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Complete List of Authors:	de Groot, Kirsten; Medical School Hannover, Dept. of Nephrology Goldberg, Carina; Hannover Medical School, Nephrology Bahlmann, Ferdinand; Phenos GmbH Woywodt, Alexander; University of Hannover Haller, Hermann; Hannover Medical School, Nephrology Fliser, Danilo; Hannover Medical School, Nephrology Haubitz, Marion; Hannover Medical School, Nephrology
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Vascular endothelial damage and repair in ANCA-associated vasculitis

K. de Groot, M.D., C. Goldberg, M.D., F. H. Bahlmann[#], M.D, PhD., A. Woywodt, M.R.C.P., H. Haller, M.D., D. Fliser, M.D., M. Haubitz, M.D.

Division of Nephrology, Department of Internal Medicine, Hannover Medical School, 30625 Hannover, Germany

[#]Phenos GmbH, 30625 Hannover, Germany

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Correspondence and reprint requests to

Kirsten de Groot, M.D.

Division of Nephrology, Department of Internal Medicine

Hannover Medical School

Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

Phone: (0049)-511-532-6319 / Fax: (0049)-511-552366

E-mail: kirsten@de-groot.de

Abstract

Objective: ANCA-associated vasculitis (AAV) is characterized by a necrotizing vessel wall inflammation, paralleled by the detachment of endothelial cells. The repair of such endothelial defects is crucial for the maintenance of a regular structure and function of the injured vessels. Bone marrow-derived endothelial progenitor cells (EPCs) are thought to play a pivotal role in the regeneration of damaged endothelium. We investigated whether EPCs are involved in vascular repair in AAV.

Patients and Methods: We assessed disease activity, CD34+ hematopoietic progenitor cells (HPCs) using flow cytometry, EPCs using an in-vitro assay and circulating endothelial cells (CECs) by immunomagnetic isolation from the peripheral blood of 31 patients with active AAV at 1, 3 and 6 months after the initiation of immunosuppressive therapy.

Results: Patients with untreated active disease had HPC and EPC numbers comparable to healthy controls (n=64). With the induction of remission, HPCs and EPCs increased significantly from 1.5 [range 0.0-7.0] to a maximum of 3.2 [0.76-9.2] cells/µl ($p<0.001$) and from 261 [171-643] to 470 [168-996] cells/high-power field ($p=0.021$), respectively. In contrast, the initially elevated CECs decreased significantly ($p<0.001$). We observed no correlation of HPC or EPC numbers with leukocyte or thrombocyte count, nor with kidney function.

Conclusion: In patients with AAV, circulating CD34+, HPCs and EPCs increase significantly after the institution of immunosuppressive therapy and disease remission. This finding points to a role of circulating CD34+ HPCs and EPCs in endothelial repair in vasculitis. Targeted stimulation of these cells might represent a new possibility of improving vascular healing in AAV.

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Key words

Endothelial progenitor cells (EPCs), circulating endothelial cells (CECs)

ANCA-associated vasculitis (AAV)

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Introduction

The histological hallmark of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is a necrotizing small vessel vasculitis which, in the majority of the patients, is associated with a crescentic glomerulonephritis. The contact of primed neutrophils and ANCA with the endothelium is considered to be the key event of endothelial injury which ultimately leads to the detachment of endothelial cells from their basement membrane. We have recently shown that these detached, mainly necrotic, endothelial cells can be detected in the circulation and their number correlates with disease activity (1).

The current treatment of AAV consists of immunosuppressive therapy to control the inflammatory process, but does not consider aspects of vascular regeneration. For the repair of endothelial defects, bone marrow-derived endothelial progenitor cells (EPCs) have been shown to play a pivotal role (2-4). They are considered to originate from CD34+ hematopoetic progenitor cells (HPCs) and circulate in the vasculature, where they home and incorporate into sites of active neovascularization (5, 6). In patients with coronary artery disease, the number of EPCs has been shown strongly to correlate inversely with cardiovascular risk factors (7). In uremic patients, a cardiovascular high-risk population, both the number and function of EPCs are greatly reduced (8). In laboratory animals and patients, EPCs expanded ex vivo from peripheral blood mononuclear cells have been shown to be of therapeutic benefit, when administered in either an autologous or allogenic setting for the treatment of ischemic conditions such as myocardial infarction (9-11). The numbers and function of EPCs can be influenced by targeted pharmacological interventions such as the administration of vascular endothelial growth factor (VEGF) (12, 13), statins (14), granulocyte-macrophage colony stimulating factor (GM-CSF) (15) and erythropoietin (8, 16).

In the present study we investigated whether EPCs could be involved in vascular repair in vasculitis patients. To test this hypothesis we assessed circulating CD34+ HPCs, EPCs and circulating endothelial cells (CECs) in 31 patients with active AAV before and, sequentially, after the initiation of immunosuppressive treatment.

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Patients and Methods

Patients

The study protocol was approved by the Hannover Medical School Ethics Committee. We studied 32 episodes of active ANCA-associated vasculitis in 31 consecutive patients before and at 1, 3 and 6 months after the initiation of immunosuppressive therapy after obtaining informed consent. Wegener´s granulomatosis (WG) was diagnosed according to the criteria of the American College of Rheumatology (ACR) (17) and the Chapel Hill Consensus Conference definition (18), microscopic polyangiitis (MPA) according to the Chapel Hill Consensus Conference definition and Churg-Strauss syndrome (CSS) according to the ACR criteria (19). Patients with malignant diseases, manifest or occult bleeding conditions, recent cardiovascular events or treatment with recombinant human erythropoietin (rHuEPO) were excluded from the study. None of the patients had received blood transfusions within 3 months before study entry. All routine laboratory measurements were carried out using certified assay methods. Vasculitis disease activity was scored using the Birmingham Vasculitis Activity Score (BVAS) (20).

