THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN THE PATHOGENESIS OF ANTI - NEUTROPHIL CYTOPLASMIC ANTIBODY - ASSOCIATED VASCULITIS

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PUBLICATIONS:

I. Publications that formed the basis of the Thesis:

- **Závada J**, Kideryová L, Pytlík R, Vanková Z, Tesar V. Circulating endothelial progenitor cells in patients with ANCA-associated vasculitis. Kidney Blood Press Res. 2008;31(4):247-54. ISSN 1420-4096, **IF 1,268**
- **Závada J**, Kideryová L, Pytlík R, Tesar V. Circulating endothelial cells and circulating endothelial progenitors in kidney disease--victims, witnesses, or accomplices? Folia Biol (Praha). 2008;54(3):73-80. Review. ISSN 0015-5500, **IF 1,140**
- **Závada J**, Kideryová L, Pytlík R, Hrusková Z, Tesar V. Reduced number of endothelial progenitor cells is predictive of early relapse in anti-neutrophil cytoplasmic antibody-associated vasculitis. Rheumatology (Oxford). 2009 Oct;48(10):1197-201. ISSN 1462-0324, **IF 4,136**

II. Further publications related to the topic of the Thesis

- Rihova Z, Honsova E, **Zavada J**.: Two familial cases of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Rheumatology (Oxford). 2006 Mar;45(3):356-7 (Letter). **IF 4,052**
- Hruskova Z, Rihova Z, Mareckova H, Jancova E, Rysava R, **Zavada J**, Merta M, Löster T, Tesar V. Intracellular cytokine production in ANCA-associated vasculitis: low levels of interleukin-10 in remission are associated with a higher relapse rate in the long-term follow-up. Arch Med Res. 2009 May;40(4):276-84. **IF 1,703**

ABSTRACT

Introduction: Circulating endothelial progenitor cells (EPC) may provide an endogenous repair mechanism to counteract ongoing endothelial damage. Antineutrophil cytoplasmic antibody - associated vasculitis (AAV) is an inflammatory disorder of small- to medium-sized vessels with relapsing/remitting progression and endothelial injury is a major feature of AAV. EPC thus may play and an important role in the pathogenesis of AAV, or serve as a useful marker for monitoring and/or prediction of outcomes in patients with AAV.

Hypothesis 1: EPC number in patients with AAV could be altered.

Hypothesis 2: The decreased capacity for endothelial regeneration paralleled by low EPC numbers could increase the risk of relapse in patients with AAV.

Patients and methods: We have measured EPC in healthy volunteers, patients with AAV, chronic kidney failure (CKD) and atherosclerosis by a colony-forming assay. We have investigated the relation between the numbers of EPC, clinical and laboratory characteristics of the patients, and long-term outcomes of patients with AAV.

Results: Patients with AAV had a significantly lower number of EPC than healthy subjects, but not than patients with CKD or atherosclerosis**.** The cumulative relapse-free survival increased stepwise across three increasing baseline levels of endothelial progenitor cells.

Conclusion: Patients with AAV have a significant and persistent deficiency of circulating EPCs. A low number of EPCs could reflect an impaired mechanism of vascular repair and may contribute to repeated relapses in these patients.

ABBREVIATIONS

AAV	ANCA-associated vasculitis
ACEi	Angiotensin-converting enzyme inhibitors
ACR	American College of Rheumatology
ANCA	Antineutrophil cytoplasmic antibody
AZA	Azathioprine
ARB	Angiotensin receptor blockers
BVAS	Birmingham Vasculitis Scoring Index
CEC	Circulating Endothelial Cell
CFU-Hill	Endothelial colony-forming units (by methodology according to Hill)
CKD	Chronic Kidney Disease
CSS	Churgï Strauss syndrome
CYC	Cyclophosphamide
DAPI	Diamidino-2-phenylindole
EC	Endothelial cell
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENT	Ears, nose, throat
EPC	Endothelial progenitor cell
EPC-CFU	Endothelial progenitor cell-Colony Forming Unit
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein isothiocyanate
GFR	Glomerular Filtration Rate
HD	Hemodialysis

MMF	Mycophenolate Mophet il
MPA	Microscopic Polyangiitis
MPO	Myeloperoxidase
MTX	Methotrexate
PAOD	Peripheral artery occlusive disease
PMN	Polymorphonuclear neutrophils
PR3	Proteinase-3
UEA-1	Ulex europaeus agglutinin-1
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
WG	Wegener's granulomatosis

ANCA-ASSOCIATED VASCULITIS

Introduction

Vasculitis is an inflammation of blood vessel walls. Diseases in which vasculitis is a primary process are called primary systemic vasculitides. They are characterized by necrotizing inflammation of blood vessels and encompass several different disease entities. Their classification, clarified at the Chapel Hill Consensus Conference [1], relies chiefly on the size of vessel involved as well as certain clinical features. Small-vessel vasculitis affects predominantly small vessels, i.e. small arteries, arterioles, capillaries, and venules and may be associated with development of anti-neutrophil cytoplasmic autoantibodies (ANCA) directed against neutrophil intracellular enzymes, myeloperoxidase (MPO-ANCA), and proteinase 3 (PR3-ANCA).

Based on their highly specific association with ANCA, three diseases of as yet unknown aetiology are coined ANCA-associated: Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) including its renal limited form (idiopathic necrotizing and crescentic glomerulonephritis), and Churgï Strauss syndrome (CSS).

Antineutrophil cytoplasmic autoantibody (ANCA)-associated necrotizing vasculitis was probably first described in 1866 by Kussmaul and Maier as ñpolyarteritis nodosaò [2]. Wegener granulomatosis was reported as an entity in 1939 by Friedrich Wegener [3], and the Churgï Strauss syndrome was named after the two pathologists, J. Churg and L. Strauss, who first described it in 1951 [4] as a disease that is similar to but clearly distinct from polyarteritis nodosa (PAN). In 1985, antibodies associated with the disease were detected and later became known as ANCA [5]. Microscopic polyangiitis (MPA) was defined during the early 1990s, following the Chapel Hill consensus statement [1]. ANCA-associated vasculitides

share a common pathology with focal necrotizing lesions, which affect many different vessels and organs; in the lungs, a capillaritis may cause alveolar hemorrhage; within the glomerulus of the kidney, a crescentic glomerulonephritis may cause rapidly progressive renal failure; in the dermis, a purpuric rash or vasculitic ulceration may occur. Wegener granulomatosis and Churg Strauss syndrome have additional granulomatous lesions.

Epidemiology

The annual incidence of the AAV as a group is similar among regions of Europe, North America, and Middle east, ranging from 11 to 47 patients per million [6-9]. However, the incidence of WG and MPA seems to differ substantially. From north to south of Europe, there seems to be a decreasing incidence of WG, complemented by an increasing incidence of MPA. In China and Japan, there is a striking preponderance of MPO-ANCAï associated vasculitis, and PR3-associated disease is relatively rare [10-12], while a recent study from New Zealand found a much higher incidence of WG than of MPA [13]. These topographic incidence differences might indicate a difference in pathogenesis between WG and MPA [6]. In almost all areas and all disease categories, the incidence is greater in men than in women [8, 9, 14] and the peak incidence of vasculitis appears to be between 65 and 74 yr of age [10, 15, 16]. Some reports found seasonal variations in peak incidences (usually reporting higher incidence of vasculitis and especially WG in winter and a lower incidence in summer [17-21] which may point to an environmental factor inducing ANCA-associated vasculitis, although this phenomenon is still subject to discussion.

Etiology

Environmental factors, such as silica [22,23], bacterial or viral infectious agents [24-28], medication [29-32], and genetic susceptibility [33-37], all have been described as being involved in either creating the environment for inducing ANCA production or inducing ANCA themselves. Although the typically advanced age at disease onset suggests an environmental cause rather than genetic factors, genetic differences have been identified between patients and control subjects. For each environmental factor, exposure does not in all cases result in ANCA-associated vasculitis, and these factors are not necessarily associated with all patients with vasculitis. The induction of ANCA-associated vasculitis seems multifactorial, with both environmental factors and genetic predisposition being involved.

Pathogenesis

Despite many years of extensive research, the etiology and pathogenesis of the ANCA-associated vasculitides remains elusive. While our present research is based on extrapolation from ex-vivo data, it is important to recognize, that especially in AAV it is very challenging to differentiate between disease- and therapy-induced alterations and this caveat pertains also to a large body of other current evidence covering the pathogenesis of AAV in humans. Interaction of neutrophils with the endothelium seems to be crucial in the pathophysiology of AAV. Scenarios concerning the interplay between neutrophils, endothelial cells and ANCA are discussed bellow. Of note, alternative explanations and other important factors (such as the role of anti-endothelial antibodies, T cells, B cells, alternative pathway of complement, infection etc.) are beyond the scope of this introductory review.

The role of polymorphonuclear neutrophils

Currently proposed paradigms emphasize the role of activated polymorphonuclear neutrophils (PMN) in the inflammatory injury to the vascular endothelium. Massive PMN infiltrates have been found in the affected organs [38], and numerous studies of patients with active AAV showed up-regulation of activation-associated receptors on PMN, expression of which declines under immunosuppressive therapy [39-42]. It is well established, that activated PMN can destroy host tissue in the context of acute inflammation through their cytotoxic and proteolytic potential. Even though the formal proof for a destructive role of PMN in ANCA-associated vasculitis is still lacking, their participation in active disease is generally accepted. However, it is still debated, what is the main cause for the activation of PMN in the pathogenesis of AAV.

The role of ANCA

In 1982, Davies *et al.* [43] was the first to report circulating ñanti-neutrophil antibodiesòthat reacted with the cytoplasm of normal neutrophils in 8 patients with pauci-immune crescentic glomerulonephritis. His observation was confirmed and extended by van der Wounde in 1985 [5], who also noticed that ANCA were closely associated with Wegenerôs granulomatosis and that their titer correlated with disease activity. ANCA are classified on the immunofluorescence appearance of fixed neutrophils (cytoplasmic or C-ANCA, and perinuclear or P-ANCA), or according to the main antigenic targets (proteinase-3 or PR3 and myeloperoxidase or MPO), which are constituents of both the neutrophil primary granule and the monocyte lysosome. There is increasing evidence that ANCA play a direct pathogenic role in inducing systemic vasculitis by interacting with PR3 or MPO on the surface of cytokine primed neutrophil granulocytes and monocytes [44, 46, 47]. Neutrophils respond by developing the capability of adhering to cytokine-activated endothelial

cells, generating a respiratory burst, releasing proteolytic granule contents, and secreting proinflammatory cytokines [45]. Furthermore, ANCA have been implicated in accelerating neutrophil apoptosis and secondary necrosis following release of reactive oxygen species. This suggests a possible self-propagating mechanism in which ANCA induce activation and apoptosis of neutrophils, which in turn causes the primed or apoptotic neutrophils to express the ANCA antigens, and allows for further ANCA binding. ANCA can directly bind to endothelial cells, reacting with PR3 or MPO antigens released from neutrophils and planted on the endothelium [46]. There are also suggestions that endothelial cells are also, under certain circumstances, capable of synthesizing PR3 [48]. Following ANCA binding, endothelial damage can result from complement activation or antibody-dependent cellular cytotoxicity. Neutrophil binding to endothelium is enhanced in the presence of ANCA, and is mediated by adhesion molecules [49]. ANCA may also induce damage by preventing the neutralization of toxic products released from activated neutrophils [50, 51]. ANCA may therefore induce damage in several ways. The role of ANCA in pathogenesis of vasculitis is suggested by animal models, in which vasculitis is found in association with the development of ANCA [52]. However, in most cases ANCA is necessary but not sufficient to induce vasculitis and other inflammatory factors being required.

