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ANCA-associated vasculitis: pathogenesis, novel markers of the disease and emerging therapies

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Much has been learned in recent years on the pathogenesis of ANCA-associated small-vessel vasculitis. The interaction of primed neutrophils with ANCA and endothelial cells is crucial to the disease. Next we gained a better understanding, from animal models, of the pathogenetic importance of the ANCA antibody. Very recent evidence provides intriguing data regarding the link between infection and vasculitis, LAMP-2 antibodies as novel markers, and NETs as a novel pathogenetic mechanism. It remains to be seen whether others are able to corroborate these findings and whether testing for LAMP-2 antibodies will become part of the clinical routine in vasculitis. Recent years also saw the emergence of various new markers of endothelial damage and the disease itself, such as circulating endothelial cells and endothelial microparticles. These novel markers correlate well with disease activity; they may well complement traditional diagnostic tools, such as ANCA testing. Preliminary evidence has provided some insight into the balance between endothelial damage and repair. Exciting preliminary data also indicate that circulating endothelial cells may not only be markers of disease activity but that these cells may have pathogenetic importance in their own right. These findings may have profound implications for the pathogenesis of vasculitis and vascular disease in general. Recent years also saw the publication of a number of seminal studies for the treatment of ANCA-associated vasculitis. We now have a much better understanding of the role of pulse intravenous cyclophosphamide and plasma exchange than ten or even five years ago. Further studies must now show whether plasma exchange is also beneficial for less severely ill patients with AASV. Finally, as ever, it is hoped that further progress in understanding the disease pathogenesis will also provide more tailored and less toxic therapies.

Key words: **Vasculitis, diagnosis – Vasculitis, pathology – Vasculitis, drug therapy.**

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Twenty years or so since the discovery of anti-neutrophil cytoplasmic antibodies [1] (ANCA), much progress has been made in understanding, diagnosing, and treating ANCA-associated small-vessel vasculitis (AASV).[2] We now know that ANCA, neutrophils and micro-vascular endothelial cells are key players during the pathogenesis of the disease. Our understanding of their interactions and the interplay with adhesion molecules has improved as well. The discussion

on the pathogenicity of the ANCA themselves is still ongoing, fuelled by recent and exciting evidence. ANCA are also a sensitive diagnostic marker of the disease at presentation although specificity is an issue. Very recently, exciting new evidence has emerged regarding potential mechanisms of molecular mimicry and new pathogenetic mechanisms, linking infection to vasculitis. In diagnosis, ANCA titres themselves remain less than ideal to guide treatment during follow up, to detect relapse, and to distinguish between vasculitic disease activity and infection. New markers of the disease have therefore long been awaited. During the last decade, novel markers of endothelial damage, such as circulating endothelial cells and endothelial microparticles have become available to assess the extent and/or acuity of vascular damage in a clinical setting. There is also the interesting question of how endothelial repair occurs and how both damage and repair evolve during the course of the disease, and in relation to treatment. There is also the intriguing question as to what effects circulating endothelial debris may have on other cell populations. Finally, progress in understanding the pathogenesis has been mirrored by considerable advances in treating vasculitis. Multicentre trials have largely set a standard for induction and maintenance of remission, respectively. One of the most interesting developments in recent years is targeting B-cells and further new treatment options may be on the horizon. In this article, we review recent developments regarding pathogenesis, discuss markers of endothelial damage and provide a brief overview of existing and emerging treatment.

Pathogenesis of ANCA-associated small vessel vasculitis

The exact etiology of AASV is not known. It is likely that genetic factors predispose to the disease while environmental factors trigger the onset of inflammation. Research over the last two decades, however, has successfully delineated pathogenetic mechanisms of the disease. This well established concept [3] focuses on the interaction of ANCA, neutrophils, and endothelium. ANCA were first described by Davies *et al.*[1] He described a series of eight patients with constitutional symptoms, focal necrotising glomerulonephritis, weight loss and arthralgia. A circulating IgG factor staining the cytoplasm of neutrophils was identified in the serum of these patients. It wasn't until 1985 when van der Woude established the link between ANCA and vasculitis.[4] Wegener's granulomatosis (WG), microscopic polyangitis (mP) and the Churg Strauss Syndrome (CSS) are nowadays regarded as the ANCA-associated vasculitides. The discussion around the continued use of the eponym "Wegener's granulomatosis" is beyond the scope of this article;[5, 6] hence we will continue to use the eponym here. Of note, WG has small vasculitis as well as granulomatous inflammation. The reason why WG shows such a distinct phenotype while otherwise showing such similarities to mP remains enigmatic. In the presence of ANCA, neutrophils and monocytes when fixed with ethanol, demonstrate diffuse granular cytoplasmic (c-ANCA), perinuclear (p-ANCA) or atypical staining pattern. Enzyme linked immunosorbent assay (ELISA) analysis reveals that c-ANCA are usually anti-proteinase 3 (PR-3) and p-ANCA are most often antimyeloperoxidase (MPO). PR-3 and MPO are two enzymes stored in the azurophilic granules of neutrophils and monocytes.[7] Alpha -1 antitrypsin is a physiological inhibitor of PR3. Deficiency of alpha-1 antitrypsin (PiZZ phenotype) has been associated with PR3 positive vasculitis [8] but a highly increased risk could not be demonstrated. Myeloperoxidase in neutrophils facilitates the formation of hypochloric acid, a strong reducing agent and defence mechanism against microorganisms and is a weak inhibitor of PR3. c-ANCA is predominant in WG (70%) and p-ANCA is predominant in CSS (60%). They are about equally distributed in MP (40% c-ANCA; 50% p-ANCA).[9] Five to 30% patients with WG, MP, and CSS are ANCA negative. It must therefore be appreciated that no ANCA test is specific for one of the diseases and that a tissue diagnosis is required. A meta-analysis of 15 studies has revealed that a positive c-ANCA by indirect immunofluorescence (IIF) has a sen-

sitivity of 34% to 92% in diagnosing of WG and a specificity of 88% to 100%.[10] IIF when combined with ELISA, increases specificity of diagnosis to nearly 100% when compared with healthy controls.[11] A potential pitfall is that atypical ANCA, *i.e.* those not directed against PR-3 or MPO, are often detected in non vasculitic conditions, such as autoimmune liver disease, inflammatory bowel disease, tuberculosis,[12] endocarditis and cystic fibrosis. Less often, cANCA is also seen in these non-vasculitic conditions. These antibodies are directed against a broad variety of other antigens, such as bacterial permeability protein (BPI) or lactoferin. On occasion, atypical ANCA are also seen concurrently with PR-3 ANCA in vasculitis.[13] Rarely c-ANCA has been reported as an epiphenomenon. An overview is provided elsewhere.[14]

ANCA and pathogenicity: an ongoing debate

The role of ANCA in the pathogenesis of systemic vasculitis has long been debated. Clinical experience dictates that some patients have persistently high ANCA titres but no clinical disease activity. More recently, however, several clinical observations as well as *in vitro* and in vivo experiments have fuelled a new discussion of the pathogenetic importance of ANCA.

Anecdotal but interesting evidence for a pathogenetic role of anti-MPO ANCA was provided by a case report of a newborn baby, who developed pulmonary renal sydrome after delivery from a mother with active microscopic polyangiitis.[15] It is believed that the transplacental transfer of IgG MPO ANCA was responsible for the clinical disease in the newborn. However this does not seem to be the rule in that healthy children without vasculitis are also seen in women with active vasculitis during pregnancy despite transplacentar transfer of MPO.[16]

A change in ANCA titres over time has been described to correlate with disease activity. In one prospective study, 92% patients showed rising ANCA titres before clinical relapse.[17] In another study, prospective monitoring of ANCA and preemptive treatment with immunosuppression for rising titres was found to prevent a clinical relapse.[18] However, it is important to note that up to 30% of patients with limited WG, are ANCA negative but tend to become seropositive with progression to systemic vasculitis. More importantly, ANCA titres are by no means infallible markers of AASV. In fact the positive predictive value of ANCA in predicting a clinical relapse in AASV is highly variable.[19, 20] This has led some authors to suggest that ANCA titres should not be used to guide treatment in the first place.[21] Nearly all experts now agree that a rise in ANCA titres without symptoms should increase awareness, but not necessarily mandate immunosuppressive treatment. New markers of the disease and its activity are therefore eagerly awaited.

Animal models of AASV

Earlier attempts of animal model of vasculitis were based on exposure to mercury chloride in Brown Norway rats.[22] This model demonstrated arthritis, caecal vasculitis, raised IgE and production of multiple autoantibodies including anti MPO antibodies. It did not, however, reflect the disease pattern of AASV in humans. Subsequent attempts to induce vasculitis in rodents with anti-MPO antibodies were unsuccessful. Neumann et al developed SCG/Kinjoh, a new strain of mouse which developed spontaneous vasculitis and crescentic glomerulonephritis with ANCA but this model was associated with significant immune deposits.[23] More recently, Xiao *et al.* prepared MPO knock-out mice and immunised them against murine MPO. Transfer of splenocytes from this immunised mice in to *RAG2*$^{-/-}$ mice induced vasculitis and pauci-immune crescentic glomerulonephritis.[24] This animal model has been regarded as compelling evidence that ANCA themselves are pathogenic.[25] In addition, Little *et al.* developed a rat model, which develops pauci-immune crescentic GN and pulmonary haemorrhage when immunised with human MPO.[26] Using a similar method to develop an animal model of PR3-associated vasculitis has shown different

results, demonstrating that multiple mechanisms are responsible for loss of tolerance to these autoantigens.[27]

Compelling as all these laboratory data may be, clinicians are still not entirely convinced and argue that ANCA alone may not be pathogenic enough or else one would not see patients with high ANCA titres and no disease activity, nor healthy children with high-titre MPO-ANCA born to women with active AASV.