Methods

Hematopoietic progenitor cells were assessed in whole EDTA blood by two-color flow cytometry (EPICS XL cytometer, Coulter Beckman) as described earlier (21). Briefly, the CD34 and CD45 expression patterns as well as the morphological qualities of progenitor cells were used for their detection following the gating strategy according to the ISHAGE guidelines (22). Whole EDTA blood was stained within 6 hours of being drawn with FITC-labeled monoclonal mouse anti-human-CD45 antibody (Coulter Beckman) and PE-labeled monoclonal mouse anti-human-CD34 antibody (Coulter Beckman) for 30 minutes. A PE-labeled mouse IgG1-antibody (Coulter Beckman) served as isotype control. Subsequent lysis of erythrocytes was performed with ammonium chloride and at least 200,000 CD45+ cells were acquired. To enumerate the number of CD34+ hematopoietic progenitor cells (HPCs) we used Trucount tubes (Becton Dickinson) containing a known number of fluorescent

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beads. The absolute number of cells per µl in the sample can be determined by normalizing to the number of acquired beads.

We assessed EPCs in a subgroup of 11 patients using an in vitro assay as described elsewhere (8). In brief, peripheral blood mononuclear cells were isolated from 14 ml of the patient's blood using density gradient centrifugation with Bicoll (Biochrome) and seeded 10^7 cells on 6- well plates coated with human fibronectin (Sigma) in endothelial basal medium -2 (Clonetics). The medium was supplemented with endothelial growth medium-2 (SingleQuots, Clonetics) containing FBS, human vascular endothelial growth factor-A, human fibroblast growth factor- B, human epidermal growth factor, insulin like growth factor-1, and ascorbic acid as indicated by the manufacturer. After 4 days of culture, non adherent cells were removed by washing the plates with PBS. The remaining adherent cells were trypsinated and reseeded 10^6 cells on fibronectin- coated 6-well plates. New media were applied, and the cell culture was maintained through to day 7. Fluorescent chemical detection was performed to determine the cell type of attached human peripheral blood mononuclear cells after 7 days in culture. To detect the uptake of 1,1(-dioctadecyl-3,3,3(,3(-tetramethylindocarbocyanine–labeled acetylated LDL (acLDL-DiI) (Molecular Probes), cells were incubated with acLDL-DiI (6 "g/ml) at 37°C for 2 hours. Cells were then fixed with 1% paraformaldehyde for 10 minutes, incubated with FITC-labeled Ulex europaeu agglutinin-1 (UEA-1, Sigma) for one hour and viewed with an inverted fluorescence microscope. Double stained cells for both UEA-1 and acLDL-DiI were defined as EPCs. Two blinded investigators counted at least four randomly selected high-power fields.

We compared HPC and EPC numbers in patients with those of 64 age- and sex-matched healthy control subjects (median age of patients 62, range 20 - 78 years, healthy controls 59, range 22 - 78 years).

Circulating endothelial cells were isolated and enumerated as described previously (23). Briefly, samples of peripheral EDTA blood were drawn with non-traumatic venipuncture. CECs were isolated with M-450 Dynabeads (Dynal) coated with an anti-CD 146 antibody

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(Biocytex). After immunomagnetic isolation, the cells were incubated with UEA-1 for 1 hour in darkness. This staining step was included in order to augment the specificity of the technique and facilitate enumeration (23). The samples were washed and the cells finally suspended in buffer. Cells were counted in a Nageotte counting chamber with a fluorescence microscope.

Statistical analysis

The values are given as medians and ranges. The statistical significance was set at $p<0.05$. Cell numbers were compared using the Mann-Whitney U test, paired Wilcoxon test and the Friedman test for comparison of different time points. Correlation was assessed by Pearson´s test.

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Results

The demographic data and disease parameters of the patients with AAV are given in Table 1. Twenty-five patients had constitutional symptoms and/or arthralgias. Kidney involvement was present in 19 patients, of those 14 had WG and 5 MPA. Extrarenal disease comprised manifestations in the upper airways in 11 patients, lung disease in 15, ocular involvement in 5, skin disease in 5, nervous system involvement in 3 and manifestations in further organs in 3 patients. Four WG patients had disease confined granulomatous manifestations of the upper and lower airways with or without constitutional symptoms. All patients showed clinically and serologically active disease at study entry (Table 1). At that time point 18 patients were without immunosuppressive therapy.

Five patients were not available for follow-up: two patients died from uncontrolled disease activity shortly after the initiation of immunosuppressive therapy, one patient was lost to follow up and two patients required rHuEPO for correction of renal anemia, which has been shown to influence EPC numbers previously (8).

After initiation of treatment (high dose steroids in all together with cyclophosphamide in 28, methotrexate in two and azatioprine in one patients) Birmingham Vasculitis Activity Scores decreased significantly from 12 [4-30] to 4 [0-21] ($p<0.001$) after 1 month of therapy and to 0 [0-4] ($p<0.001$) after 3 and 6 months of treatment (Figure 1). Circulating endothelial cells were clearly elevated before treatment (Table 1) and decreased significantly after 3 (18 [4-72] cells/ml; $p<0.005$) and 6 months (10 [0-32] cells/ml; $p<0.001$) (Figure 2). The number of HPCs at study entry (1.5 [0.0 - 7.0]/µl]) did not differ significantly in patients with AAV from that of age- and sex-matched healthy control persons (1.8 [0.4 - 7.6]/µl). After initiation of the immunosuppressive treatment, HPCs increased significantly to 2.3 [0.01 - 9.2]/µl ($p=0.025$) after 1 month and to 2.6 [0.4 - 9.0]/µl ($p=0.041$) after 3 months (Figure 3). Since the individual time to remission is variable in AAV, we compared the highest HPC number at the time points 1 or 3 months after initiation of treatment with the baseline HPC number. This maximum increment was highly significant, i.e. to 3.2 [0.76-9.2] /µl ($p<0.001$). Thereafter,

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HPCs decreased significantly to month 6 (1.7 [0.5 - 4.3]/µl ($p<0.01$). At study entry, patients with active disease had EPC numbers (261 [171-643]/high-power field) comparable to those in the age- and sex-matched healthy controls (224 [72-518]/high power field). One month after the initiation of immunosuppressive treatment, EPCs were 352 [127 – 522]/high power field, and after 3 months they had peaked to 489 [103 – 996]/high-power field. Thereafter their numbers declined to 400 [197 – 896]/high-power field at 6 months. The differences were not statistically significant due to the high time variability in peak numbers. However, when we analyzed the maximum EPC peak at months 1 to 3 in individual patients (470 [168 - 996]/high-power field) the increase in EPCs was significant ($p<0.021$, Figure 4). For eight patients in long-term remission follow-up measurements after at least 12 months did not show differences for EPCs and CECs (median: EPC 399/high power field, CEC 12/ml) compared to values at month 6.