The role of endothelium

Endothelium forms the inner cell lining of blood vessels throughout the body and has many functions; it controls vasomotor tone, blood cell trafficking, coagulation and has a major role in immune regulation. Phenotypes of endothelial cells (EC) may change over time and according to the environment in which they exist. It has been well established that chemokines and adhesion molecule expression changes with the

activation status of the endothelium [53, 54]. Endothelium in its resting state expresses little in the way of adhesion molecules but these are upregulated when activated by cytokines such as tumour necrosis factor-a (TNFa) [55]. The endothelium may thus be both a facilitator for development of vasculitis as well as a target for injury, with additional roles for switching off an ongoing inflammatory response. Endothelial cells develop an activated phenotype in ANCA-associated vasculitis with enhanced expression of adhesion molecules that promotes interaction with circulating inflammatory cells [56-61]. ANCA activation can convert rolling neutrophils to stationary adherent cells that are well placed to mediate endothelial injury [59]. Release of proinflammatory mediators, including nitric oxide, reactive oxygen species, and proteolytic enzymes, all might contribute to directly damaging the endothelial cell. Proteinase 3 and myeloperoxidase can bind to endothelial cells; indeed endothelial cells express receptors for proteinase 3 [46, 47]. Myeloperoxidase can induce endothelial cell detachment, whereas proteinase 3 can cause direct apoptosis of these cells [48]. Furthermore, ANCA can bind to the endothelial-bound antigens, inducing endothelial cell cytotoxicity. The endothelial cell is important in localizing inflammation. The important question is, why does systemic vasculitis particularly affect the lungs, upper respiratory tract and glomeruli. Various reasons have been proposed, including exposure of the particular tissue to exogenous antigens and infectious agents, variations in blood flow and endothelial shear stress, cytokine/chemokine patterns in the perivascular tissue and factors pertaining to characteristics of the endothelial cells themselves. The latter concept applies to both expression of particular endothelial cell adhesion molecules as well as ultrastructural variation in endothelial cell anatomy. For example, endothelial cells from glomeruli are very different in structure from those in skeletal muscle, the former displaying

large fenestrations (without covering diaphragm) consistent with the primary glomerular function of plasma [62]. In addition, endothelial beds from certain organs have differential expression of adhesion molecules in response to injury, such as Vascular Adhesion Protein-1 (VAP-1, strongly expressed in the inflamed kidney [63] or Vascular Cell Adhesion Molecule-1 (VCAM-1, strongly expressed in the glomerular endothelium of patients with vasculitis) [61]. Thus, the endothelium is well placed to account for some of the variation in organ injury in vasculitis; the specific question as to whether glomerular endothelial cells are particularly effective at recruiting ANCA-stimulated leukocytes is the subject of ongoing intense research.

Clinical aspects

Within ANCA-associated vasculitis (AAV) syndromes, there is a marked heterogeneity in disease severity, extent and prognosis. The most commonly affected organs are the kidneys (in 70% of AAV patients), the ear, nose and throat region and the lungs. Wegenerôs granulomatosis (WG) manifests by granulomatous necrotizing inflammatory lesions of the upper or lower respiratory tract often accompanied by rapidly progressing glomerulonephritis (RPGN). Two-thirds of patients have pulmonary involvement and one-third have RPGN. Two forms of WG are recognized: a systemic, generalized severe version seen in two-thirds of patients and a localized, limited version seen in one-third of patients. C-ANCA (anti-PR 3) is positive in over 90% of the systemic form of the disease, whereas it is seen in one-half of the limited version. Microscopic polyangiitis (MPA) is associated with P-ANCA positivity (anti-MPO) in 75% of cases. Myalgias and arthralgias are common. Renal involvement is usually seen with necrotizing, crescentic glomerulonephritis. Pulmonary alveolar hemorrhage is associated with worse outcomes. Churg-Strauss syndrome (CSS) is

characterized by appearance of eosinophilic rhinosinusitis, followed several years later by the development of severe asthma with marked peripheral blood eosinophilia, and finally the development of systemic vasculitis. Both P-ANCA and, less frequently C-ANCA, are detected in about 60% of CCS patients.
Many patients with AAV (mainly WG and MPA) suffer from repeated relapses of the disease and from consequences secondary to irreversible organ damage, and so the preservation and recovery of organ function is a major goal of therapy.

Treatment

The goals of therapy are to control disease activity and so limit organ damage, to prevent disease relapse and to minimize the risk of the late complications of organ damage. Because current therapies are toxic, an additional aim is the minimization of treatment-related damage. Treatment can be divided into 2 phases: induction of remission induction, and maintenance of remission. The cornerstone of treatment for ANCA-associated vasculitis includes induction therapy with pulse corticosteroids, followed by daily oral glucocorticoids, and the prompt institution of cyclophosphamide (CYC). Treatment with CYC is associated with risks of myelosuppression, infection, infertility and malignancy, and so current protocols aim at minimizing cyclophosphamide exposure, e.g. using regimens based on intravenous pulses rather than continuous oral cyclophosphamide [64]. More recent cyclophosphamide regimens have also reduced the dose for age and renal impairment. For less aggressive presentations, without renal impairment, methotrexate (MTX) has been demonstrated to be as good as cyclophosphamide for remission induction and is probably safer [65]. One small randomized trial of renal vasculitis found similar induction rates between mycophenolate mofetil (MMF) and cyclophosphamide [66].

Approximately 85% of patients achieve remission with induction therapy [67], but 50% of patients have a relapse in 5 years [68, 69]. Some relapses are severe, resulting in worsening end-organ damage. Most relapses respond to therapy, but patients are subjected to additional immunosuppressive or cytotoxic drugs. Fear of relapsing disease has impelled physicians to prescribe prolonged maintenance therapies in most patients. After achieving remission of the disease, less toxic treatment with either azathioptine (AZA), or other immunosuppressive drug (MTX, MMF), with lower dose of glucocortiods is instituted [70-72], however inadequate information is available to guide the duration of maintenance therapy.

Prediction of relapses

Because substantial number of patients may never have a disease relapse, use of long-term immunomodulating therapy often presents unnecessary risks and may well outweigh the benefits of preventing relapse. Little is known regarding predictors for relapse; identification of these risk factors would conceivably allow maintenance immunomodulatory therapy to be tailored to patients at high risk while sparing others unnecessary exposure to these drugs. An association between lung involvement and relapse was reported in Wegener granulomatosis [73] and AAV [81]. Persistent ANCA positivity after induction therapy is associated with almost an 80% relapse rate at 4 years compared with 20% for those who are ANCA negative [74]. This suggests that maintenance immunosuppression should be continued for several years to prevent relapse especially in those with persistently positive ANCA. Research to determine whether changes in ANCA titers predict relapses has yielded conflicting observations [75-80]. Relapse was reported to be more common in Wegenerôs granulomatosis than in microscopic polyangiitis [67], in anti-PR3 positive patients

than anti-MPO in positive patients [81], and has been associated with nasal carriage of Staphylococcus aureus in Wegener's granulomatosis [82]. Therapeutic studies that have withdrawn corticosteroid have reported a higher relapse rate than those that have not, but this question has not been directly examined. However, research has not conclusively demonstrated, whether prolonged immunosuppression prevents relapses and which medications are effective at preventing a relapse. Clearly, more information is needed to enable stratification of risk to relapsing disease in patients with AAV and to confirm the risk/benefit ratio of prolonged immunosuppressive maintenance strategies.

THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN THE ENDOTHELIAL TURNOVER

Introduction

The endothelium is the largest organ in the body consisting of endothelial cells lining every blood vessel. Far from being just a passive vessel lining, the vascular endothelium represents a rather dynamic border between circulating blood and the surrounding tissue. In addition to forming a physical barrier between the vessel wall and the lumen, endothelial cells secrete a range of compounds which modulate vascular tone, coagulation, and inflammation. Indeed, there is growing evidence that the endothelium plays a crucial role in the initiation and maintenance of inflammation, standing side by side with other major protagonists of immunological response, such as T cells and monocytes. In response to a variety of stimuli, such as changes in

shear stress and exposure to different environmental or hormonal changes, the expression of cell adhesion molecules on endothelial cells is increased, leading to the adherence of inflammatory cells. The endothelium is involved in a host of disease states, either as a primary determinant of pathophysiology or as a victim of the damage. Indeed, the state of the vasculature is now understood as representing on ongoing balance between injurious and reparative mechanisms, with chronic vascular damage due to conventional risk factors resulting in recruitment of cellular reparative components. In the setting where cellular repair capability is adequate, the stimulus to further injury is resolved, inflammation subsides, and the vessel becomes quiescent [83]. In the absence of adequate repair mechanisms, unresolved vascular damage induces an inflammatory cascade which leads to accelerated injury and overt disease.

Markers of endothelial function

Because endothelium is relatively inaccessible to direct examination, investigators have concentrated on various surrogate markers of endothelial function. One approach to the assessment of endothelial functioning relies on the changes of specific plasma markers (such as von Willebrand factor, soluble thrombodulin, tissue plasminogen activator, soluble endothelial protein C receptor, and soluble E selectin); another is by using physiological techniques (such as flow-mediated dilatation after reactive hyperaemia). More recently, an additional method for assessing vascular integrity has been developed: measurement of *circulating endothelial cells* (CEC) and *circulating endothelial progenitors* (EPC) in peripheral blood. CEC are thought to originate from sloughing off the vessel wall following some form of pathological insult and their numbers to reflect the extent of ongoing vascular injury. In contrast,

enumeration and functional assessment of circulating progenitor cells appears to be a useful technique for assessment of vascular reparative capacity.

Circulating endothelial cells (CEC)

In healthy subjects, a low basal level of endothelial turnover, or very low amounts of circulating, vessel wall-derived endothelial cells (1 to 3/mL blood), has been described [84]. Acute stress injury of the vascular endothelium, which is often followed by apoptosis of endothelial cells (ECs) rapidly enhances the number of circulating ECs. There is an expanding list of conditions associated with severe endothelial injury and elevated numbers of CECs in peripheral blood. In cardiovascular disease, the highest numbers of CECs have been found in the blood of subjects with the most severe and acute coronary artery disease [85-87]. In the field of inflammatory and connective tissue diseases, the number of CECs strongly correlated with the activity and severity of small vessel vasculitis [88], systemic sclerosis [89], and systemic lupus erythematosus [90].