Interactions between neutrophils and endothelium

The established model of AASV focuses on the interaction of ANCA, primed neutrophils and endothelial cells.[3, 28] It has been proposed that ANCA interacts with their target antigens on the surface of neutrophils and stimulate respiratory burst, when primed with inflammatory cytokines, like tumour necrosis factor α (TNFα), interleukin 1 beta (IL-1 β). This mechanism eventually leads to release of reactive oxygen species,[29] proteases and other mediators.

It is also postulated that primed neutrophils bind to primed endothelial cells, starting a cascade of inflammation, leading to tissue destruction. The interaction between neutrophils and the endothelial surface is complex.[30] Neutrophils go through different stages of attachment, rolling, arrest and transmigration through the endothelium. TNFα and IL-1β induce the expression of the adhesion molecule selectin on the endothelial surface and P-selectin glycoprotein ligand 1(PSGL-1) on the neutrophil surface. PSGL-1 binds with P-selectin which results in attachment of neutrophils to the endothelium. Chemokines, which attract neutrophils, are also expressed on the endothelial surface. PR3 itself is known to induce IL-8, a strong chemotactic and activating factor for neutrophils.[31] Further studies have confirmed that PR3 increases expression of monocyte chemoattractant protein-1 (MCP-1) and ICAM-1 at protein and m-RNA level and incubation of human umbilical vein endothelial cells (HUVEC) with PR3 for 24 hours significantly increases adhesion of neutrophils which can be blocked by monocloanal antibodies to ICAM-1 or CD18.[32, 33] Subsequently ANCA binding induces a conformational change in the integrin molecules on the neutrophil surface. Integrin binds to its ligands expressed on the endothelial surface. PR3, but not MPO interacts with endothelial cells (EC). PR3 from degranulated neutrophils is partly internalised by the EC. It has been shown that PR3 is capable of inducing a pro-apoptotic signalling pathway.[34] This observation underpins the complex interplay between PR3 ANCA and EC. Activation of neutrophils is more efficient when they are actually attached to endothelium. Adherence of neutrophils to endothelial surface is facilitated by expression and activation of adhesion molecules on both neutrophils and endothelial cells. Increased expression of α1(CD29) and α2 integrins (CD18) has been shown on neutrophils and monocytes isolated from patients with AASV comPred with normal controls.[35] In comPrison, transmigration of neutrophils through the endothelium is poorly understood. Both transmigration and adhesion are α2 integrin dependant but transmigration alone can be reduced by blocking of chemokine receptors CXCR2.[36] Recent evidence suggests that junctional adhesion molecules, CD31 and CD99 may be involved in this process.[37] Interestingly, P-selectin is absent in the normal glomeruli, possibly as a protective mechanism against the developement of glomerulonephritis. Pankhurst *et al* suggests an alternative mechanisms in the emigration of neutrophils in the inflamed glomeruli, which is independant of the usual cascade of rolling, adhesion, transmigration but probably facilitated by direct capture using α 4 integrins.[30].

Old and new mechanisms of tissue damage

Traditionally it has been postulated that the neutrophil respiratory burst and release of superoxide and other mediators at the EC is chiefly responsible for endothelial damage. However recent evidence also supports the role of serine proteases like PR3 themselves to mediate EC damage. EC can actually down-

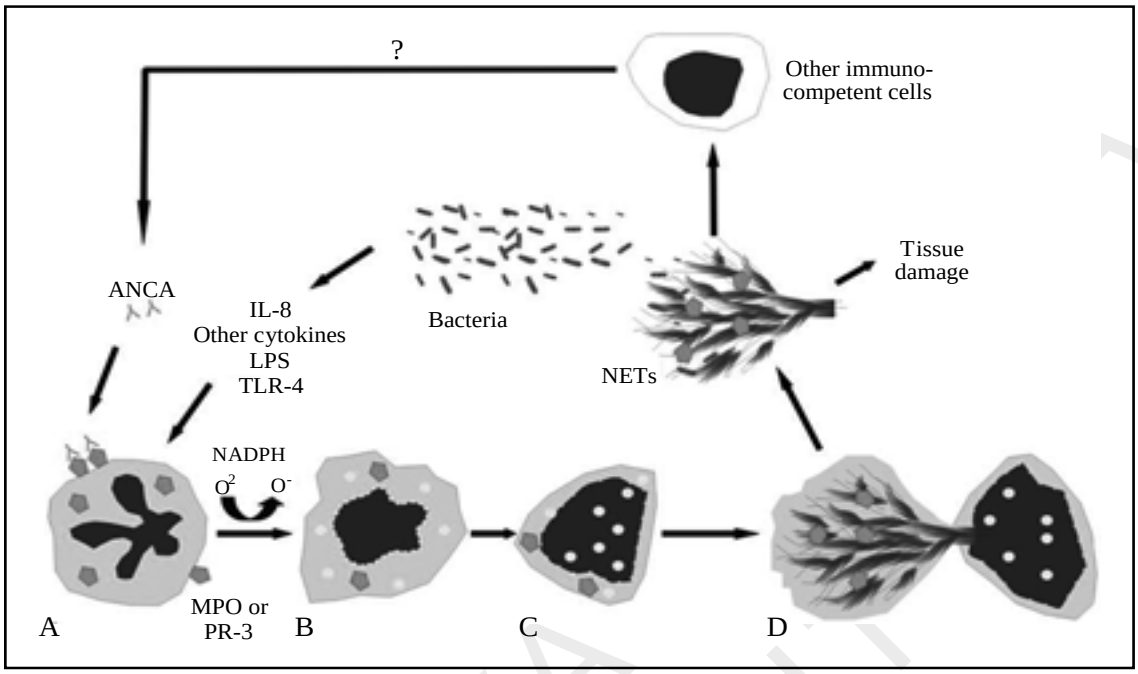

Figure 1.—NET formation and possible implications for the pathogenesis of ANCA-associated vasculitis (hypothetical). A) Activation of neutrophils leads to production of reactive oxygen species; B) Next, the nuclear membrane starts to disintegrate, leading to vesicle formation; C) The nuclear membrane continues to disintegrate and the nuclei lose their lobules. Nuclear material now fills most of the cells. D: The cells release NETs, which bind bacteria. ANCA target antigens can be trapped within extracellular NETs and may thus act as auto-antigens and this perpetuate the process of ANCA production. Partly redrawn after.[225] IL-8 denotes interleukin-8. LPS denotes lipopolysaccharide. TLR-4 denotes Toll-like receptor 4.

regulate the superoxide response of neutrophils by suppression of NADPH activity.[38] Lu *et al* proposed that EC release adenosine, which inhibits the respiratory burst of ANCA activated neutrophils and protects the endothelial surface from the potentially deleterious effects of superoxide.[39] This observation lead the authors to conclude that ANCA induced superoxide release was an unlikely mechanism for the EC injury *in vivo.*

Recently Kessenbrock *et al.*[40] provided exciting new insight into a hitherto unknown mechanism of endothelial damage in ANCA-associated vasculitis. It has been known for quite some time that neutrophils are capable of releasing gummous DNA upon cell death. This DNA in turn can trap bacteria.[41] Hence the term neutrophil extracellular traps (NET) has been coined.[41] This mechanism is stimulated by Toll-like receptor 4 (TLR4).[42] Eventually the gummous DNA can stick to the endothelium and causes tissue damage during sepsis. The release of NET thus characterizes a unique type of neutrophil-related cell death that is linked to innate immunity. Kessenbrock *et al.* were now able to demonstrate that NETs can trigger vasculitis and showed that ANCA stimulated neutrophils to release NETs, which contain MPO and PR3.[40] NETs can activate plasmacytoid dendritic cells and B cells. Interestingly, they also showed that the neutrophils ability to form NETs was enhanced by bacterial infection with *S. Aureus,* which has long been linked to vasculitis.[43] NETs in turn bind *Staphylococcus.*[41] Their results also suggest that PR-3/MPO colocalize with NETs, which may in turn present PR-3/MPO as antigens to the remainder of the immun system, thus fuelling a vicious circle.[44] The importance of these new results is twofold: First, they provide a whole new pathogenetic mechanism for vasculitis. Moreover, they provide a new link between infection and vasculitis. Figure 1 illustrates the mechanism of NET formation and speculative mechanisms linking it to vasculitis.

Endothelial cells and antiendothelial cell antibodies

Histological findings in AASV suggest that damaged EC undergo necrosis and detach from the basement membrane. These endothelial cells are detectable in peripheral blood as discussed in a separate chapter below. Could direct antibody-mediated cytolysis have a role in this? Antiendothelial antibodies are indeed detectable in patients with AASV [45, 46] and other vasculitic conditions.[47] Their existence may provide yet another mechanism of endothelial injury in vasculitis. However, there are considerable technical problems with AECA as well as a lack of consensus for their detection. Furthermore, AECA are a heterogeneous group of antibodies and are not entirely specific to endothelial cells, but may react with fibroblasts as well.

AECA when incubated with human umbilical vein endothelial cells (HUVEC), show increased expression of adhesion molecules, chemokines and cytokines and may promote leukocyte recruitment.[48] *In vitro*, AECA have also been shown to mediate complement and cell- cytotoxicity against a cultured monolayer of endothelial cells.[49] Further studies suggest that AECA promote thrombogenic events with increased production of tissue factor and von Willebrand factor.[50, 51] As of today, there are conflicting reports about the prevalence of circulating AECA in AASV and the reported prevalence ranges from 8% to 100%.[45, 46, 50] Recent experiments using glomerular endothelial cells as a specific substrate comPred with HUVEC, showed a low prevalance of AECA (14% in WG and none in MP) in AASV.[52] Others describe that AECA have considerable pathogenetic importance in AASV, suggesting a kinase-dependent mechanism.[53] For now, it is probably fair to say that the role of AECA remains controversial and ill-defined. A reasonable next step could be a multi-centre effort towards technical consensus and standardisation of AECA.