There was no difference in numbers of HPCs, EPCs or CECs between AAV patients with and without renal involvement or patients with WG or MPA at any time point, although patients with WG showed a trend towards higher maximum HPC (3.91 numbers/µl versus 2.74 /µl in patients with MPA; $p=0.143$).

There was no correlation of leukocyte and thrombocyte counts, and creatinine clearance (Cockroft-Gault) with EPCs or HPCs. However, we found a significant correlation between the maximal individual increase of HPCs and the initial disease activity assessed with the BVAS ($r=0.430$; $p<0.025$).

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Discussion

Microvascular endothelial injury is a major feature of ANCA-associated vasculitis (24-26), and an important factor for chronic morbidity. Despite the achievement of remission in the majority of patients (27, 28) permanent morbidity develops in about 90 % of the patients with AAV; this includes chronic renal insufficiency, hearing or visual loss, pulmonary fibrosis, peripheral motor or sensory neuropathy and treatment-related problems like diabetes, bone marrow failure and premature infertility. Most of the damage occurs early in the course of the disease (29), i.e. mainly during the first 6 months. Therefore, beside the early induction of remission, a swift healing process of the endothelial damage is important.

In our study, EPCs were normal in patients with active AAV and increased with the achievement of remission. The time kinetics of HPCs and EPCs were in an inverse relationship to CECs (Figure 5), the latter being a sensitive marker of endothelial damage. These results permit the conclusion that EPCs could be involved in the repair of vascular defects in patients with AAV. A similar time course of EPCs has been reported in patients with Kawasaki disease (30). Nakatani et al. found that EPCs were highest in the subacute phase, i.e. significantly higher than in the acute disease or in the remission phase (30). The authors suggested that EPCs might be involved in both the repair of endothelial damage and in the neovascularization that can be observed in patients with Kawasaki disease. In contrast to our findings, Holmen et al. (31) reported reduced numbers of EPCs in patients with active vasculitis. However, their comparatively small control group was considerably younger than their patients with AAV, and this fact could explain the conflicting results, at least in part. It is known that the EPC numbers decrease with senescence (32). In addition, we presented a time course of HPC and EPC numbers during the induction of remission, whereas Holmen et al. studied only one time point in their patients. This may also confound the results because we could show that the induction of remission and, in parallel, EPC numbers have a highly variable time course. The former is known from other studies in patients with AAV (28), the latter is a new finding.

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With respect to repair of endothelial damage, the rapid endothelisation of denuded injured vessels is essential to avoid severe complications, such as bleeding and thrombosis. Individual susceptibility to vasculitic damage and scarring may therefore depend on the ability to mobilize EPCs. This concept is supported by data in patients with systemic sclerosis and patients after pneumonia. Endothelial progenitor cells were reduced in systemic sclerosis and patients with extremely low cell numbers were likely to have profound vascular scars and ulcers (33). In patients with bacterial pneumonia, the number of circulating EPCs significantly increased after the resolution of pneumonia; EPC levels seem to predict the ability for lung repair as patients with low numbers developed persistent fibrotic changes after recovery from pneumonia (34). Beside the positive effects of early endothelial healing with regard to preservation of organ function, rapid reconstitution of vessels by EPCs may lead to the restoration of endothelial function and thus prevent accelerated arteriosclerosis by inhibiting neointimal formation (35). These positive effects of EPC-induced vascular regeneration have been documented in animal models with the use of statins (36). Moreover, it has been hypothesized that, in patients with SLE, reduced circulating EPCs contribute to increased cardiovascular risk (37). Therefore, mobilisation of EPCs may have therapeutic implications in patients with AAV. Statins, rHuEPO and various growth factors such as VEGF and GM-CSF have been shown to increase EPC numbers in vivo (8, 12, 14). Unfortunately, GM-CSF, as used in experimental models by Takahashi et al. (15) in order to mobilize stem cells for re-endothelialization of the damaged vessel wall, cannot be used in patients with AAV, because the resulting neutrophilia may trigger a relapse (38). Nevertheless, in future treatment of ANCA-associated vasculitis may not only include adequate immunosuppressive medication, but also strategies to increase EPC numbers or function in order to facilitate endothelial repair mechanisms. Further studies are warranted with respect to the influence of immunosuppressive treatment and the course of disease activity on EPCs, since it is conceivable that immunosuppressive treatment per se affects EPC proliferation. In line with this assumption, recently we could demonstrate that the supplementation of cell culture media with prednisolone or cyclosporin A led to a clear and statistically significant reduction

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in EPC numbers (39). The relation between EPCs and CECs and the residual organ damage after treatment is another interesting question and should be taken into consideration in further studies with a more homogenous patient cohort regarding organ involvement.

We could not find significant differences in numbers and kinetics of all three cell types neither between patients with WG or MPA, nor between patients with or without renal involvement. However, the number of patients was too small to allow meaningful subgroup analysis. An aspect that deserves future attention is the precise characterization of EPCs, which is still a field of ongoing research. Up to today, there is no “standard” in vitro protocol for EPC isolation, and there are also no surface markers that identify circulating EPCs unambiguously, possibly different subsets of EPCs exist. In fact, the protocol used to isolate EPCs in the present study is currently a widely applied method by the most experienced groups in the field (40-42), and the thus generated EPCs have been shown to have functional properties of endothelial cells.

In conclusion, in patients with AAV circulating CD34+ HPCs and EPCs increase significantly after the institution of immunosuppressive therapy and disease remission. This finding points to a potential role of circulating EPCs in endothelial repair in vasculitis patients. The targeted (pharmacological) stimulation of these regenerative cells might represent a new possibility of improving vascular healing in AAV.