Circulating endothelial progenitors (EPC)

Endothelial damage can be compensated for by endothelial repair. In the past, such a regeneration of the injured endothelium has been attributed to the migration and proliferation of the neighbouring endothelial cells. More recent studies, however, indicated that additional repair mechanisms help to restore endothelial integrity. In the early studies [91, 92], implanted Dacron grafts and the surface of ventricular assist devices were shown to be rapidly covered by bone marrow-derived cells. Asahara was the first to describe cells circulating in peripheral blood that expressed markers of haematopoietic stem cells and were able to differentiate *in vitro* into endothelial cells [93]. These cells were later named "endothelial progenitor cells"

(EPCs), showed expression of various endothelial markers, and incorporated into neovessels at sites of ischemia. In vivo experiments have shown that EPCs can improve neovascularization. The power of progenitor cell mediated repair was demonstrated in a mouse model of atherosclerosis in which the development of atherosclerosis was delayed by augmentation of progenitor cell mediated repair via regular administration of bone marrow cells obtained from healthy animals [94]. In mouse and rabbit models of hind limb ischaemia, mobilization of EPCs remarkably promoted new blood vessel formation in the injured areas, enhanced perfusion, and supported recovery of ischaemic tissue [95, 96]. Ex vivo expanded EPCs, isolated from peripheral blood mononuclear cells, incorporated into the foci of myocardial neovascularization [97], whereas intracoronary infusion of peripheral blood or bone marrow derived progenitors in patients with acute myocardial infarction was shown to associate with significant benefits in post-infarction remodelling [98, 99]. While a basal level of circulating EPCs is present in normal, healthy individuals, their number increases in response to vascular injury [100]. However, in patients with persistent vascular damage the EPC counts are usually lowered. Hill *et al.* and Vasa *et al.* demonstrated that EPCs (measured in peripheral blood using a colony forming unit assay) had an inverse relationship to the Framingham risk score in healthy patients [101], to the number of coronary artery disease (CAD) risk factors in patients with coronary disease [102], and to alterations in brachial reactivity [101]. Others have related EPC as defined by culture techniques to hypertension [103], chronic renal failure [104], congestive heart failure [105,106], diabetes [107], hypercholesterolemia [108], coronary artery disease [109, 110] and cerebrovascular disease [111].

To conclude, endothelial progenitor cells are believed to promote vascular repair. Exhaustion of these reparative capacities likely plays a significant and antecedent role in the development of clinically evident vascular disease. Endothelial damage ultimately represents a balance between the magnitude of injury and the capacity for repair, and the measurement of the number and function of endothelial cells and their precursors circulating in the peripheral blood is becoming an essential part of the current paradigms concerning endothelial function and turnover.

THE PRESENT INVESTIGATION

General remarks

Since resident endothelial cells infrequently proliferate [112], it has been postulated that there are other sources of vascular replenishment in response to continuous damage [113]. Recent studies have identified a population of presumably bone marrow-derived cells called endothelial progenitor cells (EPCs) that circulate in peripheral blood, express a variety of endothelial surface markers, incorporate into sites of neovascularization and home to sites of endothelial denudation [114-121]. EPC counts proved to be a useful biomarker of vascular function and injury [101, 102], and to be predictive of clinical outcome in different types of disease [122ï 128]. ANCA-associated vasculitis (AAV) is a group of inflammatory disorders, characterized by inflammation and necrosis of blood vessels and relapsing remitting progression. Patients with AAV suffer from ongoing microvascular damage, which can be halted by immunosuppressive treatment. However, this treatment is frequently toxic to bone marrow and thus may decrease the number of EPCs in patients in AAV. A decreased number of EPCs could result in inadequate microvascular repair and contribute to repeated flares of the disease observed in these patients. The role of EPCs in AAV is unclear at present. We sought to investigate the numbers of EPC in patients with AAV and 1) to compare EPC counts measured in patients with AAV, other vasculopathies and helathy volunteers, 2) to analyze the relation of EPC level to the clinical and laboratory paremeters of AAV, and 3) to evaluate the possible role of EPC as a predictive marker for long term outcome of AAV.

Specific aims of the study

Hypothesis 1: AAV patients are exposed to a number of factors that may decrease the pool of EPCs (acute systemic inflammatory response, widespread endothelial damage, treatment inducing bone marrow suppression, kidney dysfunction with retention of uremic toxins). We hypothesized that the EPC number in these patients could be severely altered.

Aim 1: To perform a cross-sectional study to assess the number of circulating EPCs in patients with AAV, in patients with other types of vascular injury (end-stage renal failure on maintenance hemodialysis, peripheral occlusive artery disease), and healthy volunteers. To analyze EPC counts in AAV patients with respect to their activity/remission status, level and type of ANCA antibodies, renal function and institution of immunosuppressive treatment.

Hypothesis 2: Decreased pool or impaired mobilization of EPCs could hamper the mechanism of endothelial repair and may contribute to repeated relapses in AAV. We hypothesized that low EPC numbers could predict the propensity to early relapse in patients with AAV.

Aim 2: To analyze the long-term outcome of AAV patients with respect to the number of EPC at baseline.

Methodological considerations for the assessment of endothelial progenitor cells

Overview of the available methodologies

Despite the immense interest in this field, there is still neither standard definition for EPCs, nor accepted methodology for their assessment. The main obstacle to assess EPC is because these cells are extremely rare in unmobilized peripheral blood and there is no easily performed ógold standardô assay (such as the hematopoietic reconstitution after lethal radiation used to determine hematopoietic stem cells). Currently used methods for quantification of EPCs are either culture-based [101, 102], or fluorescence activated cell sorting (FACS)-based measurements. The culture-based protocols may differ on the necessity for a preplating step, medium used, time of culture, and types of cells counted, while assays based on FACS may vary in the markers used. In most studies based on FACS-based measurements, a combination of CD34, vascular endothelial growth factor receptor-2 (VEGFR-2), and/or CD133 is applied. In addition, another EPC identification method was recently proposed [129] based on aldehyde dehydrogenase (ALDH) enzymatic activity responsible for the resistance of hematopoietic progenitors to chemotherapeutic injury, cyclophosphamide especially [130, 131]. This may be of importance for the topic of our thesis, because cyclophosphamide may be used for stem cell purification as well as for the treatment of ANCA-associated vasculitis.

Culture based procols

Concering culture-based protocols, two most used methods for isolation of EPCs from the peripheral blood have been described. First, isolated monocytic cells are seeded onto fibronectin-coated plates and cultured in the presence of growth factors that promote EPC outgrowth and form colonies after 5ï 7 days. These colonies consist of a central cluster of round cells surrounded by multiple spindle-shaped cells (endothelial cell colony-forming units, CFU-EC). Second, monocytic cells from peripheral blood plated onto collagen-I-coated plates in endothelial growth media (EGM-2) can give rise to CFU-EC after 14ï 21 days. According to the cultivation assay used and the resultant cell population properties mentioned above, two distinct phenotypes of EPCs have been described, the early and the late outgrowth EPCs.

Endothelial outgrowth cultivation method according to Hill

In 1997, Asahara et al. [93] isolated CD34+ mononuclear cells from human peripheral blood, cultured them on fibronectin, then tested them for the expression of leukocyte (CD45) and endothelial cell (CD34, CD31, Flk-1, Tie-2, E selectin, and endothelial nitric oxide synthase) markers to confirm that the cultured cells developed an endothelial cell-like phenotype. When cultured on fibronectin-coated dishes, the enriched CD34+ cells formed "blood island-like" clusters of differentiating endothelial cells. Ito et al [132] modified the assay conditions to screen for nonadherent mononuclear blood cells (after 24 hours of preplating) that formed the same kind of cluster formation and counted these as EPC-derived clusters. Hill et al [101] modified the assay further by preplating the blood mononuclear cells for 48 hours and then replating the nonadherent cells and observed over several days for the

appearance of the EPC colony forming unit cell that was interpreted as a quantitative readout of the EPCs. In our case, we have used an early outgrowth colony forming assay as introduced by Hill and coworkers [101] which is commercialy produced now by Stem cell technologies.

Limits of the available assays

It is important to note, that recent studies have shown most assays not to correlate with each other well [133, 134] and have highlighted the impact of different cell isolation protocols on the functional capacity of these progenitors [135]. Indeed, there may be considerable heterogenicity of EPC population concerning phenotype, cell lineage of origine, proliferative capacity and role in vascular reparative processes. The early outgrowth EPCs are probably derived from monocytic cells, have low proliferative capacity and adopt characteristics of ECs such as expression of eNOS. Although these cells may incorporate into the endothelial monolayer, they fail to form perfused vessels in vivo. The late outgrowth EPCs seem to have a high proliferation rate and can be maintained in culture extensively. These cells may play a key role in neoangiogenesis in vivo as experimental data indicated that they are vessel-forming EPCs. Interestingly, although infusion of EPCs into ischaemic limbs of immunocompromised mice can remarkably improve perfusion and recovery from injury, only low numbers of EPCs incorporated into the new capillaries can be identified [136-139]. This suggests that EPCs may release angiogenic factors in a paracrine manner. This supportive function of EPCs may be crucial in ensuring the survival of tissue-residing cells and enhancing blood vessel formation and tissue repair. Of note, early outgrowth EPCs appear to produce higher levels of growth factors compared with late outgrowth EPCs, suggesting a diverse role in

neovascularization for the two phenotypes. [140] Consequently, it could be suggested that although early outgrowth EPCs exhibit a low proliferative capacity by themselves, they may act to secr ete angiogenic growth factors stimulating the proliferative capability of the late outgrowth EPCs or resident mature endothelial cells.

The methodology of endothelial earl y-outgrowth cultivation was repeatedly used by different groups [101, 122, 125-127] and its results were shown to well correlate with physiological markers of endothelial function and cardiovascular outcomes. Therefore, we believe that the early endothelial outgrowth cultivation method to represent a well-established method to assess a functionally important population of EPCs, and to be a useful surrogate marker of endothelial repair and function.

Patients and Controls

A cohort of 41 patients with a diagnosis of AAV according to the international consensus definitions [1] were recruited from the Department of Nephrology, Charles University Hospital, Prague in 2004ï 05. At the same time, 25 healthy volunteers, 15 non-vasculitis patients on chronic hemodialysis and 13 patients with angiographically documented peripheral arterial occlusive disease (PAOD) were enrolled as control population samples. The EPCs were measured in all these patients by a colony-forming unit assay cross-sectionally at that time. 10 AAV patients were sampled twice throughout their treatment period or after reaching remission ï these second samples were included just in the subgroup analysis of AAV patients, but not in the comparison between different patient groups. The AAV cohort was then prospectively followed until September 2008, when the analysis of long-term outcomes was performed. At least once in their history, all patients were ANCA positive and 95% of them had histologically proven pauci-immune glomerulonephritis. WG was diagnosed according to the criteria of the ACR [141] and the Chapel Hill Consensus Conference definition [1], microscopic polyangiitis (MPA) was diagnosed according to the Chapel Hill Consensus Conference definition. Baseline characteristics of the patients are shown in Table 1.