T lymphocytes

For a long time, the majority of research into AASV had been focused on neutrophils and ANCA whereas in limited disease with only granulomatous lesions the pathogenic role of T lymphocytes has always been more evident. However more recently the role of lymphocytes is emerging. Immunohistological studies as early as 1983 showed involvement of T cells in the perivascular lymphoid infiltrate in patients with WG.[54] Clinical observations of induction of remission with anti T cell antibodies also support the pathogenic role of T cells in AASV.[55, 56] Isotype switching between IgG1 and IgG4, the two predominant forms of ANCA, is dependant on IL-4, a T cell cytokine. Furthermore, disease activity of WG correlates well with concentration of IL-2, which is a marker of T cell activation.[57] T cells from patients of AASV, whether active or in remission, show significant proliferation when cultured with ANCA antigens (PR3 and MPO).[58] The cytokine secretion profile of T cells from granulomatous lesions in WG shows a Th1 pattern.[59] Other studies have also confirmed the increased production of interferon by T cells in patients of WG, suggesting a Th1 pattern.[60] Of note, T cell activation requires help of co-stimulatory pathway. In WG patients, it has been shown that expression of CD28 is significantly low and expression of B7 is increased.[61] CD28 costimulation is associated with Th2 type of response and in absence of this T cells default to mount a Th1 type of response.[62] Evidence suggests that T cells remain activated in WG, even in remission state.[63] It is tempting to think that this may be related to the response to superantigens produced by *Staphlococcus aureus*,[64] as infection with these bacteria has long been linked to WG. In addition, a defect in the suppressor function of natural regulatory T cells (T_{Reg}) has been proposed in patients with AASV, leading to persistant autoreactivity of T cells.[65] Finally, genetic polymorphisms in Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) are known to negatively regulate T cell responsiveness and thus offer an explanation for T cell hyperreactivity in AASV.[66] Recent reasearch has also focussed on IL-17 producing $CD4^+$ T cells, termed Th 17 cells, as a crucial cellular subset in the pathogen-

esis of autoimmune diseases.[67] It is already known that T cells are important in granuloma formation in WG and a relative increase of PR3 specific Th17 cells has been seen in ANCA positive WG patients, in comparison with ANCA negative WG and healthy controls.[68] This suggests a crucial role of Th17 cells in autoantibody production in WG.[68] Finally, the role of $CD4^+$ T cells is further supported by their appearance in the renal tissue and urinary sediments [69] in patients with active WG.

B lymphocytes

B cells are direct precursors of antibody producing plasma cells that must eventually secrete the ANCA. In addition, B cells are likely to contribute to pathogenesis of AASV by other mechanisms, such as co-stimulation and antigen presentation within granuloma.[70] Some now believe that the granuloma in WG may be the site of ANCA synthesis.[71] PR-3 positive cells in the vicinity of antigen-presenting cells are also observed in granulomas.[72] This observation also suggests the granuloma as the site of the continued immune response against PR-3. Single-cell analysis of B lymphocytes seems to confirm this hypothesis.[70] B cells are also highly effective in antigen presentation and cytokine production (IL6, IL10 and TNFα). It has been shown that there is increased level of B cell activating factor (BAFF/BLYS) in patients of WG.[73] The recent marked success of anti B cell directed therapy with rituximab further strengthens the pathogenic association between B cells and AASV as discussed in greater detail below.

CCR5

CCR5 is a member of the beta chemokine receptors. Its ligand is CCL5, previously known as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted). An important role of CCL5/CCR5 has been described in several autoimmune diseases.[74] Current concepts suggest a role of CCR5 during the signalling pathway in the recruitment of T cells and macrophages. It is also known that CCR5 is expressed mainly on Th1 type of cells.[75] Presence of macrophages and T cells in granuloma of WG and Th1 type of response in WG makes CCR5 important in the pathogenesis of WG. Zhou et al showed in an immunohistological study of lung biopsies that patients with WG have increased expression of RANTES and an increased number of CCR5+ve cells in inflamed lung tissue compared to adjacent normal lung tissue. Interestingly, numerous CCR5+ve cells were found tethering to pulmonary vascular endothelium. In comparison, cells within the lumen were predominantly CCR5-ve, suggesting CCR5 to be involved in transmigration of cells across endothelium. A 32 basepair deletion in CCR5 gene (CCR5 Δ32) confers resistance to human immunodeficiency virus (HIV) as CCR5 is essential for membrane binding cell penetration by HIV.[76] When compared with ANCA status, this deletion is significantly under-represented in ANCA negative patients.[77] Conversely, CCR5Δ 32 was associated with persistent ANCA positivity. Lack of this deletion in ANCA negative patients suggest importance of CCR5 in pathogenesis of WG and it can be postulated that absence of functional CCR5 pathway and negative ANCA will dramatically reduce development of WG.[77]

What induces ANCA production?

The true cause of ANCA production is not known to the present day. Environmental studies suggested a connection with silica exposure [78] but these data remain conflicting at present. There is surely a genetic background although clusters of AASV in a family are rare.[79] In this regard, it is of particular interest that membrane expression of PR-3 is genetically determined.[80] It is also known that having a large subset of neutrophils expressing membrane PR3 is a risk factor for vasculitis.[81] NET release by neutrophils during infection may present ANCA antigens to the remainder of the immune system and render them auto-antigenic as discussed

above. Previous data have also implicated bacterial superantigens provoking autoantibody production.[82] Pendergraft and co-workers serependitously discovered another novel mechanism that causes ANCA production: They describe an immune response against a peptide that is antisense or complementary to the auto-antigen, which then induces anti-idiotypic antibodies (auto-antibodies) that cross-react with the auto-antigen.[83]

Another tempting hypothesis includes a molecular mimicry between microbial antigens and host proteins. Recently Kain *et al.* proposed molecular mimicry between lysosomal membrane protein 2 (LAMP2) and bacterial adhesin FimH. In neutrophils it is integrated in to membranes of MPO and PR3 containing vescicles, hence LAMP2 is another and previously recognised target antigen for ANCA.[84] Their new findings suggest that exposure to FimH induces autoantibodies to human LAMP2, which initiate pauci-immune glomerulonephritis in rats immunised with FimH. The authors also showed that prevalence of these autoantibodies to human LAMP2 was more than 90% in patients with active pauci-immune glomerulonephritis. As onset of vasculitis is often preceded by infections with FimH expressing bacteria, they conclude that FimH triggers the autoimmunity to human LAMP2, which is responsible for the tissue injury in vasculitis syndrome.[85] Of course, these findings need corroboration in further studies. Also, a standard for the detection of LAMP-2 antibodies needs to be established. Larger multi-centre studies should then establish the prevalence of these antibodies in the vasculitides. It would also be of interest whether these antibodies are capable of inducing NET formation.[44]

ANCA negative crescentic glomerulonephritis

Although not part of the AASV spectrum of diseases, ANCA-negative pauci-immune crescentic glomerulonephritis is still of considerable interest to the field, not least due to the similarity of the lesions in histological specimens. It is probably fair to say that the disease is poorly understood with a lack of pathogenetic concepts as well as paucity of therapeutic guidelines. Up to 20% patients with crescentic glomerulonephritis have no other underlying disease and never have circulating ANCA.[86] Mei Ding's experiment offers a novel hypothesis for an alternative mechanism in such ANCA negative patients. In their mouse model experiment, deletion of the Von-Hippel-Lindau gene (Vhlh) from intrinsic glomerular cells from mice was sufficient to initiate crescentic GN.[87] Vhlh is a negative regulator of Hypoxia inducible factor (HIF) which is a master regulator of oxygen homeostasis, angiogenesis and vascular remodelling.[88] Loss of Vhlh leads to stabilisation of HIF and up-regulation of the hypoxia response downstream. Furthermore, Ding *et al.* also found strikingly increased expression of the chemokine receptor CXCR4 in podocytes. CXCR4 binds to stromal derived factor 1 (Sdf1), which is a growth factor secreted by mesangial cells. Increased expression of Sdf1 was also noticed in glomeruli. Increased Sdf1 production may recruit inflammatory cells and contribute to formation of glomerular crescents.[89] Interestingly treatment of mice with neutralising antibodies to CXCR4 results in delayed onset and reduced severity of glomerulonephritis in these mice. Ding *et al.* add further evidence to these findings by demonstrating increased expression of HIF1 target genes and CXCR4 protein in glomeruli from humans with pauciimmune RPGN. This study provides an important road map for further evaluation to determine if increased HIF expression and its targets are critical in pathogenesis of pauci-immune crescentic glomerulonephritis. Finally, it will be interesting to see whether blocking antibodies to CXCR4 or HIF1 may have a therapeutic role.