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References

1. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small- vessel vasculitis. Lancet 2003;361(9353):206-10.
2. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85(3):221-8.
3. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275(5302):964-7.
4. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95(3):952-8.
5. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, et al. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 2000;87(9):728-30.
6. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97(7):3422-7.
7. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;89(1):E1-7.
8. Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, et al. Erythropoietin regulates endothelial progenitor cells. Blood 2004;103(3):921-6.
9. Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103(5):634-7.

For Peer Review

10. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation 2002;106(24):3009-17.

11. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 2004;364(9429):141-8.

12. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. Embo J 1999;18(14):3964-72.

13. Kalka C, Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T, et al. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg 2000;70(3):829-34.

14. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 2001;108(3):391-7.

15. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5(4):434-8.

16. Bahlmann FH, de Groot K, Duckert T, Niemczyk E, Bahlmann E, Böhm S, et al. Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. Kidney International 2003;64(5):1648-1652.

17. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum. 1990;33:1101 - 1107.

18. Jennette JC, Falk RJ, Andrassy K, Bacon P, Churg J, Gross WL, et al. Nomenclature of systemic vasculitides: The proposal of an International Consensus Conference. Arthritis Rheum. 1994;37:187-192.

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19. Masi AT, Hunder GG, Lie JT, Michel BA, Bloch DA, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis). Arthritis Rheum 1990;33(8):1094-100.

20. Luqmani RA, Bacon PA, Moots RJ, Janssen BA, Pall A, Emery P, et al. Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. Q J Med 1994;87:671 - 678.

21. de Groot K, Bahlmann FHB, Sowa J, Koenig J, Menne J, Haller H, et al. Uremia causes endothelaial progenitor cell deficiency. Kidney Int 2004;66:641-646.

22. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 1996;5(3):213-26.

23. Woywodt A, Goldberg C, Scheer J, Regelsberger H, Haller H, Haubitz M. An improved assay for enumeration of circulating endothelial cells. Ann Hematol 2004;83(8):491-4.

24. Raza K, Thambyrajah J, Townend JN, Exley AR, Hortas C, Filer A, et al. Suppression of inflammation in primary systemic vasculitis restores vascular endothelial function: lessons for atherosclerotic disease? Circulation 2000;102(13):1470-2.

25. Filer AD, Gardner-Medwin JM, Thambyrajah J, Raza K, Carruthers DM, Stevens RJ, et al. Diffuse endothelial dysfunction is common to ANCA associated systemic vasculitis and polyarteritis nodosa. Ann Rheum Dis 2003;62(2):162-7.

26. Bacon PA. Endothelial cell dysfunction in systemic vasculitis: new developments and therapeutic prospects. Curr Opin Rheumatol 2005;17(1):49-55.

27. Hoffman GS, Kerr GS, Leavitt RY, Hallahan CW, Lebovics RS, Travis WD, et al. Wegener's granulomatosis: an analysis of 158 patients. Ann. Intern. Med. 1992;116(6):488 - 498.

For Peer Review

28. Reinhold-Keller E, Beuge N, Latza U, de Groot K, Rudert H, Nolle B, et al. An interdisciplinary approach to the care of patients with Wegener's granulomatosis: long-term outcome in 155 patients. Arthritis Rheum 2000;43(5):1021-32.

29. Exley AR, Carruthers DM, Luqmani RA, Kitas GD, Gordon C, Janssen BA, et al. Damage occurs early in systemic vasculitis and is an index of outcome. Qjm 1997;90(6):391-9.

30. Nakatani K, Takeshita S, Tsujimoto H, Kawamura Y, Tokutomi T, Sekine I. Circulating endothelial cells in Kawasaki disease. Clin Exp Immunol 2003;131(3):536-40.

31. Holmen C, Elsheikh E, Stenvinkel P, Qureshi AR, Pettersson E, Jalkanen S, et al. Circulating inflammatory endothelial cells contribute to endothelial progenitor cell dysfunction in patients with vasculitis and kidney involvement. J Am Soc Nephrol 2005;16(10):3110-20.

32. Imanishi T, Moriwaki C, Hano T, Nishio I. Endothelial progenitor cell senescence is accelerated in both experimental hypertensive rats and patients with essential hypertension. J Hypertens 2005;23(10):1831-7.

33. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. Lancet 2004;364(9434):603-10.

34. Yamada M, Kubo H, Ishizawa K, Kobayashi S, Shinkawa M, Sasaki H. Increased circulating endothelial progenitor cells in patients with bacterial pneumonia: evidence that bone marrow derived cells contribute to lung repair. Thorax 2005;60(5):410-3.

35. Kong D, Melo LG, Mangi AA, Zhang L, Lopez-Ilasaca M, Perrella MA, et al. Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells. Circulation 2004;109(14):1769-75.

36. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002;105(25):3017-24.

For Peer Review

37. Luijten RK, Hoefer IE, Koomans HA, Derksen RH, Verhaar MC. Endothelial progenitor cell availability is impaired in subjects with systemic lupus erythematosus. J Am Soc Nephrol 2005;16:527A.

38. Hellmich B, Csernok E, Trabandt A, Gross WL, Ernst M. Granulocyte-macrophage colony-stimulating factor (GM-CSF) but not granulocyte colony-stimulating factor (G-CSF) induces plasma membrane expression of proteinase 3 (PR3) on neutrophils in vitro. Clin Exp Immunol 2000;120(2):392-8.

39. de Groot K, Bahlmann FH, Bahlmann E, Menne J, Haller H, Fliser D. Kidney graft function determines endothelial progenitor cell number in renal transplant recipients. Transplantation 2005;79(8):941-5.

40. Aicher A, Heeschen C, Sasaki K, Urbich C, Zeiher AM, Dimmeler S. Low-energy shock wave for enhancing recruitment of endothelial progenitor cells: a new modality to increase efficacy of cell therapy in chronic hind limb ischemia. Circulation. 2006;114(25):2823-30. Epub 2006 Dec 4.

41. Galasso G, Schiekofer S, Sato K, Shibata R, Handy DE, Ouchi N, Leopold JA, Loscalzo J, Walsh K. Impaired angiogenesis in glutathione peroxidase-1-deficient mice is associated with endothelial progenitor cell dysfunction. Circ Res. 2006;98(2):254-61. Epub 2005 Dec 22.

42. Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T, Dimmeler S, Zeiher AM. Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl J Med. 2006;355(12):1222-32.

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Table 1: Epidemiological data and disease specific parameters of 31 patients with active ANCA-associated vasculitis.

		Range
No. of patients	31	
No. of episodes of active vasculitis New diagnosis of AAV Relapse	32 14 18	
WG/ MPA/CSS	24/6/1	
Median age [years]	62	20-78
Median BVAS at start	13	4-30
Median circulating CD34+ cells (HPCs)/µl at start	1.5	0-7.0
Median circulating endothelial cells (CEC) /ml at start	44	8-724
Median CRP at start [mg/l]	42	1-290
Median EPCs/high-power field at start (n=11)	261	171-643
Median creatinine at start [µmol/l]	117	57 - 720

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Figure legends

Figure 1: Disease activity as assessed the Birmingham vasculitis activity score in 32 episodes of active disease in 31 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile

Figure 2: Circulating endothelial cells (CECs) in 32 episodes of active disease in 31 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile.

Figure 3: CD34+ hematopoietic progenitor cells (HPCs) in 32 episodes of active disease in 31 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile.

Figure 4: Endothelial progenitor cells (EPCs) in 11 episodes of active disease in 11 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile.

Figure 5: Exemplary course of CECs, HPCs, EPCs in one patient with AAV.

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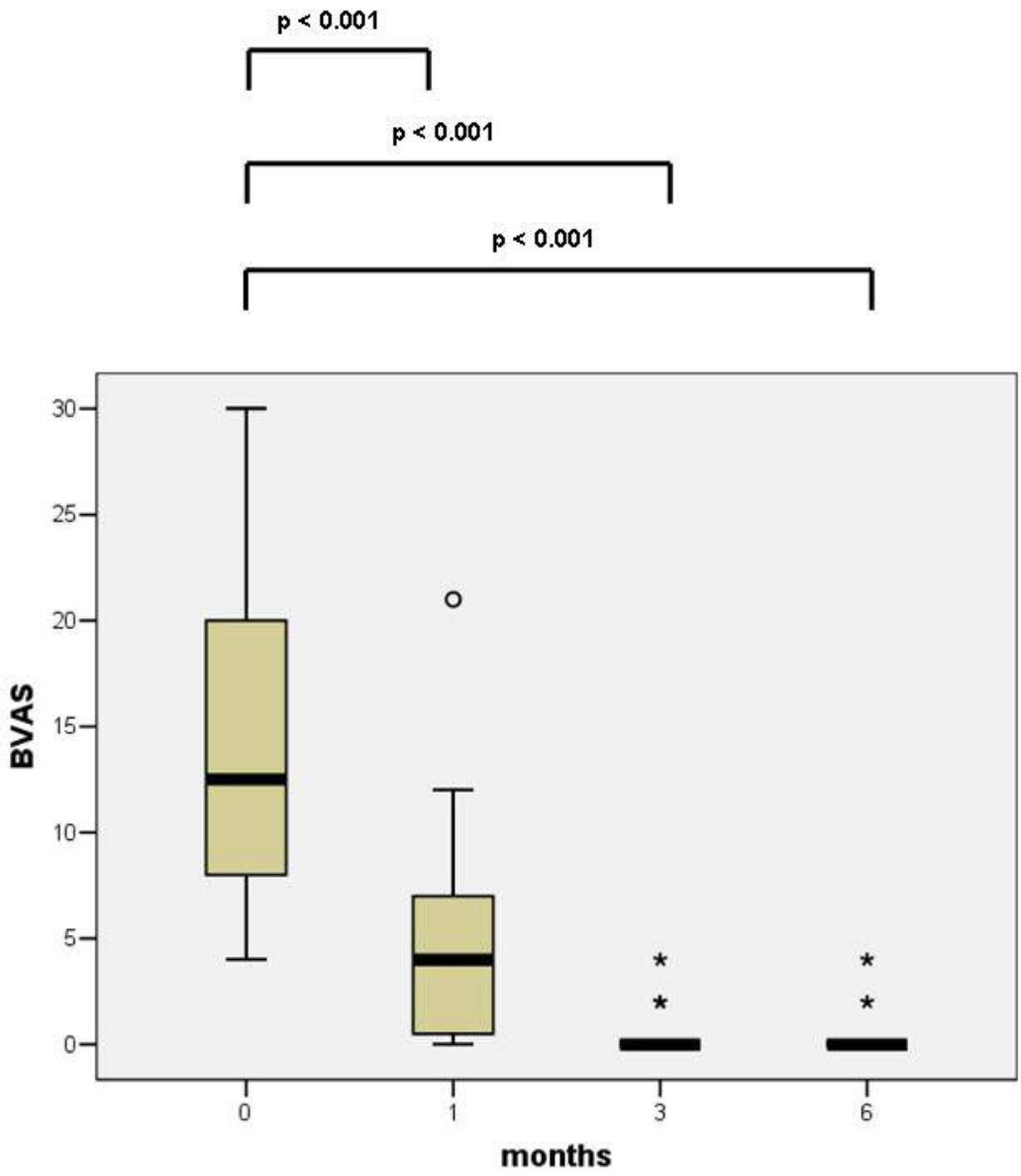