Definition of relapses, follow-up

Disease activity was scored with the use of the validated Birmingham Vasculitis Scoring Index (BVAS). Remission was indicated by a complete absence of clinical activity using the BVAS item list. Relapse could only occur in patients who reached remission lasting at least one month. Relapse was defined as vasculitic signs and symptoms in any organ system with BVAS > 1. Major relapse required recurrence or

new appearance of one of the following major organ involvement attributable to active vasculitis: an increase in serum creatinine of > 30% within a period of 3 months or clinical, radiologic or bronchoscopic evidence of pulmonary hemorrhage or granulomata. Minor relapse was defined as recurrence of disease activity of lesser severity, such as epistaxis, crusting, myalgia, arthralgia, arthritis, mouth ulcers, rash, episcleritis, scleritis, and pulmonary symptoms without or with minor radiologic changes.

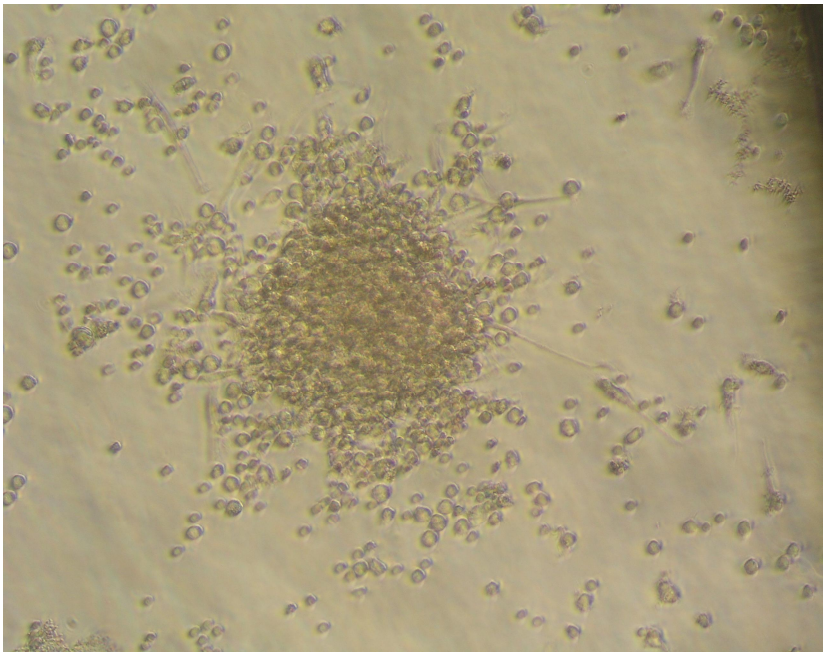
Quantification of circulating endothelial cells

Circulating EPCs were quantified by the colony-forming unit assay as described by Hill et al [101]. Briefly, blood was taken by venepuncture, and after discarding the first portion, 20 ml of blood anticoagulated with heparin was centrifuged on Ficoll-Hypaque (Ammersham, Upsalla, Sweden) to obtain the mononuclear fraction. Peripheral blood mononuclear cells were washed in phosphate-buffered saline and counted on hemocytometer. 10^7 of recovered cells were resuspended in 4 ml of the Endocult™ medium (StemCell Technologies, Vancouver, Canada) and divided into two equal portions. Each of the portions, containing $5x10^6$ cells in 2 ml of Endocult™ was seeded in one well of the BD BioCoat™ Gelatin 6-well plate (BD Biosciences, Franklin Lakes, NJ, USA) and cultivated for 48 hours at 37°C in atmosphere with 5% CO_2. Nonadherent cells were then removed together with the rest of the medium, counted, and $2x10^6$ cells from each portion were seeded in two wells of the BD BioCoat™ Fibronectin 24-well plate (i.e. from each sample 4 wells containing 10^6 nonadherent mononuclear cells were seeded). After another 72 hours, endothelial colonies (EPC-CFU) were scored under inverse microscope. Only colonies with at

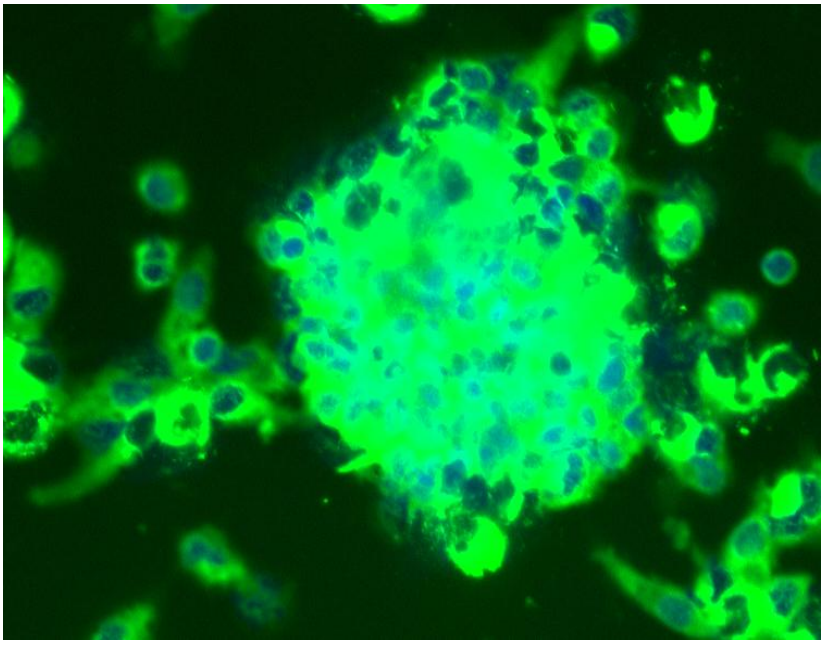
least 20 cells, containing rounded cells in the middle and elongated cells at the periphery were considered as endothelial colonies.

The phenotype of EPC colonies was determined in five subjects. EPC colonies were grown on fibronectin coated glass chamber slides. CD31 (clone HC1/6, Chemicon, Temecula, CA, USA), VEGFR-1 (clone FLT-19, Sigma Saint Louise, Missouri, USA), VEGFR-2 (clone 89106, R&D Systems, Minneapolis, USA) and von Wildebrandt factor (clone 2F2-A9 BD Biosciences) positivity were determined by immunofluorescent (FITC resp. PE) labeled antibodies. Similarly, UEA-1 lectin binding was evaluated with FITC-labeled UEA-1 lectin. Cell nuclei were visualized with DAPI (Sigma-Aldrich, Germany) staining, which enabled to discriminate endothelial colonies from scattered adherent cells. As this procedure is a quite laborious one, after confirming that EPC colonies are positive for the above mentioned markers, we did not repeat these studies in other subjects.

EPC-CFU



EPC-CFU stained by FITC labelled antibodies for VEGFR-2



Other biochemical and hematological tests and ANCA detection

Biochemical and hematological tests were performed in Central Laboratories of General Teaching Hospital, Prague, according to the institutional guidelines. ANCA antibodies were determined by ELISA (Orgentec, Mainz, Germany) and by indirect immunofluorescence microscopy.

Statistical Analysis

We tested the data for normality by the Kolmogorov-Smirnov and Lillefors tests. As most of the data did not show normal distribution, we used non-parametric tests for the respective statistical calculations and continuous variables were generally expressed as medians and ranges. As there was an excellent correlation (Spearman

r = 0.94ï 0.98) among the number of EPC colonies per 10^6 seeded mononuclear cells, per 10^6 circulating white blood cells and per 1 ml of blood, CFU-Hill number per ml of blood was used in all subsequent calculations. Correlations of CFU-Hill/ml with age, BVAS score, level of ANCA, creatinine, GFR, CRP, albumin, and number of involved organs were calculated using the Spearman test. Comparisons of different subgroups of AAV patients according to sex, treatment category, subtype of ANCA, various thresholds of GFR, and concurrent illnesses or drug use (immunosuppressive drugs, statins, erythropoietin, ACE inhibitors), as well as comparisons between AAV patients and control groups were performed with Mann-Whitney U test when comparing two independent groups, Wilcoxon signed-rank test for paired samples and Kruskal-Wallis test with Dunnôs multiple post-test comparisons when more than two independent groups were compared. Chi square test or its appropriate modification was used for comparison of categorical variables.

In the second part of the project, the association between baseline levels of EPCs (measured in 2004ï 05) and the occurrence of relapse was evaluated (in September 2008). The levels of EPCs were analysed as categorical variables - patients were divided into three pre-specified, numerically equal groups corresponding to patientsô EPC counts (low, medium and high) at the time of enrolment. Survival (time to the first relapse) was determined with the use of the Kaplanï Meier method and the Cox regression analysis. The log-rank test was used to determine statistical differences in terms of survival. Patients were censored if they had not relapsed at their last follow-up visit or if they died of other cause than AAV activity. Because of a relatively small number of patients we were not able to perform a robust multivariate Cox proportional analysis, but we performed univariate analyses and a multivariate analysis with three putative predictors of relapse included: type of ANCA (anti-MPO

vs anti-PR3), type of disease (WG vs MPA) and EPC group (low, medium and high EPC counts). The hazard ratio for the EPC group represents the predicted change in the hazard for a unit increase in the predictor (e.g. an increase from low to medium or from medium to high in the number of EPCs). Statistical significance was assumed when a null hypothesis could be rejected at P<0.05 (all P-values are two-sided). Statistical analyses were performed with the use of GraphPad Prism Version 4.03 for Windows (GraphPad Software Inc., San Diego, Calif., USA) and SPSS software, version 11.5, for Windows (Chicago, IL, USA).

Results I.

Basic Characteristics of Patients and Controls

Characteristics of AAV patients and controls are shown in table 1 . There was no statistically significant difference in the age of healthy subjects and patients of all three groups (Kruskal-Wallis test, p = 0.33), men and women were equally represented in the respective groups save for PAOD group, where males predominated (p = 0.02). Among patients in the AAV group, 100% had kidney involvement, 71% had lung involvement and 45% involvement of ear, nose and throat area. The median creatinine level at diagnosis was 325 εmol/l (range 88ï 927), median BVAS score was 23 (range 12ï 36), and the median level of ANCA antibodies was 84 U/ml (9ï 100, normal range 0ï 5). Patients with anti-MPO and anti-PR3 type of ANCA antibodies were equally represented (49 and 51%).