Circulating endothelial cells as markers of endothelial damage

Circulating endothelial cells (CEC) are not a new concept to measure vascular damage. They were in fact first described almost 40 years ago [90] on the basis of light microscopy

in smears of peripheral blood. A more widespread use of CEC was only possible with the discovery of the S-Endo 1 antigen [91] and immuno-magnetic isolation.[90] This approach was originally devised for the enumeration of rare cells in body fluids. CECs are, even in the most acute vascular disorder, rare cells in peripheral blood; hence immuno-magnetic isolation is well suited for this purpose. More recently, fluorescence-activated cell sorting (FACS) has also been employed.[92] Immunomagnetic isolation uses morphological criteria although the technique is time-consuming and cumbersome.[93,94] At first glance, multiparametric FACS [95] appears user-friendlier. FACS can avoid false positive counting of leukocytes (a typical problem of the immunomagnetic approach). The technique also holds some potential to elucidate the phenotype of CEC. In this regard, analysis of CEC with six-colour FACS and demonstration of apoptosis has recently been described.[96] However, the technique does not permit direct observation of the cell morphology.[92] There is ongoing debate as to which of the two may be preferable. A standard for immunomagnetic isolation and enumeration of CECs has been proposed already [97] but there is no uniformly accepted consensus across the field.[90] Despite the lack for consensus CEC have been found to be reliable markers that correlate with clinical disease activity across a variety of vascular disorders.[90] For obvious reasons, cardiovascular disease has received much attention [98, 99] while current studies focus on phenotypic analysis, concurrent enumeration of CEC and EPC, and the clinical utility of CEC.

Circulating endothelial cells in ANCA-associated vasculitis

It is clear from the pathogenesis of ANCA-associated small-vessel vasculitis that endothelial cells are damaged and eventually dislodged from the basement membrane. We thus became interested in circulating endothelial cells (CEC) in vasculitis and hypothesized that cell count would reflect disease activity. Another hope was that we could glean further insight into the pathogenesis from phenotypic analysis of these cells. Even in the first couple of patients we encountered very high numbers of CEC in ANCA vasculitis. Given that damage to and death of endothelial cells is the hallmark of the disease, we were not surprised that cell numbers correlated extremely well with disease activity.[93]

Phenotypic analysis, however, proved more difficult than anticipated. We also attempted laser capture microdissection to further characterise the cell phenotype. However very few if any intact CEC remained for analysis and further characterisation of the cells was just not feasible. In retrospect, one of the crucial obstacles in this endeavour and in our attemps to characterise CEC by immunocytochemistry was the fact that even in acute vasculitis CEC are rare in peripheral blood. Hence the absolute number of CEC available for any given analysis is always pitifully small.

It is quite clear from the concept of small vessel vasculitis that CEC cannot be specific to ANCA vasculitis and elevated CEC numbers have since been demonstrated in other vasculitides, such as aortoarteritis [100] and Kawasaki disease.[101] CEC are also elevated in systemic lupus erythematosus.[102] A very recent study demonstrated elevated numbers of CEC and soluble markers in rheumatoid arthritis [103] and thus raised questions about the pathogenesis of the disease. In a broader sense, CEC are not even specific for vasculitis per se. They are also markedly elevated in other, non-vasculitic, forms of widespread acute vasculopathy. We studied CEC in thrombotic microangiopathy. Not surprisingly, CEC numbers were also markedly elevated and a decline in cell numbers indicated successful plasma exchange.[104] Therefore one has to remember that elevated CEC are not specific to vasculitis, let alone to its ANCA-associated variant. Hence we don't envision them to replace traditional diagnostic tools, such as tissue biopsy and ANCA testing, but to complement them.

In this regard a notoriously difficult clinical scenario is the patient with known AASV who becomes unwell during immunosuppressive treatment. Distinguishing between

disease activity and infection can be difficult to say the least. The limitations of ANCA testing have been discussed above. We found CEC very useful to monitor treatment and to distinguish between relapse and infection in difficult cases.[105] We also studied patients with limited granulomatous disease and with relapse.[106] Patients with vasculitic relapses had markedly elevated numbers of circulating endothelial cells and indeed similar cell numbers when comPred to patients at their initial vasculitic presentation. Patients with limited disease due to granulomatous disease had only slightly elevated cell numbers, which were similar to those seen in remission. These findings gave us confidence in the clinical use of CEC in vasculitis [94, 105] although our findings need to be corroborated by others. Finally, it would be interesting to see prospective monitoring of CEC in vasculitis patients.

Endothelial microparticles in ANCA-associated vasculitis

In general, microparticles (MP) are submicrometric fragments derived from plasma membranes. Increased numbers occur in response to a variety of events, such as activation, injury, or apoptosis. Loss of phospholipid asymmetry is a crucial event during this process.[107, 108] On their surface microparticles express antigens that reflect their cellular origin. These surface markers permit their enumeration and characterisation by flow cytometry. Microparticles have attracted considerable interest in vascular disease although a consensus definition of these particles and a uniformly accepted protocol for their enumeration is still lacking.[109] In this regard the situation resembles that of CEC whereby several techniques compete and progress is hampered by lack of standardised criteria for definition and enumeration. Furthermore, endothelial microparticles only represent a small subgroup of all MP found in human plasma.[110] Specific endothelial microparticles were first described in 1990 by Hamilton and colleagues.[111] We studied endothelial microparticles (EMP) by FACS analysis and found elevated EMP in active vasculitis.[112] Similar results had previously been obtained in a paediatric cohort of vasculitis patients.[107] Particle counts also correlated with disease activity.[112] It is probably safe to assume that CECs and microparticles do not reflect the same disease process. In other words, CEC, EMP and also soluble markers, such as thrombomodulin, each reflect different facets of vascular activation and damage although some degree of overlap may exist. Finally, EMP may also have pathogenetic importance in vasculitis. They are now regarded as crucial players at the interface of atherosclerosis and inflammation.[113, 114] Leukocyte MP induce endothelial IL-6 and MCP-1 production.[115] It has been demonstrated that EMP are tissue-factor positive [116] and very recent evidence suggests that they can also convert plasminogen into plasmin.[117] Finally, elegant studies in flow chambers have demonstrated that MP enhance leukocyte rolling.[118] Taken together, current data suggest that EMP may not only be a surrogate marker of vasculitis but that they may contribute to the proinflammatory and procoagulant status of the endothelium.

Endothelial progenitor cells in ANCA-associated vasculitis

The role of endothelial progenitor cells (EPC) [119] in vascular disease and their potential role for therapy [120] have been reviewed recently.[121] Of note, the field of EPC is particularly hampered by lack of standardisation.[122, 123] Our knowledge about the kinetics of CEC detachment and EPC mobilisation as well as their interaction is equally limited. Very recently, the margins between endothelial progenitor cells and haematopoietic stem cells became somewhat blurred after proof that endothelial cells can be hematopoietic in mice.[124] We studied numbers of circulating CD34+ progenitor cells and EPCs in vasculitis and demonstrated that these cells increased significantly after the institution of immunosuppressive therapy and with disease remission.[125] Others have previously described an increase in EPCs in inflammatory vascular

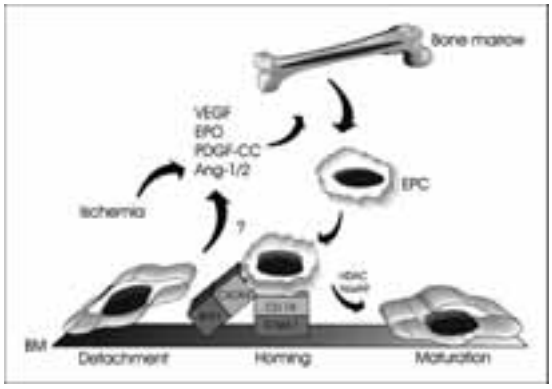

Figure 2.—Mechanisms of endothelial repair. BM, basement membrane; EPC, endothelial progenitor cell. ANG: angiopoetin; EPO: erythropoetin; PDGF: platelet-derived growth factor; VEGF: vascular endothelial growth factor; SDF: stromal-cell derived factor; CXCR: chemokine motif receptor; HDAC: histone deacetylase activity; HoxA9: homoeobox transcription factor A9; D: cluster of differentiation 18; ICAM: intercellular adhesion molecule. From Woywodt A, Erdbruegger U, Haubitz M,[119] with permission.

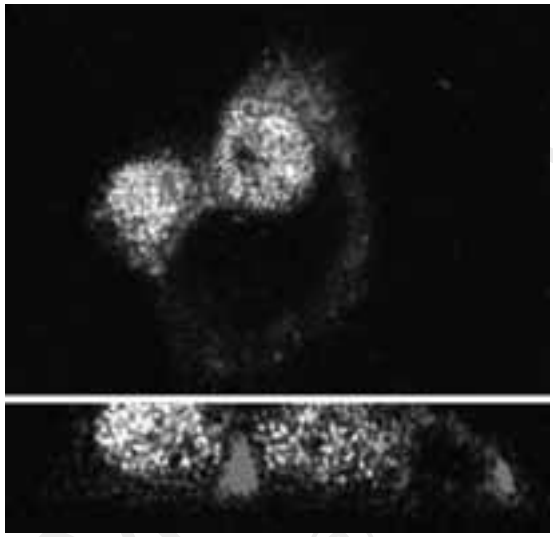

Figure 3.—Engulfment of apoptotic endothelial cells (green) by untreated human umbilical vein endothelial cells (HUVEC). From Woywodt A, Kirsch T, Haubitz M,[226] with permission.

diseases: Avouac *et al.*, for instance, described increased EPC numbers in scleroderma.[126] In contrast to de Groot *et al.*,[125] other studies postulate an imbalance between CECs and EPCs in patients with vasculitis.[127, 128] What make these studies so difficult to comPre is, again, the lack of standardisation and the use of different assays and surface markers. Therefore, these studies provide interesting food for thought but require independent confirmation. What stimulates EPCs in reaction to ischemia or other forms of insult? As a nephrologist, it is worthwhile to remember that erythropoietin (EPO) regulates EPCs.[129] Hence EPO treatment must always be corrected for when EPCs are measured in renal patients. Statins also influence EPC numbers.[130] Other factors that have been implicated as regulators of EPCs include vascular endothelial growth factor (VEGF), the angiopoetins, and platelet-derived growth factor CC (PDGF-CC). EPCs are capable of homing in to sites of vascular damage. Mechanisms include CD18/ICAM-1 and sdf-1/CXCR4. Endothelial commitment requires histone deacylase (HDAC) activity and depends on the expression of the homoeobox transcription factor HoxA9.[131] It is probably fair to say that EPCs will receive further scientific attention in vasculitis while a standard as to their definition and enumeration is eagerly awaited. Figure 2 provides an overview of mechanisms of endothelial repair.