Figure 1

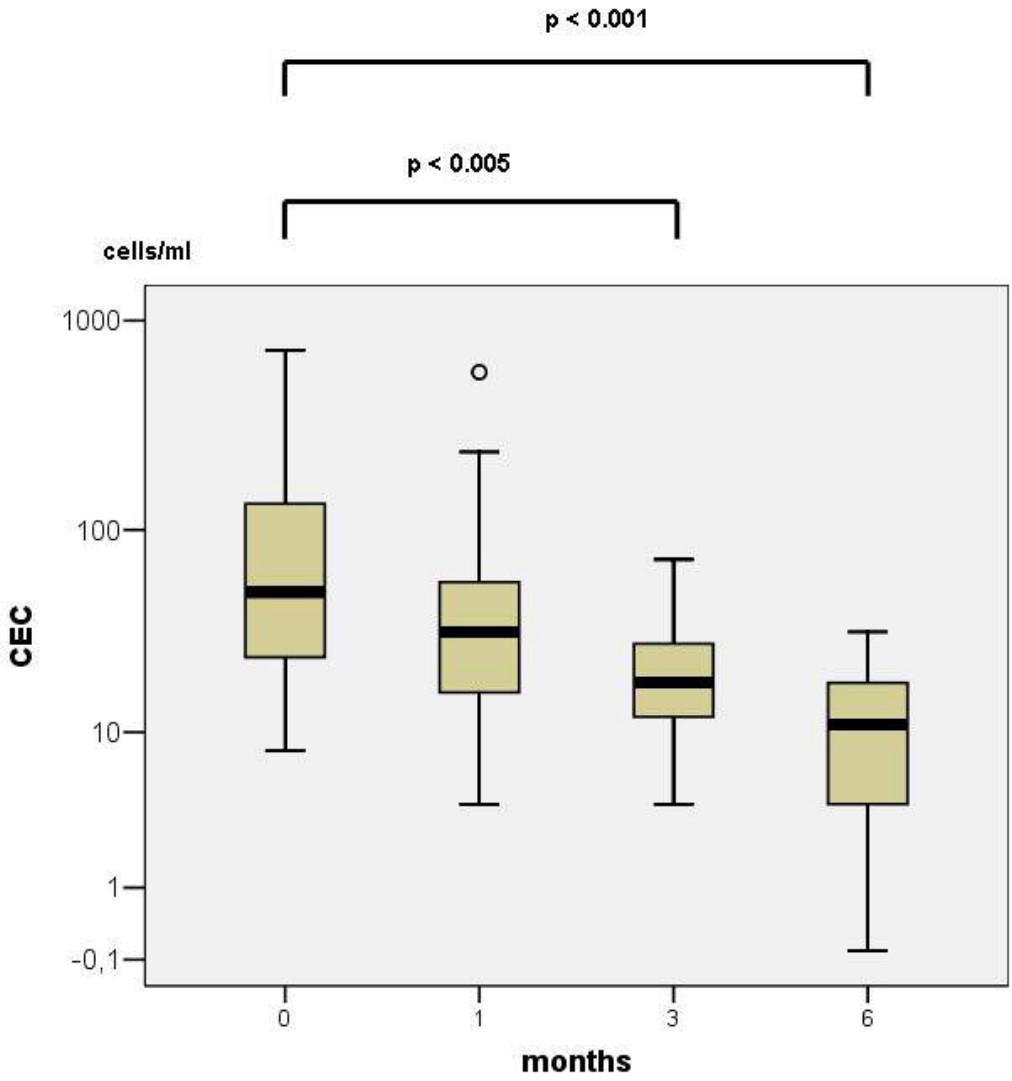

Figure 2

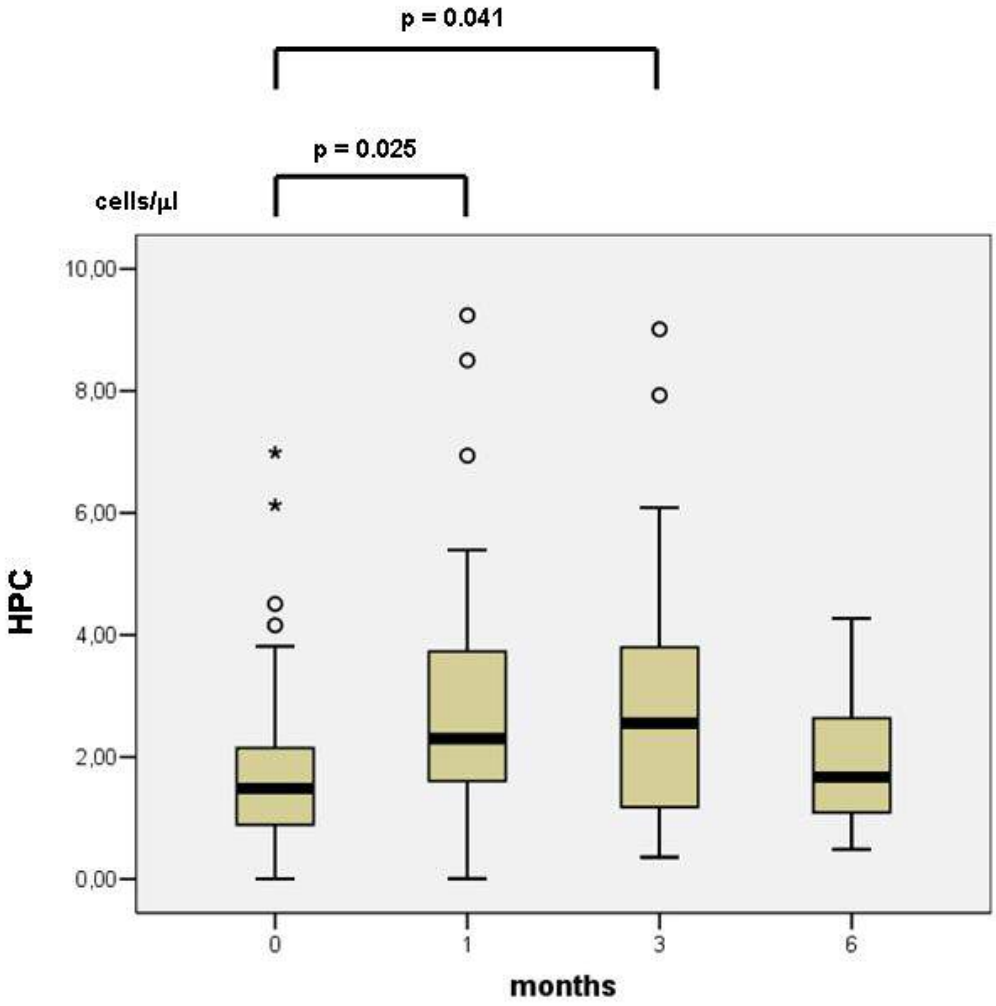

Figure 3

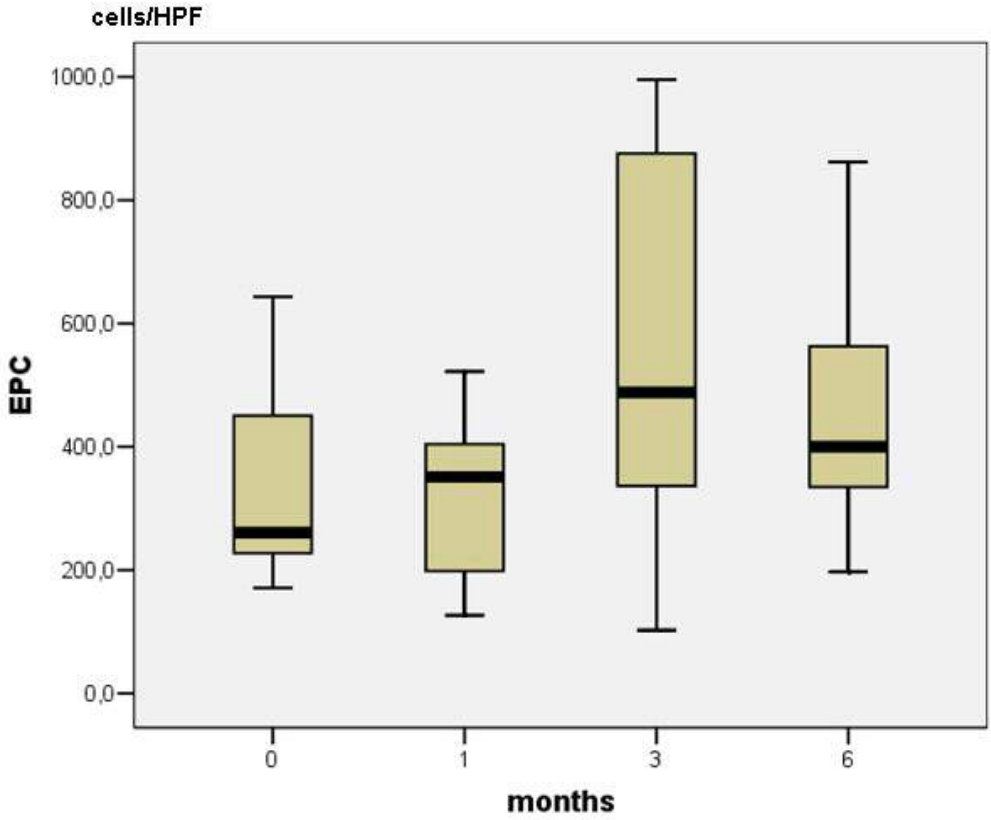

Figure 4

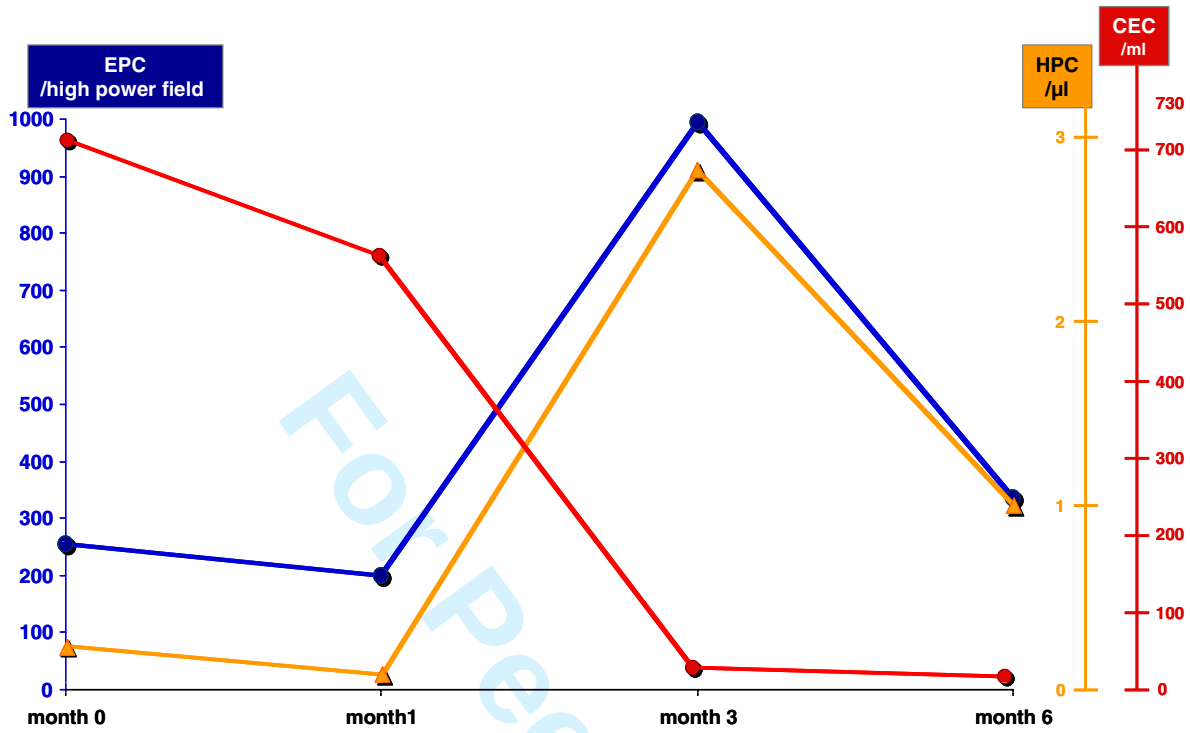

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Figure 5

Vascular endothelial damage and repair in ANCA-associated vasculitis

K. de Groot, M.D., C. Goldberg, M.D., F. H. Bahlmann[#], M.D, PhD., A. Woywodt, M.R.C.P., H. Haller, M.D., D. Fliser, M.D., M. Haubitz, M.D.

Division of Nephrology, Department of Internal Medicine, Hannover Medical School, 30625 Hannover, Germany

[#]Phenos GmbH, 30625 Hannover, Germany

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Abstract	250
Main body	2252

Correspondence and reprint requests to

Kirsten de Groot, M.D.