Table 1. Patientsôcharacteristics

	All AAV[a]	Active AAV before treatment	Active AAV on induction treatment	AAV in remission	Dialysis patients	PAOD patients	Healthy controls
Number	41	19	18	14	15	13	25
Male	20 (49%)	10 (53%)	12 (67%)	4 (29%)	9 (60%)	11 (85%)	9 (36%)
Female	21 (51%)	9 (47%)	6 (33%)	10 (71%)	6 (40%)	2 (15%)	16 (64%)
Age, years	58 (23–82)	59 (31–73)	54 (34–82)	56.5 (23–68)	59 (24–81)	64 (26–85)	55 (45–71)
Creatinine, µmol/l[b]	262 (57–927)	325 (88–927)	144 (57–224)	138 (63–409)		83 (69–154)	
Glomerular filtration rate, ml/min[c]	16.6 (4.2–121)	16.6 (4.2–70)	26.7 (10–88)[c]	21 (10–121)[c]	10[c]		
CRP, mg/l	17 (0–210)	64 (2–210)	2.5 (2–156)	3 (0–147)			
Albumin, g/l	34 (21–44)	30.5 (21–39)	35 (21–40)	39 (30–44)			
BVAS	12 (0–41)	23 (12–36)	6 (3–41)	0 (0%)			
ANCA level	32 (0–107)	84 (9–107)	19.5 (0–100)	0 (0–32)			
ANCA type							
Anti-MPO	20 (49%)	15 (79%)	7 (39%)	8 (57%)			
Anti-PR3	21 (51%)	4 (21%)	11 (61%)	6 (43%)			
Immunosuppressive treatment	0 (0%)	0 (0%)	18 (100%)	13 (93%)			
Dialysis treatment	9 (22%)	0 (0%)	4 (21%)	5 (36%)	15 (100%)	0 (0%)	
Statin use	14 (34%)	2 (10%)	6 (33%)	9 (64%)	3 (20%)	6 (46%)	
Erythropoietin use	9 (22%)	0	5 (28%)	4 (28%)	13 (87%)	0	
ACEi/ARB use	19 (46%)	6 (32%)	10 (55%)	9 (64%)	12 (80%)	7 (54%)	
Organ involvement							
Kidney	41 (100%)	19 (100%)	17 (94%)	14 (100%)			
Lung	30 (71%)	17 (79%)	16 (89%)	7 (50%)			
ENT	19 (45%)	8 (42%)	6 (33%)	6 (43%)			
Number of CFU-Hill	0.3 (0–39)	1.2 (0–39)	0.5 (0–7)	0.15 (0–29)	1.9 (0–136)	3.5 (0–260)	19.6 (0–119.4)

All continuous variables are expressed as median and ranges. Categorical variables are expressed as numbers and percentages. AAV = ANCA-associated vasculitis, PAOD = peripheral artery occlusive disease, CRP = C-reactive protein, BVAS = Birmingham Vasculitis Activity Score, ACEi/ARB = angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, ENT = ear, nose, throat, CFU-Hill = endothelial colony-forming units.

[a] Ten AAV patients were sampled twice throughout their treatment period or after reaching remission – these second samples were included just in the subgroup analysis of the AAV patients, but not in the comparison between different patient groups.

[b] For non-dialyzed patients.

[c] For patients on dialysis, arbitrary glomerular filtration rate 10 ml/min was ascribed.

Number of Circulating Endothelial Progenitors Are Lower in AAV Patients than in Healthy Controls, as well as in Hemodialysis Patients vs. Healthy Controls

In the four group comparisons (AAV, HD, PAOD patients and healthy volunteers) there was a significant difference between all groups ($p < 0.0001$, Kruskal-Wallis) and in subsequent post-test analysis (Dunnôs comparison) there were significant differences in the number of circulating endothelial precursors (expressed as CFUHill/ ml blood) between AAV patients and healthy subjects ($p < 0.0001$) and between hemodialysis patients and healthy subjects ($p < 0.05$). The difference between PAOD patients and healthy volunteers did not reach statistical significance

in the multiple group comparison (p = 0.075), as well as there were no statistical differences between the respective patient groups.

Fig. 1

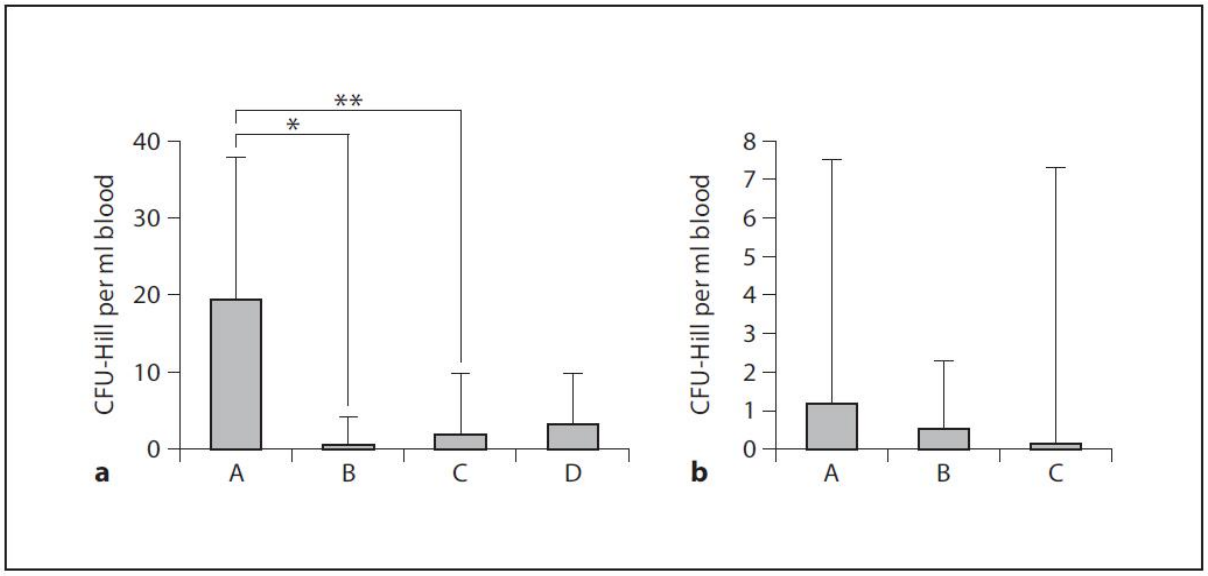


Fig. 1 Circulating endothelial precursor cells in AAV patients and controls. **a** The number of CFU-Hill colonies per ml blood is shown for healthy controls (A), patients with AAV (B), patients without AAV on dialysis treatment (C), and patients with peripheral vascular disease (D). Results are depicted as medians (columns) and 75% percentiles (bars). On Kruskal-Wallis comparison for all groups, the resulting difference is highly significant (p < 0.0001) and on Dunnôs comparison, healthy controls have also significantly more colonies per ml blood than patients with AAV and patients on dialysis without AAV. * p < 0.001, * * p < 0.05. **b** Statistically insignificant changes over the course of disease are shown in patients with active AAV at diagnosis (A), active AAV on treatment (B), and AAV in remission (C). p = 0.61 (Kruskal-Wallis).

Number of Circulating Endothelial Progenitors Do Not Correlate with Markers of Inflammation and Do Not Recover during Treatment

As expected, during the course of AAV treatment, the BVAS score decreased significantly (p < 0.001), as did other parameters of activity too (ANCA level, p < 0.001, CRP, p = 0.003 in Kruskal-Wallis comparison among all three treatment subgroups). The GFR improved only insignificantly (p = 0.39), as 4 patients in the active subgroup on treatment and 5 patients in remission required chronic dialysis treatment. However, neither did we find any significant difference between the number of circulating endothelial precursors in untreated patients with active disease,

treated patients with active disease and patients in remission (fig. 1 b), nor there was any significant correlation between the number of circulating endothelial precursors and markers of activity (fig. 2). There was a trend towards a higher number of CFU-Hill/ml in patients without immunosuppressive treatment (median 2.2 vs. 0.6 CFU Hill/ml), but it did not reach statistical significance (p = 0.16). It was also irrelevant if patients were on corticosteroids only or received cytotoxic agents (cyclophosphamide or azathioprine, data not shown). Concerning 10 patients where paired samples were available, there was no difference in the number of EPCs during the course of treatment and the pairing has not given any additional information compared to the unpaired testing (data not shown).