Circulating endothelial cells as potential mediators of disease

Several lines of evidence have long suggested that CEC themselves could be pro-inflammatory.[132] Damaged eukaryotic cells have been shown to release a variety of pro-inflammatory factors, to initiate a Toll-like-receptor-2/NFκB-dependent reaction in monocytes and fibroblasts.[133] Evidence has also emerged that ANCA accelerate apoptosis in neutrophils and imPired clearance of apoptotic neutrophils has been described [134]. Kirsch et al. showed in a recent study that apoptotic and necrotic endothelial cells and their fragments are rapidly internalized by healthy endothelium (Figure 3). Support for these findings came from other studies demonstrating the phagocytic capability of endothelial cells.[135] Kirsch *et al.* could also show that endothelial cells exposed to apoptotic and necrotic cells exhibit enhanced adhesion properties for leukocytes and that

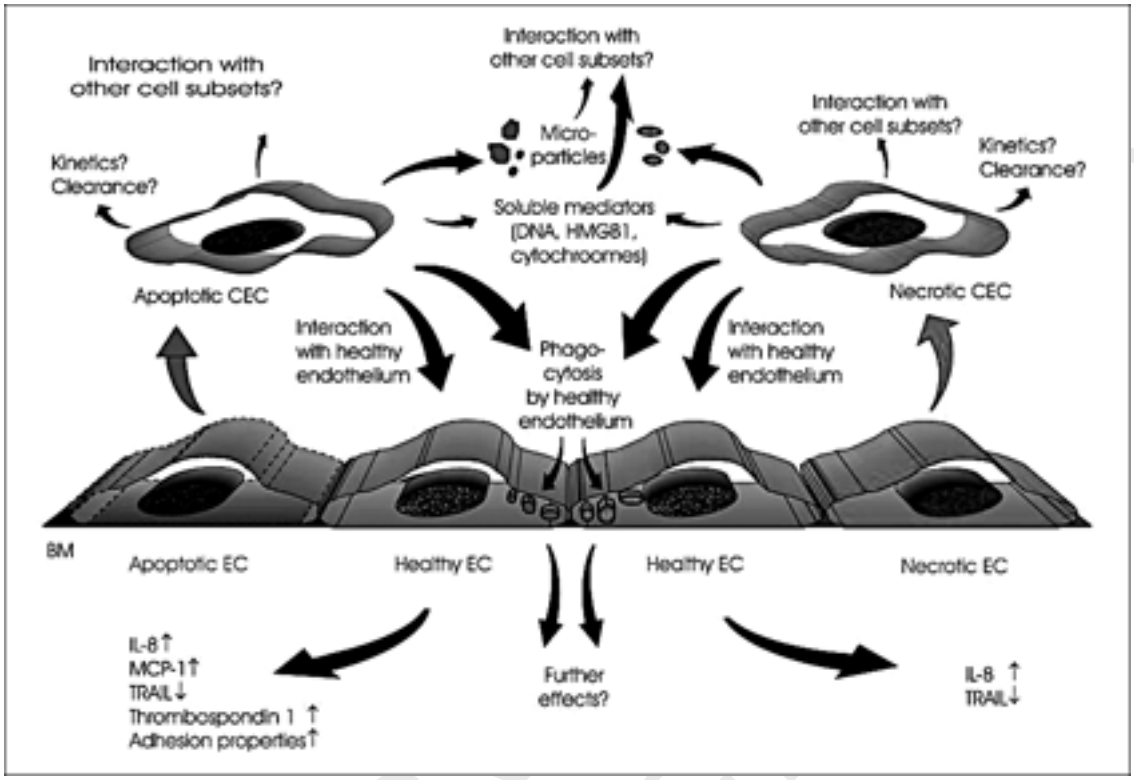

Figure 4.—Interactions of damaged circulating endothelial cells with healthy endothelium. IL-8 denotes interleukin-8. MCP-1 denotes Monocyte chemotactic protein-1. HMGB denotes High-mobility group box 1. TRAIL denotes tumor necrosis factor-related apoptosis-inducing ligand. Note that some interactions, for example those with other cell subsets in peripheral blood, are as yet unproven [226].

isolated CECs from patients with vasculitis even aggravated these effects.[136] These effects on binding properties could be explained, at least in part, by release of the proinflammatory chemo-attractants IL-8 and MCP1 which serve as chemo-attractants. Interestingly, apoptotic and necrotic cells induced different patterns of effects in healthy endothelium. Enhanced IL-8 and MCP1 levels in serum have been detected in patients with active vasculitis.[137] Endothelial synthesis of these mediators triggered by ANCA [138] and circulating endothelial cells [136] may contribute to the pro-inflammatory state associated with vasculitis. Kirsch *et al.* have recently investigated this topic further and became interested in thrombospondin (TSP-1). This multidomain, multi-functional glycoprotein modulates cell adhesion and proliferation.[139] Kirsch *et al.* were able to show that apoptotic cells induce enhanced expression of TSP-1 in human endothelial cells and demonstrated that TSP-1 facilitates engulfment of apoptotic cells by phagocytes.[140] It is tempting to speculate that endothelial-derived elevated TSP-1 may serve as a signal for phagocytes promoting enhanced clearance of apoptotic cells. Figure 4 summarizes proposed interactions of circulating endothelial cells with healthy endothelium.

Therapy of ANCA-associated vasculitis

General principles

A common feature of the ANCA-associated vasculitides is marked heterogeneity regarding disease manifestation, severity and prognosis, which affects treatment strategies. Two different classifications, namely the Chapel Hill system and the American College

of Rheumatology criteria, are in use concurrently. A unified classification has been proposed but is not yet widely used.[141] The European Vasculitis Study group (EUVAS) has previously proposed definitions of disease activity and stages to guide therapy and clinical studies (Table I).[142] Briefly, non-organ or non-life-threatening disease, which can be localized or early systemic, is distinguished from organ-threatening disease, which can be generalized, severe or refractory. Other instruments are also in use to assess disease activity. Merkel *et al.* correlated these different tools and found them highly correlated and reliable.[143] A second generation of such tools for the assessment of disease activity is currently under development.[144]

Treatment strategies have evolved since the introduction of glucocorticoids in 1948. Methotrexate, azathioprine and cyclophosphamide were introduced in the 1960's, and plasma exchange in 1976. It is worthwhile to remember that long-term survival and remission only became feasible with the introduction of Cyclophosphamide by Fauci.[145] Before the introduction of cyclophosphamide-based therapeutic regimens, mortality of Wegener's granulomatosis at 1 year was 80%.[146] Since then, much has been learned through numerous studies, including multi-centre, international and prospective trials over the last 20 years. It is through this body of evidence that ANCA-associated vasculitis has now become a chronic and treatable disease. 5-year survival rates now approach 80% [147] although relapses are not uncommon.[148] Finally, it is noteworthy that elderly patients still have a poor prognosis.[149]

The cytotoxic drug regimens currently used have a significant toxicity, which can contribute to morbidity and mortality. A considerable share of the current mortality is indeed due to infections. Malignancy is of similar concern and cyclophosphamide is usually implicated whereby the cumulative dose seems to be of greatest importance. A large study from Scandinavia reported a twofold increased risk of cancer when compared to the general population.[150] Cancer of the urothelium and skin as well as lymphoma and leukemia are most common.[150] Life-long vigilance regarding malignancy, and urothelial neoplasms in particular, is advised in all patients who have been treated with cyclophosphamide. However prospective studies on screening strategies in these high risk patients are lacking.

TABLE I.—*Definitions of disease stages of ANCA-associated vasculitis.*[156]

Category	Definition
Localised	Upper and/or lower respiratory tract disease without any other systemic involvement or constitutional symptoms
Early systemic	Any, without organ or life-threatening disease
Generalized	Renal or other organ threatening disease, serum creatinine <500 µmol/litre (5.6 mg/dl)
Severe	Renal or other vital organ failure, serum creatinine >500 µmol/litre (5.6 mg/dl)
Refractory	Progressive disease unresponsive to glucocorticoids and cyclophosphamide

Immunosuppression also increases the risk of infertility, whereby the risk is particularly high with cyclophosphamide. In the NIH study, cyclophosphamide therapy in women resulted in a 57% incidence of amenorrhea lasting more than one year or inability to become pregnant.[151] The risk relates to cumulative dose and regimen and pulse cyclophosphamide is probably less detrimental to fertility.[152] These days counselling should be mandatory, particularly in younger patients. Data in men treated with cyclophosphamide are not nearly as good in vasculitis but studies using cyclophosphamide to treat tumors show there is no reason to believe their fertility should be spared by alkylating agents such as cyclophosphamide in vasculitis. Cryopreservation of sperm should therefore be considered if appropriate.

Inflammatory vascular disease also confers an increased risk of cardiovascular disease [153] although the evidence is stronger for lupus [154] than for ANCA-associated vasculitis.[155] It is not quite clear whether the increased risk relates to the disease, its treatment, or both. Anti-hypertensive therapy is crucial, as is treatment of risk factors such as hyperlipidemia.

