti-C5 monoclonal antibody before the administration of anti-MPO IgG in mice prevented the development of NCGN [150].

The importance of the complement anaphylatoxin C5a, a potent chemoattractant and agonist of neutrophils, has also been highlighted. Schreiber et al [80], in an in vitro study, demonstrated that either the production of C5a by supernatants from ANCA-stimulated neutrophils and the C5a mediated priming effects via its specific neutrophil receptor **(C5aR)** allowed neutrophils to generate ROS in response to ANCA. Then, the study was extended in an animal model of ANCA-induced NCGN, where mice lacking C5aR were protected from developing NCGN [80]. The role of C5a and C5aR was further defined by Xiao et al [151] in a mouse model of anti-MPO-induced NCGN. C5aR deficient mice had a mild disease, whereas the deficiency of another receptor for C5a, C5a-like receptor 2 **(C5L2)**, had the opposite effect, indicating that C5aR enhances and C5L2 suppresses inflammation. In addition, oral administration of a small compound antagonist of the human C5aR, CCX168 **(Avacopan)**, markedly ameliorated anti-MPO induced NCGN in mice

expressing human C5aR in a dose-dependent manner [151]. The beneficial effects of Avacopan in patients with AAV have just been published [152] in a randomized, double-blind, placebo-controlled, phase II trial (CLEAR trial; ClinicalTrials. gov, identifier: NCT01363388), which demonstrated that inhibition of C5aR may be an alternative to the use of oral glucocorticoids, and that C5a is an important inflammatory mediator in AAV.

Biopsy specimens from patients with AAV showed deposition of a certain degree of AP components, but no staining for C4d, a marker of the classic and the lectin pathway [153-156]. In accordance with these findings, elevated serum levels of terminal and AP components with normal levels of C4d have been reported in patients with active AAV [157]. Interestingly, plasma levels of Bb, considered being a marker of activation of the AP, correlated with disease activity according to the Birmingham Vasculitis Activity Score **(BVAS)** [157]. Conversely, other studies have documented deposition of C3c and C1q in biopsies of AAV, indicating an activation of the classical pathway of complement [155,156,158,159].

Recently, Sethi et al [160] performed laser microdissection and proteomic analysis of the glomeruli of patients with AAV and ANCA-negative pauci-immune GN to determine further the complete profile of all complement factors involved. A predominant C3 deposition was found in both PR3 and MPO-positive patients and even more pronounced in the ANCA-negative patients, revealing activation of the AP. No factor B or properdin was observed, indicating that the AP activation takes place in the fluid phase and not locally in the tissues. However, small amounts of C4 and immunoglobulins were detected, particularly in the PR3-positive patients, indicating that minimal classical pathway activation also occurs.

All these findings confirm the involvement of complement in the pathogenesis of ANCA-AAV and suggest that the inhibition of the complement system, particularly by targeting C5a, is a promising therapeutic strategy.

CONCLUSIONS

In this review, we documented that on the basis of genetic and epidemiological evidence PR3-AAV and MPO-AAV are distinct autoimmune syndromes. In addition, although the pathogenesis of AAV remains incompletely understood, we have reported recent research developments in this field. To date, the importance of ANCAs in the pathogenesis of the disease is evident by clinical and experimental observations from in vitro and in vivo studies. Furthermore, our knowledge regarding the aforementioned mechanisms of immunogenesis of ANCAs and concerning the sequence of the pathogenic events which cause endothelial vascular damage have significantly improved in the last decade.

These findings could now be tested in future clinical trials using new therapeutic strategies based on novel biological drugs in well defined homogenous patient populations according to their ANCA specificity.

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