Fig. 2 a

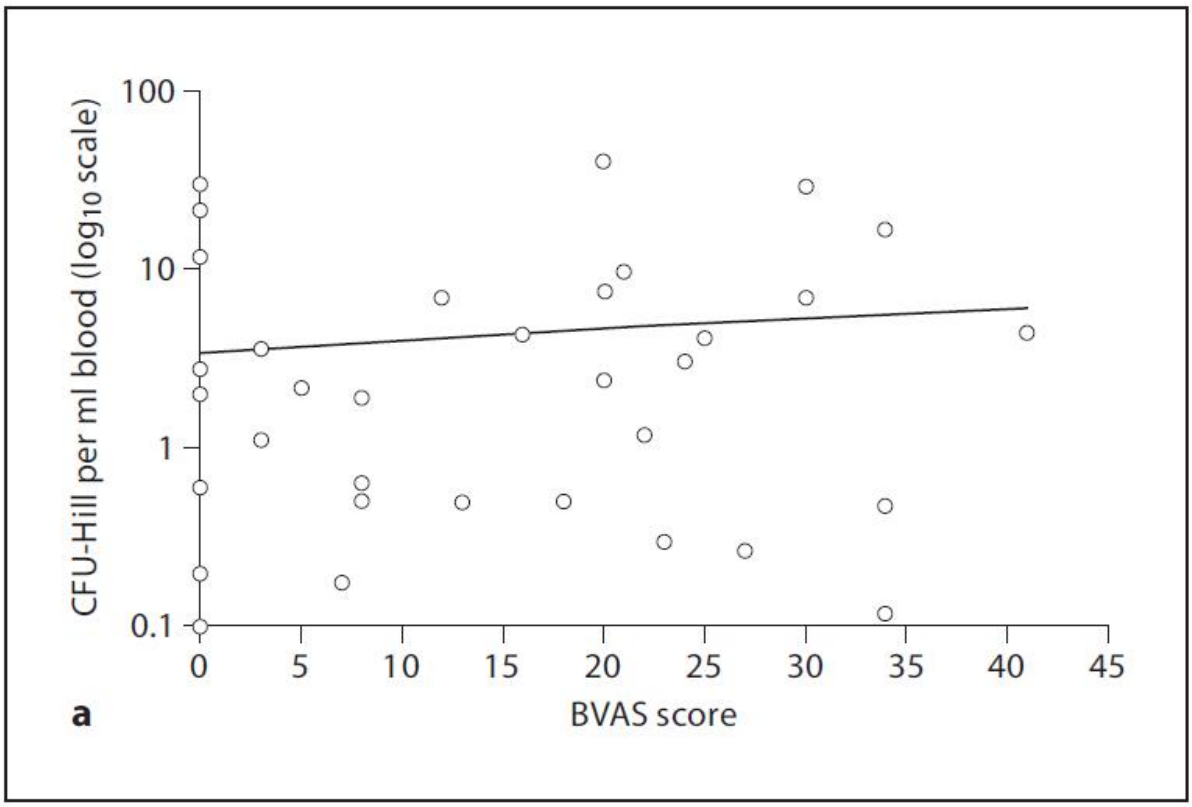


Fig. 2 b

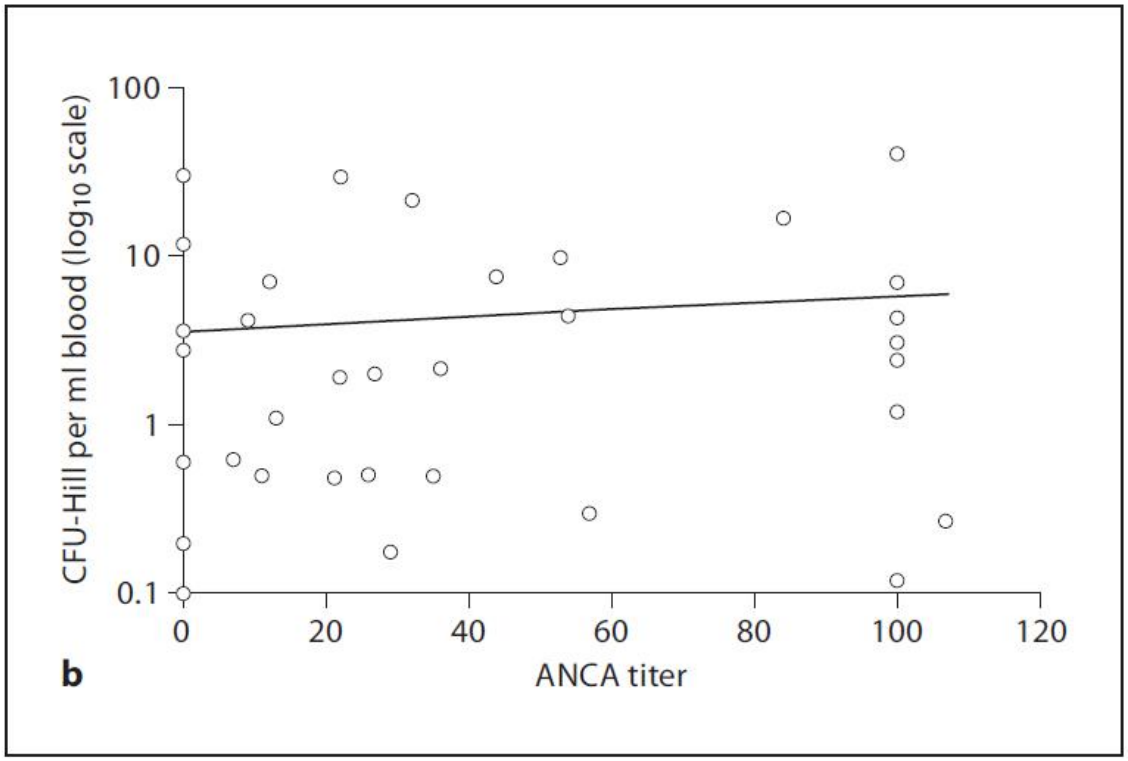


Fig. 2 c

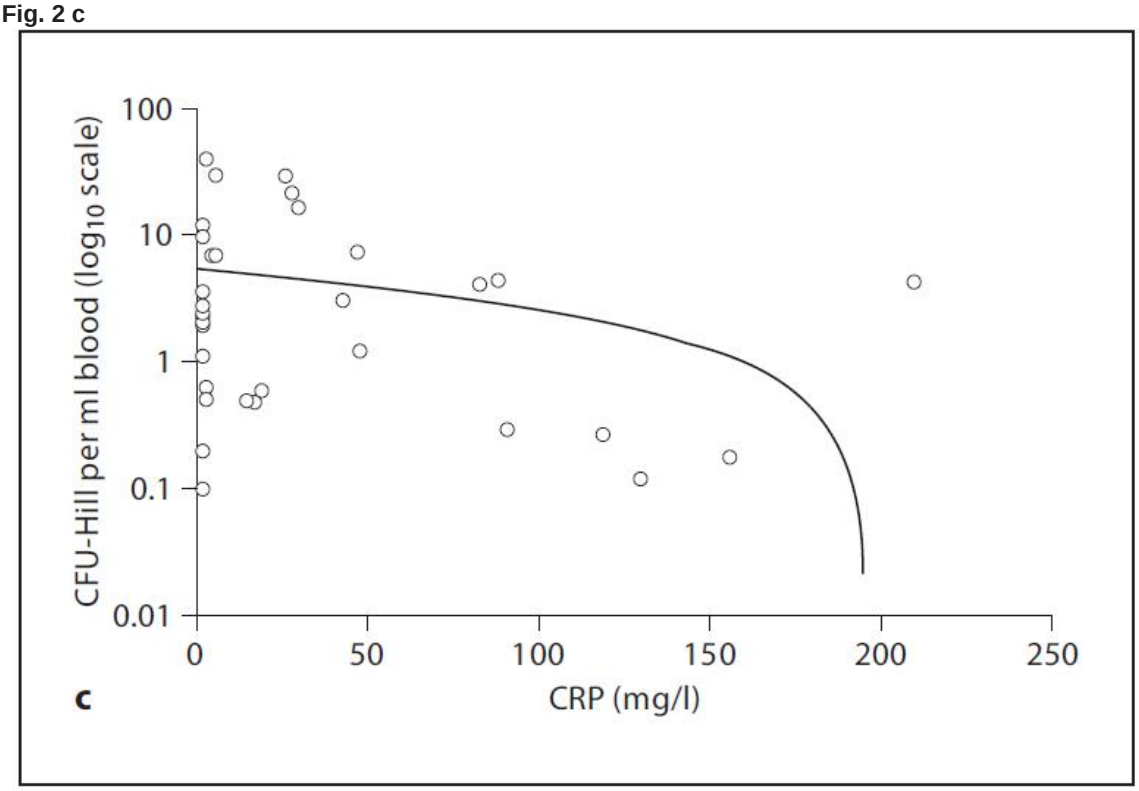


Fig. 2. Circulating endothelial precursor cells in AAV patients do not correlate with markers of disease activity. The lack of correlation between the number of endothelial cells and BVAS score is shown (**a** ; Spearman r = 0.199, p = 0.21), ANCA titer (**b** ; Spearman r = 0.159, p = 0.33) and CRP (**c** ; Spearman r = ï 0.118, p = 0.46).

Number of Circulating EPCs Depends on the Type of ANCA and on the Severity of Renal Impairment in AAV Patients

As mentioned above, there was no significant correlation between EPC counts and levels of ANCA. However, when patients with different types of ANCA were compared, there was a trend towards higher EPC counts in patients with ANCA anti-MPO antibodies compared to patients with anti-PR3 (Mann-Whitney U test, median 3.1 vs. 0.2 CFU-Hill/ml, p = 0.06, fig. 3). When we performed the Spearman correlation between creatinine level or GFR and the number of CFUHill colonies/ml blood, we did not find any significant differences (not shown). However, when we compared two groups according to preset thresholds of GFR, we found a significantly lower number of CFU-Hill colonies in patients with a very low GFR or who were on dialysis treatment (corresponding to stage V chronic kidney disease according to K/DOQI), i.e. when patients with GFR < 15 and > 15 ml/min were compared (median 0 vs. 1.6 CFU-Hill/ml, p = 0.015; fig. 4 a). Because of the lower number of CFU-Hill/ml in patients with AAV and significantly impaired renal function, we were interested if this lowering of circulating EPCs is not merely a surrogate marker for renal insufficiency. However, when patients with AAV and GFR < 15 ml/min or on hemodialysis were compared with patients on dialysis without vasculitis, the number of CFU-Hill/ ml were still lower for the AAV patients (median 0 vs. 1.9 CFU-Hill/ml, p = 0.03; fig. 4 b).

Fig. 3

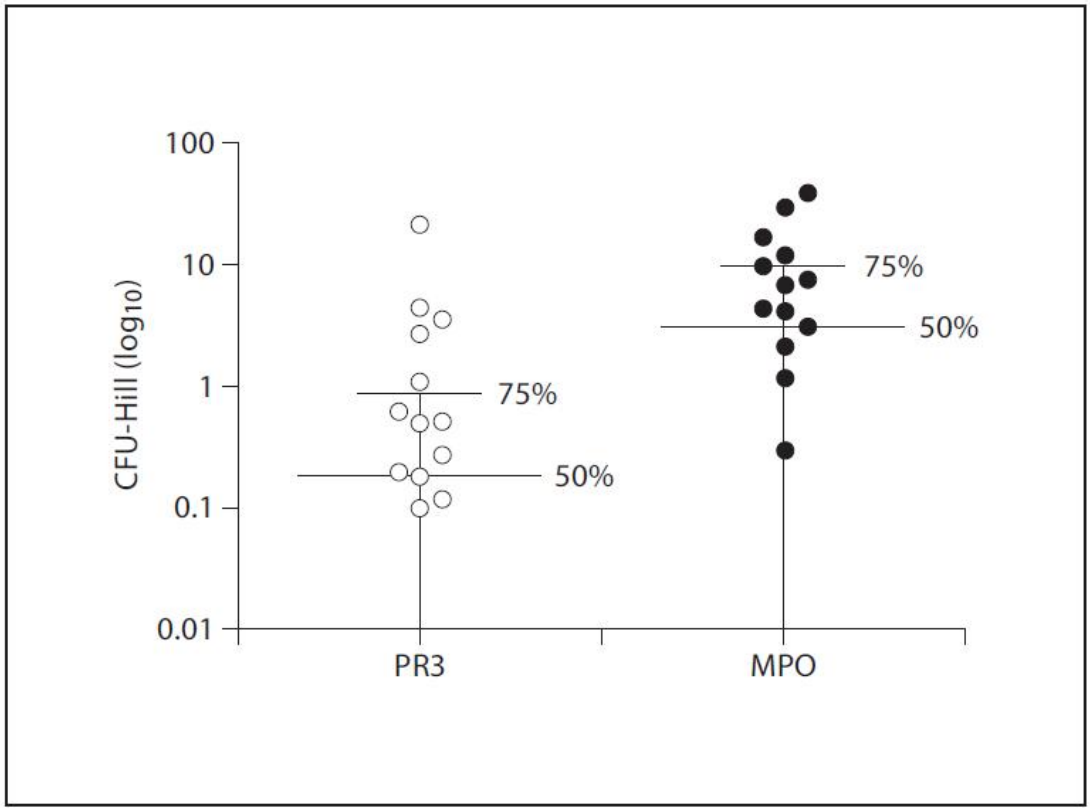


Fig. 3 Patients with anti-MPO ANCA positivity have a higher number of circulating endothelial precursor cells than patients with anti-PR3 positivity. Median 0.2 CFU-Hill/ml (anti-PR3) vs. 3.1 CFU-Hill/ml (anti-MPO). p = 0.06 (Mann-Whitney U test).