TABLE II.—*Treatment of ANCA-associated vasculitis, modified after.*[156]

Category	Induction	Maintenance
Localized	Methotrexate and steroids	Azathioprine or methotrexate plus low dose steroids
Early systemic	Steroids and either methotrexate or cyclophosphamide	—
Generalized	Cyclophosphamide (preferably intravenous for 3-6 months; oral 2mg/kg body weight/day) and steroids (initially intravenous boli, followed by 1mg/kg/day for 1 month)	Steroids and either Azathioprine, Methotrexate (in pa-tients with normal renal function) or alternatively Leflunomide
Severe	Cyclophosphamide (intravenous or oral) and steroids plus plasma exchange	MMF in patients with intolerance to Azathioprine
Refractory	Rituximab, steroid pulses, infliximab, immunoglobulins, Deoxyspergualin, anti-thymocyte globulin	No consensus

Given this considerable toxicity of treatment, more recent studies have attempted to establish treatment strategies with reduced toxicity that are more tailored to the individual patient's characteristics. Therapeutic concepts are also changing from permanent cytotoxic treatment to sequential treatment strategies using less toxic drugs after remission has been achieved. This approach has surely reduced mortality and morbidity and must be used as a reference to test the efficacy and safety of newer drugs. A recent paper from the European Vasculitis Study Group has summarised current recommendations for the management of ANCA-associated small and medium vessel vasculitis.[156] Nevertheless it remains sobering that some forty years after the introduction of cyclophosphamide this toxic drugs still remains the mainstay of treatment in severe disease. Finally the issue of prophylaxis of treatment toxicity, for example with trimethoprim/sulphamethoxazol to prevent pneumocystis pneumonia or with mesna during cyclosphosphamide treatment, is as important as the therapy itself but beyond the scope of this article.

Induction of remission in generalized vasculitis

Oral cyclophosphamide (Cyc) and glucocorticoids represented the first treatment to permit long-term survival in generalized and organ-threatening disease (including renal failure with serum creatinine >500 μmol/L).[151, 157] However oral cyclophosphamide is associated with considerable toxicity from treatment. In the seminal NIH study haemorrhagic cystitis occurred in 50%, bladder cancer in 5.6%, myelodysplasia in 2%, and lymphoma in 0.7%.[151] Subsequent studies were performed to determine whether intravenous pulse cyclophosphamide was equally effective for the induction of remission.[158] Three randomized-controlled trials showed that both regimens have similar efficacy in achieving remission when compared to oral cyclophosphamide (Table II).[159-161] However, intravenous cyclophosphamide resulted in lower cumulative doses (over 50% reduction) and less side effects such as infection and leucopenia.[161] A meta-analysis concluded that intravenous cyclophosphamide was equally effective but associated with a higher relapse rate as compared to oral cyclophosphamide.[157] A very recent randomized trial, published this year, showed that intravenous pulse cyclophosphamide was equally effective in inducing remission when compared to oral cyclophosphamide.[162] These data give us further confidence to adopt intravenous pulse cyclophosphamide as the new standard for induction of remission with less drug toxicity. However this issue remains debated and longer follow-up remains desirable. Several

variants of intravenous pulse cyclo-phosphamide are in use and doses are around 0.5 to 1.0 g/m^2 body surface area. Many clinicians use 750 mg/m^2 as standard dose. Some adjust the dose according to the leukocyte nadir at 14 days; newer regimens also adjust the dose for age and renal impairment.[163] The duration of intravenous cyclophosphamide treatment is also unclear. Traditionally most clinicians would treat for one year and then switch to Azathioprine but now the tendency is towards shorter duration, for example three to six months. There is currently no firm evidence in this regard and most clinicians will make an individual decision in each case, based on the extent and severity of disease and the risk of immunosuppression. Of note, we have learned from CYCAZAREM [164] and WEGET [165] that most patients achieve remission within six months after diagnosis.

Plasma exchange has long been used in severe cases of ANCA-associated small-vessel vasculitis, particularly those with pulmo-renal syndrome and diffuse alveolar haemorrhage. An earlier small retrospective study in 20 patients with alveolar hemorrhage syndrome showed resolution of hemorrhage in all patients using plasma exchange.[166] More convincing evidence has now emerged. In the recent MEPEX study, plasma exchange has been shown to improve renal recovery for patients with ANCA-associated vasculitis presenting with renal failure (serum creatinine >500 μmol/L): this trial showed a 69% renal recovery rate in patients with AAV with serum creatinine >500 μmol/L treated with plasma exchange comPred to 49% with methylprednisolone.[167] All patients received continuous cyclophosphamide and oral prednisolone as well. On that basis, the role of plasma exchange in severe ANCA-associated small-vessel vasculitis seems to be well established. However, plasma exchange is not a risk-free treatment. Increased heparin exposure and volume overload may prove detrimental in patients who are dialysis-dependent already and especially those with pulmonary haemorrhage. Whether the issue of heparin exposure can be circumvented in by citrate anticoagulation remains unproven. Use and timing of plasma exhange will for now require considerable experience in these patients. Smaller centres may also struggle with the logistics, particularly out of hours. Thus, it remains to be seen whether plasma exchange is also advantageous in less severe cases (serum creatinine <500 μmol/L) and further studies, such as PEXIVAS, are awaited. Finally, in MEPEX the mortality of severe ANCA-associated with renal failure vasculitis remained high, *i.e.* around 25%, regardless of whether steroid pulse or plasma exchange was used.[168] This may be attributed to the use of high steroid dosis and oral cyclophosphamide in an old patient population.

Induction of remission in non-organ-threatening and granulomatous disease

Early disease of the upper respiratory tract can be managed with steroids and oral trimethoprim/sulphamethoxazole although this treatment option has become somewhat less popular in recent years. Topical treatment is also crucial especially if severe rhinitis is present.[168] Nasal oil and saline nose spray are useful as well as topical Mupirocin if *Staphylococcus* is present.

Induction of remission in non-organ-threatening disease without renal imPirment can be achieved with low-dose once weekly methotrexate (MTX), which is somewhat less toxic than Cyclophosphamide although not entirely without problems. The NORAM study confirmed its efficacy in early systemic disease but also showed a high rate of relapse.[169] Furthermore, the drug is contra-indicated in renal failure. There is also a need for proper counselling in once weekly oral treatment is begun: Fatal leukopenia may ensue if patients take the drug daily. Pulmonary and hepatic toxicity is another matter of concern. Additionally, one small, randomized trial of moderate renal vasculitis found similar induction rates with mycophenolate mofetil compared to cyclophosphamide [170] although these data require confirmation in larger studies. For now, methotrexate remains an alternative to cyclophosphamide in mild disease without renal involvement.

A feared manifestation of WG is bronchial/

laryngeal stenosis.[171, 172] These lesions can develop largely independent of all other disease activity and prove be very refractory to treatment. These cases require a multi-disciplinary approach involving interventionalists from other fields. Intra-lesional steroids have been used with good success in active disease and repeated dilation of chronic stenoses is feasible in experienced hands.[173]

Maintenance of remission

It is well known that remission can be maintained with oral cyclophosphamide and tapering doses of prednisolone, albeit at the cost of substantial drug toxicity and side effects. Newer drug regimens using sequential drug therapy have aimed to maintain remission with less toxicity. In the CYCAZEREM trial in 2003,[164] 144 AAV-patients (WG/MP or renal limited variant) with generalized disease (Crea <500 μmol/L) received oral cyclophosphamide plus glucocorticoids and were randomized to either continue oral cyclophosphamide or to switch to azathioprine (Aza) after induction of remission. No difference in relapse rates, renal function and progression to end-stage renal disease was seen after 18 months (13.3% vs. 15%) . These data suggested that a switch to azathioprine after remission is effective and safe. Are there alternatives to azathioprine? In the WEGENT trial, published in 2008, 126 patients (WG or mP) were randomized to receive either MTX or Aza for 12 months after successful induction of remission with intravenous cyclophosphamide.[174] There was no difference in relapse free survival and toxicity between theses two drugs, demonstrating that methotrexate can also be used as an alternative maintenance drug.[174] Therefore methotrexate may be regarded as an alternative in patients who cannot tolerate azathioprine or who are younger, as one would like to avoid the effects on skin and development of secondary malignancy. However one must bear in mind that somewhat higher relapse rates have previously been reported with the drug.[175] Are there further alternatives? Mycophenolate mofetil (MMF) can also be used as an alternative, but data from uncontrolled prospective and retrospective studies also show a higher relapse rate.[176, 177] Preliminary data from a EUVAS study (IMPROVE) showed earlier relapses in patient treated with MMF than in the azathioprine group. Others have used a novel drug, Leflunomide, an immuno-modulatory drug inhibiting dihydroorotate dehydrogenase, to maintain remission.[178] They subsequently demonstrated that leflunomide was more effective than methotrexate [175] although the drug has frequent side effects. Neuropathy is of particular concern.[179]

To maximize the effectiveness of maintenance regimens, the treatment strategy may have to be individualized according to the clinical context. For example, maintenance Azathioprine has been associated with significantly higher relapse rates in patients that had positive ANCA levels at the time of switching.[180] Also, patients with WG have a higher tendency to relapse than patients with MP. Earlier studies have linked relapse in WG to nasal carriage of staphylococcus aureus.[64] Interestingly, relapse rates in patients with respiratory tract involvement could be reduced by adding sulfamethoxazole/trimethoprim to the maintenance regimen.[181] More recently, this strategy has been somewhat forgotten and no recent studies have addressed this therapeutic approach.

The strongest predictor of relapse is withdrawal of immunosuppression.[162] There is presently no consensus on the duration of maintainance therapy, and recommendations range from 18 months to 5 years in patients with persistent ANCA positivity. This issue is addressed in another ongoing trial (REMAIN). This interesting study will evaluate whether prolonged maintenance therapy with low-dose prednisolone and azathioprine reduces long-term morbidity in systemic vasculitis, by reducing the frequency of relapse, when compared with cessation of therapy in the second year.