Fig. 4

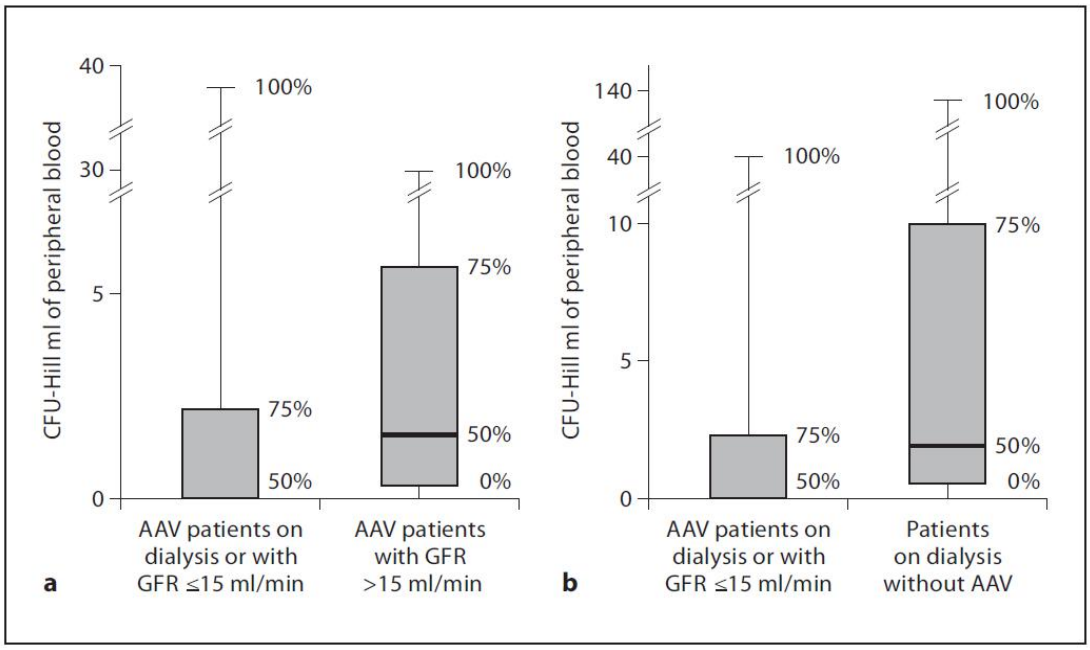


Fig. 4 Dependence of circulating EPC numbers on severity of renal damage. Patients in AAV with terminal renal failure have less circulating endothelial progenitors than patients with better preserved renal function (**a** ; median 0 vs. 1.6, p = 0.015, Mann-Whitney U test), and less circulating EPCs than patients with terminal renal failure without vasculitis (**b** ; median 0 vs. 1.9, p = 0.03, Mann-Whitney U test).

Results II

Cohort description

31 AAV patients with active disease and 10 patients in remission were enrolled to initial EPC sampling and prospectively followed-up. 10 patients were sampled twice during the course of their disease, either with active disease both before and after institution of immunosuppressive treatment (IST), or before and after reaching remission of the disease. As mentioned above, we have not found any significant difference in EPCs counts between these paired measurements, nor a significant difference in EPCs numbers between the 3 subgroups of AAV patients (active before IST, active after IST, in remission. The average time from EPC measurement to censorship or relapse was 21 months. Cohort characteristics with respect to baseline EPC-counts are shown in table 2. There were no significant differences in the baseline characteristics between the EPC groups, except for the type of ANCA antibodies, as the anti-MPO positive patients were underrepresented in the medium EPC level group and overrepresented in the high EPC level group. The number of endothelial progenitor cell colonies (EPC-CFU) per ml of blood ranged from 0 to 39 with a mean of 4.2 CFU/ml. Group 1 represents patients with virtually no growth of endothelial progenitor cell colonies (EPC-CFU<0.01/ml), group 2 patients with intermediate numbers of EPC-CFUs (0.1-3 EPC-CFU/ml), and group 3 patients with high numbers of EPC-CFUs (>3 EPC-CFU/ml).

Table 2. Baseline patient characteristics

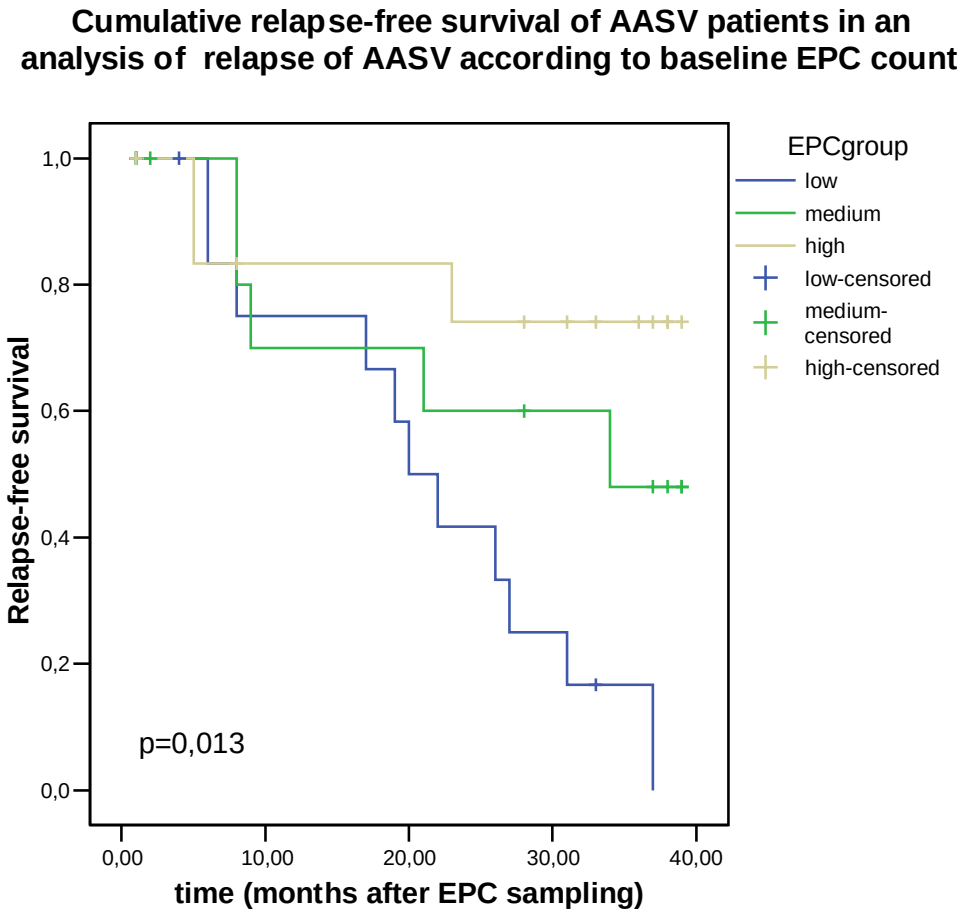
Characteristics	Group 1 (low EPC level)	Group 2 (medium EPC level)	Group 3 (high EPC level)	*P*-value
Number of patients	14	14	13	
Age, years[a]	63 (50–67)	52 (42–57)	60 (57–65)	0.1
Gender, *n* (%)				0.63
Male	8 (57)	7 (50)	5 (38)	
Female	6 (43)	7 (50)	8 (62)	
Type of disease, *n* (%)				0.11
WG	8 (57)	10 (71)	4 (31)	
MPA	6 (43)	4 (29)	9 (69)	
Type of ANCA, *n* (%)				**0.02**
Anti-PR3	7 (50)	11 (79)	3 (23)	
Anti-MPO	7 (50)	3 (21)	10 (77)	
Organ involvement, *n* (%)				
Kidney	14 (100)	14 (100)	12 (92)	0.33
Lung	10 (71)	10 (71)	9 (69)	0.99
Ear–nose–throat area	5 (36)	9 (64)	4 (31)	0.16
Serum creatinine, µmol/l[a]	373 (166–500)	251 (117–500)	290 (119–500)	0.45
Patients on dialysis, *n* (%)[a]	3 (21)	3 (21)	3 (23)	0.56
EPC measurement, *n* (%)				0.66
In active disease	10 (71)	10 (71)	11 (85)	
In remission	4 (29)	4 (29)	2 (15)	
Medication[a], *n* (%)				
ACEi/ARB	5 (36)	6 (43)	6 (46)	0.85
Statins	5 (36)	5 (36)	4 (31)	0.95
Erythropoietin	4 (29)	3 (21)	1 (8)	0.39

Categorical data are presented as number (percentage of total within groups), and continuous data are expressed as medians (interquartile range). *P*-values <0.05 are marked in bold. [a]At the time of the EPC measurement.

Impact of baseline EPC counts on Relapse

A total of 19 patients (46%) relapsed during the study period: 11 in the low EPC group, 5 in the medium group, and 3 in the high EPC group (p=0.01). This trend was most striking with respect to renal relapse, when 7 patients (50%) in the low-EPC group but none in the medium and high-EPC group suffered from renal relapse (p<0.001). There was no significant difference in the relapse rate in other organs between the EPC-groups. Cumulative relapse-free survival increased in a stepwise fashion across increasing levels of EPC-CFUs (see figure 5).

Fig. 5



In univariate analyses, both the EPC group (p=0.008) and type of disease (WG vs. MPA, p=0.02) were found to be associated with a propensity to earlier relapse. There also was a trend toward early relapse in patients with anti-PR3 antibodies as compared to anti-MPO antibodies (p=0.09). When these 3 suggested risk factors were combined in the risk analysis (type of ANCA, type of disease, EPC group), just the EPC group was independently associated with the time to relapse (hazard ratio for relapse of 0.42 (CI 0.21, 0.85) with an increase from low to medium or medium to high EPC group, (p=0.02). The frequency of outcomes and results of subsequent analyses are shown in Tables 3 and 4. Similar results were obtained when analyzing

solely the 31 patients *sampled in active disease*: 9 (90%), 4 (40%), and 3 (27%) of these patients relapsed (p=0.01), and 7 (70%), 0 (0%), and 0 (0%) patients suffered from renal relapse (p<0.001) in the low, medium and high EPC groups respectively. The survival analysis for time to any relapse in this smaller subgroup was not significant (p=0.1), but was significant for time to renal relapse (p<0.001).