Emerging therapies

Empiric cytotoxic treatment has improved the survival of patients with AAV significant-

ly and newer regimens have reduced treatment-related toxicity and morbidity. However by today's standards many of these drugs, such as cyclophosphamide, seem crude and not targeted on the pathogenetic mechanisms of vasculitis. In addition, relapsing and refractory disease remain challenging to treat. Newer treatment strategies are therefore eagerly awaited. It must be assumed that preventing the initiation of the inflammatory cascade is the most effective treatment in vasculitis. If this is impossible, the goal is to attenuate subsequent immune responses by targeting crucial pathogenetic mechanisms or inflammatory mediators. Directing therapies at these sequential targets will go beyond non–specific immunosuppression and hopefully expose patients to less toxicity. Most of the newer drugs are used either as additional or adjunctive treatment, only some of them are investigated as an alternative to the standard of care.

Therapy directed at B cells

One of the most exciting new developments in the treatment of ANCA-associated vasculitis is the emergence of a new target for treatment, the B lymphocyte. B-cells play an important role during the pathogenesis of the disease as they differentiate to plasma cells that must eventually produce the ANCA antibodies. These ANCA-secreting B cells are believed to mature and reside within granulomatous lesions in WG.[70] Rituximab, an anti-CD20 chimeric monocloncal antibody, has allowed partial or complete remissions in relapsing or refractory AAV in 70-100% in small case series or case reports.[182] The largest retrospective multicenter study of 65 patients in the UK showed at least partial response in 99% of all cases.[183] Clinical relapses occurred frequently, approximately 30% within the first 2 years. Most relapses occurred after reconstitution of peripheral B cells. Leucopenia and infectious complications such as potential reactivation of hepatitis B virus [182] and cytomegalovirus is a matter of concern although clinically the drug is remarkably well tolerated. Progressive multifocal leukoencephalopathy has also been reported.[184]

Some have reported that granulomatous head and neck manifestations of WG are also amenable to rituximab.[185] Others describe failure of rituximab treatment in granulomatous disease.[186] It is probably fair to say that these findings highlight the need for a better understanding of the interplay between granulomatous lesions and B cells. Furthermore, the exact mechanism of action of rituximab in ANCA vasculitis is incompletely understood, since CD20 is not expressed on plasma cells themselves, but ANCA titres decrease following rituximab treatment. Another unresolved problem is the timing of re-treatment. A recent multi-centre survey, while confirming the efficacy of rituximab, showed that ANCA or B cell levels are not ideal to guide re-treatment.[183] Potentially, the effect can be explained by the interference of rituximab with B-cell proliferation in the setting of high plasma-cell turnover,[187, 188] or being located in anatomical niches. Despite our limited understanding regarding its mechanism of action and concerns about long-term toxicity, rituximab has probably been the most significant addition to our therapeutic armamentarium in the last five years. Two large controlled studies (RITUXVAS and RAVE) gave first very promising results regarding its use as a first line therapy with the chance to reduce (RITUXVAS) or avoid (RAVE) cyclophosphamide.

Newer B-cell depleting therapies such as ocrelizumab (humanized anti CD20), ofatumumab (human anti-CD20) and epratuzumab (humanized anti-CD22) have been developed. Some of these agents have already been used in systemic lupus erythematosus and rheumatoid arthritis while no data have yet been reported in vasculitis. Another potential target of therapy is the B lymphocyte stimulator (BAFF/BLYS), a member of the TNF superfamily that is critical to B-cell development.[189] The crucial role of BAFF/BLYS in lupus is only just emerging.[190] TNF can increase the release of BAFF/BLYS from neutrophils [191] and BAFF/BLYS serum levels have been found to be increased in patients with vasculitis.[73] Therapeutic manipulation is already available through Belimumab (humanized anti-BAFF/BLYS). It remains to be seen

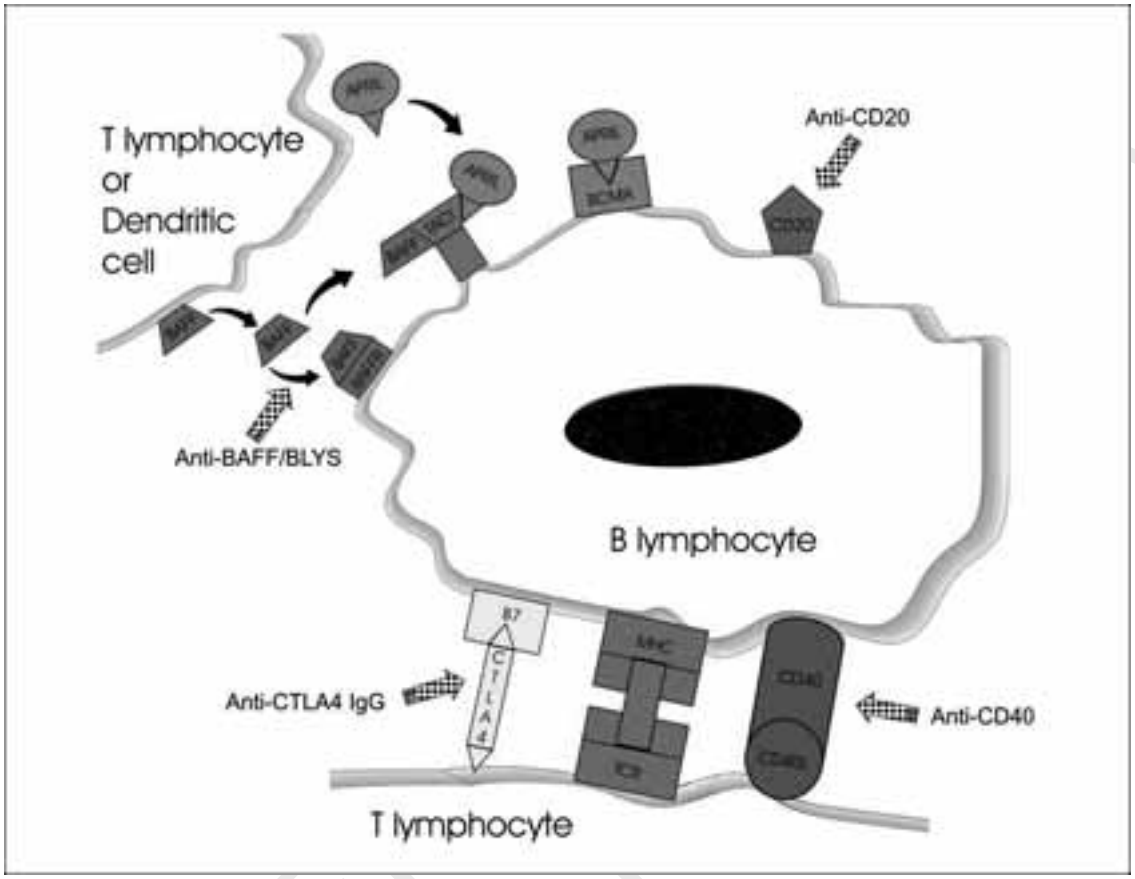

Figure 5: B cells as current and future targets for therapeutic intervention. BAFF denotes the BAFF/BLYS factor. BAFFR denotes BAFF/BLYS receptor. CTLA4 denotes Cytotoxic T-Lymphocyte Antigen 4 and B7 its receptor. APRIL denotes a proliferation-inducing ligand. TACI denotes transmembrane activator and CAML interactor, the receptor for BAFF/BLYSS and APRIL. BCMA denotes B cell maturation antigen. TCR denotes T cell receptor. CD40 denotes cluster of differentiation 40 and CD40L its ligand. CD 20 denotes cluster of differentiation 20. Note that BAFF/BLYS and APRIL promote survival of B lymphocytes. BAFF/BLYS and APRIL work via TACI whereas APRIL can also activate via BCMA. Therapeutic manipulation of BCMA and TACI is not yet available. Anti-CD40 is also available (Dacetuzumab).

whether BAFF/BLYS could become a therapeutic target in vasculitis. Figure 5 provides an overview of current and future therapeutic interventions directed at B cells.

Therapy directed at T cells

The production of ANCA is dependent on T cells. T cells are highly activated with increased expression of HLA-DR and CD25 and predominantly show a Th1 cytokine phenotype. They are found in affected tissues, in granulomas and vasculitic lesions.[192] Effector memory T cells are increased and possibly not adequately controlled by regulatory T cells, leading to chronic inflammation in AASV.[65] Many of the established drug treatments, such as azathioprine or mycophenolate mofetil (MMF), affect T lymphocytes albeit in a non-specific manner. 15-deoxyspergualin (gusperimus) is a novel T cell directed drug.[193] It inhibits the interleukin-2-stimulated maturation of T cells and the polarization of T cells into IFN-gamma-secreting Th1 effector T cells resulting in growth of activated naive CD4 T cells. Deoxyspergualin also inhibits cytokine and monocyte activation. It has been effective in a small group of patients resistant or intolerant to cyclophosphamide.[194] Leukopenia is a significant side effect, but seems to be

manageable.[195] Another issue is that the drug is administered by subcutaneous self-injection, which may not be feasible in each and every patient. Unfortunately, disease flares were frequent after the drug was withdrawn.