Table 3 Outcomes of the AAV patients with respect to their baseline EPC levels

Characteristics	Group 1 (low EPC level)	Group 2 (medium EPC level)	Group 3 (high EPC level)	*P*-value
Death from any cause	2 (14)	4 (29)	3 (23)	0.65
Relapse	11 (79)	5 (36)	3 (23)	**0.01**
Major relapse	8 (57)	4 (29)	2 (15)	0.06
Minor relapse	3 (21)	1 (7)	1 (8)	0.43
Organ(s) involved in relapse				
Kidney	7 (50)	0 (0)	0 (0)	**<0.001**
Lung	4 (28)	4 (28)	2 (15)	0.65
Ear–nose–throat area	1 (7)	1 (7)	0 (0)	0.61
Others	1 (7)	0 (0)	1 (7)	0.58
Serum creatinine, μmol/l[a]	206 (133–500)	235 (96–500)	185 (109–371)	0.56
Overall change in serum creatinine	−14 (−160–0)	−4 (−18–22)	−46 (−132–0)	0.16
Change in serum creatinine per month of follow-up	−1.1 (−3.5–0)	−0.2 (−0.4–1.6)	−2.9 (−6.6–0)	0.70
Patients on dialysis, *n* (%)[a]	6 (43)	5 (36)	3 (23)	0.55

Categorical data are presented as number (percentage of total within groups) and continuous data are expressed as medians (interquartile range). *P*-values < 0.05 are marked in bold. [a]At the time of the last follow-up visit.

Table 4 Cox-proportional analysis of putative risk factors for relapse of AAV

	Unadjusted hazard ratio (95% CI)	*P*-value	Adjusted[a] hazard ratio (95% CI)	*P*-value
Type of disease (WG *vs* MPA)	3.10 (1.17, 8.19)	**0.023**	1.73 (0.50, 5.93)	0.39
Type of ANCA (anti-PR3 *vs* anti-MPO)	2.27 (0.89, 5.79)	0.087	1.35 (0.41, 4.46)	0.62
EPC group (high, medium and low)	0.43 (0.23, 0.80)	**0.008**	0.42 (0.21, 0.85)	**0.02**

P-values <0.05 are marked in bold. [a]Adjusted for the other two variables.

Impact of baseline EPC counts on death from any cause and renal outcome

There was no significant difference in terms of incidence of death of any cause or measures of renal outcome (such as the mean time-averaged change in serum creatinine or dependence on dialysis treatment) with respect to baseline EPC levels (see Table 2).

Discussion

AAV is characterized by a widespread microvascular injury. Although the histopathological appearance of established, clinically evident, systemic vasculitis is usually one of frank trans-mural necrosis of the blood vessel wall, the early pathological lesion is detachment of endothelial cells from the basement membrane progressing through endothelialitis [142]. Accordingly, high numbers of circulating detached, apoptotic or necrotic endothelial cells (CEC) were found in several studies in patients with active disease [63, 88] and these numbers lowered when patients reached remission with appropriate treatment [88]. However, most schemes used to describe and clarify the pathogenesis of vasculitis do not consider aspects of vascular regeneration. In the present study, we investigated whether EPCs could be involved in vascular repair in patients with AAV. In the time when our study was iniated no data concerning the role of EPC in AAV were published. Since then 2 other studies [63, 143] concerning EPC in AAV were published except for the data from our group. In a study by Holmen et al. [63] EPC were investigated in 36 patients with PR3 positive WG and low numbers of EPCs have been found in those with active disaese. Furthermore, *in vitro*, inflammatory endothelial cells (IECs) produced soluble factors, which had a significant negative effect on the proliferation, migration and endothelial nitric oxide synthases (eNOS) expression of EPCs. Next, De Groot et al [143] have measured serially EPC in 11 patients with AAV and found a trend toward an increase of EPC counts in parallel with decrease in disaese activity. Our results bring new insights in the predictive role of EPC for long term outcomes of patients with AAV.

Comparison of EPC in AAV, other vascular disorders, and healthy volunteers

First of all we were interested, whether the level of EPC is lower in AAV patients then in healthy volunteers. Moreover, while AAV patients are exposed to a number of factors that may decrease the pool of EPCs (such as acute systemic inflammatory response, widespread endothelial damage, treatment inducing bone marrow suppression, kidney dysfunction with retention of uremic toxins), it was conceivable that number of EPC may be lower in patients florid systemic vasculitis, than in patients with slowly progressive types of vascular injury (such as atherosclerosis or chronic uremia) or than AAV patients in remisssion of the disease. In our hands, the number of EPCs was extremely low in AAV patients and very low in the patients with other vasculopaties as well, while in healthy volunteers we found the expected results. However, we were not able to find significant differences in EPC numbers between the three groups of patients with different types of endothelial dysfunction/damage (vasculitis, uremia, atherosclerosis). This could have been caused by a rather low number of patients in each group and/or possibly lower sensitivity of CFU-Hill assay in patients with an extremely low number of EPCs.

EPC numbers of AAV patients in the context of clinical and laboratory data

In contrast to the results of Holmén et al. [63] who had studied a similar number of AAV patients (although all patients in this study were classified as Wegenerôs granulomatosis and were uniformly anti-PR3-positive), we did not find a higher number of endothelial cells in patients on treatment or in remission. In fact, there was a tendency towards a lower number of these cells in treated subgroups, which did not reach statistical significance due to large interindividual variabilities among

patients (fig. 1 b). There are two factors that may pertain to the low number of EPCs found in our patients with AAV ï kidney dysfunction and immunosuppressive treatment. It is well known that renal failure may decrease the level of EPC [144ï 147] and our study supports this notion: first, patients with AAV and advanced renal failure (stage 5 according to the K/DOQI classification) or dialyzed patients had an even lower number of circulating EPCs than patients with better preserved renal function (fig. 4 a). Second, non-vasculitis patients with end-stage renal failure also had a lower number of endothelial cells than healthy subjects. However, the assumption that a low number of circulating EPCs in patients with vasculitis is not attributable only to renal damage is supported by the fact that patients with AAV and extremely poor renal function had even lower number of EPCs than patients on dialysis, but without vasculitis (fig. 4 b). Another factor possibly contributing to the low number of circulating EPCs is the effect of immunosuppressive medication. Not only patients with active disease, but also the vast majority of patients in remission of the disease were on maintenance immunosuppressive treatment.

Immunosuppressive therapy has been shown to have an inhibitory effect on the proliferation of EPCs. Butzal et al. [148] have shown that rapamycin inhibits the proliferation and differentiation of human EPCs in vitro. They have also observed that rapamycin induced apoptosis of EPCs. Data from Bertolini et al. [149] have shown that low-dose metronomic cyclophosphamide in mouse lymphoma model lowers the number of EPCs. The influence of immunosuppression was not significant in our analysis, though there was a trend towards a lower number of EPCs in patients on immunosuppressive treatment. The lack of significance might have been caused by the small number of patients studied. Also, the possible adverse effect of cytotoxic

drugs on EPC pool in the bone marrow could be counterbalanced by the suppression of the inflammatory process.

The role of EPC for prediction of long term outcomes of AAV

The second part of our study extends our previous findings, and demonstrates that in patients with ANCA-associated vasculitis reduced number of circulating endothelial progenitor cells is associated with increased risk of early relapse of the disease. The results of our study confirm the previous reports suggesting that circulating endothelial progenitor cells can be used as a predictive biomarker for cardiovascular risk and vascular injury. The levels of circulating endothelial progenitor cells in the peripheral blood correlate with measures of endothelial function [101, 102], and trials aimed to increase the number of EPCs at the site of tissue damage have shown therapeutically meaningful results [150-152]. EPC counts have also been shown to predict clinical outcomes in cardiovascular disease and stroke, as well as in acute lung injury or sepsis [122-126]. In AAV, both low and normal EPC level were reported, as well as inverse or no correlation with markers of activity of the disease by our group and others [63,143, 153]. Differences in the techniques of EPC measurement as well as in the characteristics of studied patient cohorts (mainly with respect to the level of renal dysfunction) could have been involved in these discrepancies. In the initial analysis of our cohort [153] we did not find significant associations between the number of EPC-colonies and the activity of the disease scored with the use of the validated Birmingham Vasculitis Scoring Index (BVAS), the number of involved organs or the level of CRP and ANCA antibodies in these

patients. The interplay of factors, such as the level of disease activity, CRP, renal dysfunction, types and dose of immunosuppressive drugs and other medications (such as statins, ACE inhibitors or erythropoietin) could have an impact on the level of EPCs [154, 155]. Despite the potential of these confounding influences, very low yield of EPC-CFUs seems to signal impaired intrinsic potential to microvascular repair and increased propensity to relapse of the disease. It is well documented that the endothelium is the primary target of injury in AAV [156, 157]. The contact of primed neutrophils and ANCAs with the activated endothelium is considered to be the key event of the vascular inflammatory damage resulting in the detachment of endothelial cells from their basement membrane. The initiating triggers of the disease flares are not well defined, but it is conceivable that micro-endothelial damage from the effects of hypertension, smoking, ischemia or infection may trigger or amplify vasculitic injury.

Therefore, the endothelium may be both a facilitator for development of vasculitis as well as a target for injury, with substantial role in modulating an ongoing inflammatory process [158]. Importantly, EPCs can mediate vascular repair even in the continued presence of vascular injury [94]. EPCs could oppose the perpetuation of endothelial damage and prevent activation of endothelial cells, acting mainly through their direct incorporation into the damaged endothelium and by production of angiogenic factors stimulating neighboring endothelial cells and other endothelial precursors [159]**.**

Limitations of the study

The limited number of patients in our cohort does not allow for a robust multivariable regression analysis and precludes making any definitive assumptions. Clearly, the nature of our study does not permit us to dissect the individual components leading

to decreased EPC-CFU counts or to elucidate the exact mechanism how EPCs protect against the relapse. Also, It would have been helpful to assess EPCs in all AAV patients serially and complement the cultivation method with FACS based methodogy.

Conclusion

We have conducted a pilot study to examine the numbers of EPC in patients with AAV. In the first part of our project, we have shown that contrary to circulating (detached) endothelial cells, the putative endothelial progenitor cells are significantly lower in patients with AAV than in healthy subjects. This could be caused by the widespread endothelial damage (with resulting depletion of EPC pool), systemic inflammatory response, uremic toxins, and other factors, such as the ANCA subtype and/or immunosuppressive treatment.

In the second part of our project we sought the investigate the potential of baseline EPC level to predict long-term outcomes in AAV patients. Our results suggest that assessment of endothelial progenitor cells in patients with ANCA-associated vasculitis may be helpful in the identification of patients at an increased risk for early relapse of the disease. Previous studies [67, 68] have identified a diagnosis of WG or PR3-ANCA at diagnosis as risk factors for relapse. Consistently, in our cohort both these factors proved to have significant role in univariate analyses, but in a combined analysis just EPC level remained to be predictive. EPC level may thus represent a conjoint surrogate marker of the balance between protective and pathogenic vascular

mechanisms. If the inverse relation between EPC and risk of relapse is confirmed in further studies, measurement of EPC could become a useful tool in clinical practice, e.g. to tailor the strategy of induction and maintenance immunosuppressive treatment, and to adjust the frequency of follow-up visits according to risk stratification based on the EPC levels.

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