Another unspecific T cell directed drug is antithymocyte globulin, which represents a collection of polycloncal antibodies directed against the surface antigens of activated T cells. In a small study in 15 patients with WG resistant to cyclophosphamide it has been shown to be effective.[196] The monoclonal anti-CD52 antibody alemtuzumab [197] is a more selective T cell drug. Alemtuzumab has been used earlier to treat patients with refractory or relapsing AASV.[56] In the largest available study conducted in 71 patients, 65% went into clinical remission and 20% had a significant improvement. Disease relapse and infectious complications, however, were quite common.[198]

T cell activation is thought to be due to a "two-signal model" in CD4+ T cells. Activation of CD4-positive T cells occurs through engagement of the T cell receptor and co-stimulation of CD28 on the T cell surface by the major histocompatibility complex peptide and B7 family members on the antigen presenting cell. Cytotoxic-T-lymphocyte-associated antigen 4 (CTLA 4) can prevent "delivery" of the second co-stimulatory signal required for complete activation of T-cells. The gene encoding CTLA-4 has multiple polymorphisms and a positive association between WG and longer alleles of (AT)n in the CTLA-4 gene has been demonstrated.[199] In addition, CTLA4-IG has prevented disease progression in an animal model of crescentic glomerulonephritis.[200] Unfortunately, a trial investigating CTLA-4 IG as an adjunctive to MTX to induce and maintain remission in early systemic ANCA-associated vasculitis was terminated, apparently due to low recruitment and supply issues with the sponsoring manufacturer. Finally, it has to be said that the catastrophic experience with the anti-CD28 Monoclonal Antibody TGN1412 [201] has dampened the initial enthusiasm regarding therapeutic manipulation of co-stimulatory signals.

Therapy directed at individual cytokines

In vitro and *in vivo* evidence demonstrates that TNF-α plays a central role during the pathogenesis of ANCA-associated vasculitis. TNF-α mediates granuloma formation, endothelial activation and neutrophil priming in ANCA-mediated vascular damage. TNF-α is also increased in serum of patients with active vasculitis.[202] In two animal models antibodies against TNF-α prevented or attenuated ANCA induced crescentic glomerulonephritis.[203, 204] Pilot studies showed a benefit of treatment with Etanercept, is a recombinant, soluble, human, TNF receptor fusion protein.[205, 206] A larger randomized, placebo-controlled trial (WGET) evaluated the efficacy of Etanercept in inducing and maintaining remission in 180 WG patients as an adjunct to standard therapy. No additional effect of Etanercept was found and there were more adverse-events.[164] The increased incidence of malignancy is of particular concern.[207] Infliximab, a chimeric anti TNF monoclonal antibody has been studied in open-label trials including 51 patients with AASV.[205, 208-210] In this heterogenous group Infliximab induced remission in 70-88% of patients with refractory vasculitis. However an increased rate of serious infections has been seen with the drug,[211] most notably tuberculosis.[212] As of today, no randomized trial of infliximab in patients with AASV exists. Infliximab may possibly also help in resistant granulomatous disease, as efficacy against granulomatous inflammation has been shown in patients with Crohn's disease.[213]

Interleukin-5 (IL-5) can also be targeted by an antibody (mepolizumab) which reduced dosage of glucocorticoids in the hypereosinophilic syndrome [214] and is currently investigated in patients with Churg Strauss Syndrome. IL-5 is intimately linked to the eosinophil inflammatory response. Its utility may therefore be limited to the Churg Strauss syndrome, in which eosinophils play a crucial role.

IL-6 is another pro-inflammatory cytokine. Its role in other inflammatory diseases, such as inflammatory bowel disease, is well characterised although its importance in vasculi-

tis is not quite clear. IL-6 can be targeted by tocilizumab, a fully humanized monoclonal anti-IL-6 antibody. Tocilizumab did show benefit in patients with rheumatoid arthritis [216]. Animal data and studies in systemic lupus erythematosus show some favorable effect on extrarenal manifestations [216] but its efficacy in vasculitis remains unclear.

Therapy directed at other targets

Intravenous immunoglobulin (IVIG) is an immune-modulatory drug with multiple effects. It is thought to interact with the inhibitory Fc receptor. A few open-label studies have shown that IVIG is effective in refractory ANCA-associated vasculitis and in early active disease.[217] IVIG is a potential treatment alternative if standard care is not feasible, *e.g.* in the context of active infection or pregnancy.

Furthermore, recent evidence also suggests a crucial role for the alternative pathway of complement activation.[218] Traditionally, the ANCA-associated vasculitides have been classified as pauci-immune, suggesting that complement plays no major role in the disease process. More recently, however, it has emerged that complement may be more important than initially believed. Accordingly, monoclonal antibodies against the complement component C5 can significantly attenuate MPO-ANCA crescentic glomerulonephritis in mice.[203] Also, C5a and its receptor have beed described as an amplification loop for ANCA-mediated neutrophil activation.[219] Eculizumab, a humanized monoclonal antibody can inhibit C5 cleavage. The drug has been used in hemolytic uremic sydnrome [220] but no data are currently available in vasculitis.

A common theme in recent years has been an increased understanding of the pathways of neutrophil signaling in vasculitis. More and more of these pathways are therefore becoming potential targets for treatment. Imatinib mesylate, a tyrosine kinase inhibitor used in malignancies as chronic myeloid leukemia, has been used in vitro on cells from patients with active vasculitis. These isolated cells showed less expression of CD25, and decreased conversion of T cells to memory T cells in vitro. Its use in experimental models of rheumatoid arthritis [221] also suggest a possible benefit in other autoimmune diseases, although data in vasculitis are lacking. Another crucial pathway seems to be that of an isoform of the protein kinase PI3K, which suppresses ANCA-mediated neutrophil chemotaxis and activation. It might be another treatment target.[222] Inhibition of this kinase can decrease the inflammation in SLE and RA.[223, 225] P21ras is also crucial to superoxide production and vascular necrosis, two salient features of ANCa-associated vasculitis. Farnesylthiosalicylic acid is a selective inhibitor of mediator p21ras. The inhibition of this downstream mediator p21ras could diminish superoxide production and vascular necrosis. Nevertheless, the use of kinase inhibitors as an antiproliferative agent has still to be proven in clinical studies.

Conclusions

Much has been learned in recent years on the pathogenesis of ANCA-associated small-vessel vasculitis. We already knew that interaction of primed neutrophils with ANCA and endothelial cells is crucial to the disease. Next we gained a better understanding, from animal models, of the pathogenetic importance of the ANCA antibody although clinicians remain sceptical, given the fact that some patients have high titres but no disease. Very recent evidence provides intriguing data regarding the link between infection and vasculitis, LAMP-2 antibodies [85] as novel markers, and NETs as a novel pathogenetic mechanism. It remains to be seen whether others are able to corroborate these findings and whether testing for LAMP-2 antibodies will become part of the clinical routine in vasculitis. Recent years also saw the emergence of various new markers of endothelial damage and the disease itself, such as circulating endothelial cells and endothelial microparticles. Available data suggest that these novel markers correlate well with disease activity and that they may well complement traditional diagnostic tools, such as ANCA test-

ing. Preliminary evidence has provided some insight into the balance between endothelial damage and repair. Exciting preliminary data also indicate that circulating endothelial cells may not only be markers of disease activity but that these cells may have pathogenetic importance in their own right. These findings may have profound implications for the pathogenesis of vasculitis and vascular disease in general. Recent years also saw the publication of a number of seminal studies for the treatment of ANCA-associated vasculitis. We now have a much better understanding of the role of pulse intravenous cyclophosphamide and plasma exchange than ten or even five years ago. Further studies must now show whether plasma exchange is also beneficial for less severely ill patients with AASV. Finally, as ever, it is hoped that further progress in understanding the disease pathogenesis will also provide more tailored and less toxic therapies.

Riassunto

Vasculiti da ANCA-positive: patogenesi, nuovi marker di malattia e terapie emergenti

Negli ultimi anni la patogenesi delle vasculiti dei piccoli vasi ANCA-positive è stata oggetto di molti studi. L'interazione dei neutrofili con gli ANCA e le cellule endoteliali è cruciale nella patogenesi della malattia. Successivamente studi su modelli animali hanno permesso di capire l'importanza patogenetica degli anticorpi ANCA. Evidenze recenti hanno mostrato nuovi modelli patogenetici quali la relazione fra le infezioni e le vasculiti, gli anticorpi LAMP-2 e NET. Rimane solo da stabilire se altri studi siano in grado di corroborare questi risultati e se il dosaggio degli anticorpi LAMP-2 possa essere utilizzato come indagine di routine nelle pratica clinica. Negli ultimi anni sono stati introdotti nuovi marker di danno endoteliale della malattia, come cellule endoteliali circolanti e microparticelle endoteliali. Questi nuovi marker sono correlate con la attività della malattia e possono essere utilizzati come mezzi diagnostici oltre a quelli tradizionali come i test per gli ANCA. Alcuni studi preliminari hanno osservato una correlazione fra il danno e la ripazione endoteliale. Sono stati riporati risultati promettenti che indicano che le cellule endoteliali circolanti non sono marker della attività della malattia, ma presentano comunque un ruolo fondamentale nella patogenesi della malattia stessa. Questi risultati hanno implicanze nella patogenesi delle vasculiti e delle malattia vascolari in generale. Negli ultimi anni è stato osservato un incremento nelle pubblicazioni di studi in merito al trattamento della vasculiti ANCA-positive. Rispetto a cinque o dieci anni fa, conosciamo meglio il ruolo della ciclofosfamide somministrata per via endoveosa e dei plasma exchange. Sono tuttavia necessari ulteriori studi al fine di valutare gli effetti dei plasma exchange nelle forme di vasculiti meno gravi. Infine si spera che in un futuro prossimo si possano capire meglio i meccanismi patogenetici della malattia al fine di poter garantire terapie più specifiche e meno tossiche.

Parole chiave: Vasculiti, diagnosi – Vasculiti, anatomia patologica – Vasculiti, terapia farmacologica.

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