Division of Nephrology, Department of Internal Medicine

Hannover Medical School

Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

Phone: (0049)-511-532-6319 / Fax: (0049)-511-552366

E-mail: kirsten@de-groot.de

Abstract

Objective: ANCA-associated vasculitis (AAV) is characterized by a necrotizing vessel wall inflammation, paralleled by the detachment of endothelial cells. The repair of such endothelial defects is crucial for the maintenance of a regular structure and function of the injured vessels. Bone marrow-derived endothelial progenitor cells (EPCs) are thought to play a pivotal role in the regeneration of damaged endothelium. We investigated whether EPCs are involved in vascular repair in AAV.

Patients and Methods: We assessed disease activity, CD34+ hematopoietic progenitor cells (HPCs) using flow cytometry, EPCs using an in-vitro assay and circulating endothelial cells (CECs) by immunomagnetic isolation from the peripheral blood of 31 patients with active AAV at 1, 3 and 6 months after the initiation of immunosuppressive therapy.

Results: Patients with untreated active disease had HPC and EPC numbers comparable to healthy controls (n=64). With the induction of remission, HPCs and EPCs increased significantly from 1.5 [range 0.0-7.0] to a maximum of 3.2 [0.76-9.2] cells/µl ($p<0.001$) and from 261 [171-643] to 470 [168-996] cells/high-power field ($p=0.021$), respectively. In contrast, the initially elevated CECs decreased significantly ($p<0.001$). We observed no correlation of HPC or EPC numbers with leukocyte or thrombocyte count, nor with kidney function.

Conclusion: In patients with AAV, circulating CD34+, HPCs and EPCs increase significantly after the institution of immunosuppressive therapy and disease remission. This finding points to a role of circulating CD34+ HPCs and EPCs in endothelial repair in vasculitis. Targeted stimulation of these cells might represent a new possibility of improving vascular healing in AAV.

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Key words

Endothelial progenitor cells (EPCs), circulating endothelial cells (CECs)

ANCA-associated vasculitis (AAV)

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Introduction

The histological hallmark of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is a necrotizing small vessel vasculitis which, in the majority of the patients, is associated with a crescentic glomerulonephritis. The contact of primed neutrophils and ANCA with the endothelium is considered to be the key event of endothelial injury which ultimately leads to the detachment of endothelial cells from their basement membrane. We have recently shown that these detached, mainly necrotic, endothelial cells can be detected in the circulation and their number correlates with disease activity (1).

The current treatment of AAV consists of immunosuppressive therapy to control the inflammatory process, but does not consider aspects of vascular regeneration. For the repair of endothelial defects, bone marrow-derived endothelial progenitor cells (EPCs) have been shown to play a pivotal role (2-4). They are considered to originate from CD34+ hematopoetic progenitor cells (HPCs) and circulate in the vasculature, where they home and incorporate into sites of active neovascularization (5, 6). In patients with coronary artery disease, the number of EPCs has been shown strongly to correlate inversely with cardiovascular risk factors (7). In uremic patients, a cardiovascular high-risk population, both the number and function of EPCs are greatly reduced (8). In laboratory animals and patients, EPCs expanded ex vivo from peripheral blood mononuclear cells have been shown to be of therapeutic benefit, when administered in either an autologous or allogenic setting for the treatment of ischemic conditions such as myocardial infarction (9-11). The numbers and function of EPCs can be influenced by targeted pharmacological interventions such as the administration of vascular endothelial growth factor (VEGF) (12, 13), statins (14), granulocyte-macrophage colony stimulating factor (GM-CSF) (15) and erythropoietin (8, 16).

In the present study we investigated whether EPCs could be involved in vascular repair in vasculitis patients. To test this hypothesis we assessed circulating CD34+ HPCs, EPCs and circulating endothelial cells (CECs) in 31 patients with active AAV before and, sequentially, after the initiation of immunosuppressive treatment.

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Patients and Methods

Patients

The study protocol was approved by the Hannover Medical School Ethics Committee. We studied 32 episodes of active ANCA-associated vasculitis in 31 consecutive patients before and at 1, 3 and 6 months after the initiation of immunosuppressive therapy after obtaining informed consent. Wegener´s granulomatosis (WG) was diagnosed according to the criteria of the American College of Rheumatology (ACR) (17) and the Chapel Hill Consensus Conference definition (18), microscopic polyangiitis (MPA) according to the Chapel Hill Consensus Conference definition and Churg-Strauss syndrome (CSS) according to the ACR criteria (19). Patients with malignant diseases, manifest or occult bleeding conditions, recent cardiovascular events or treatment with recombinant human erythropoietin (rHuEPO) were excluded from the study. None of the patients had received blood transfusions within 3 months before study entry. All routine laboratory measurements were carried out using certified assay methods. Vasculitis disease activity was scored using the Birmingham Vasculitis Activity Score (BVAS) (20).

Methods

Hematopoietic progenitor cells were assessed in whole EDTA blood by two-color flow cytometry (EPICS XL cytometer, Coulter Beckman) as described earlier (21). Briefly, the CD34 and CD45 expression patterns as well as the morphological qualities of progenitor cells were used for their detection following the gating strategy according to the ISHAGE guidelines (22). Whole EDTA blood was stained within 6 hours of being drawn with FITC-labeled monoclonal mouse anti-human-CD45 antibody (Coulter Beckman) and PE-labeled monoclonal mouse anti-human-CD34 antibody (Coulter Beckman) for 30 minutes. A PE-labeled mouse IgG1-antibody (Coulter Beckman) served as isotype control. Subsequent lysis of erythrocytes was performed with ammonium chloride and at least 200,000 CD45+ cells were acquired. **To enumerate the number of CD34+ hematopoietic progenitor cells (HPCs) we used Trucount tubes (Becton Dickinson) containing a known number of**

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fluorescent beads. The absolute number of cells per µl in the sample can be determined by normalizing to the number of acquired beads.

We assessed EPCs in a subgroup of 11 patients using an in vitro assay as described elsewhere (8). **In brief, peripheral blood mononuclear cells were isolated from 14 ml of the patient's blood using density gradient centrifugation with Bicoll (Biochrome) and seeded 10^7 cells on 6- well plates coated with human fibronectin (Sigma) in endothelial basal medium -2 (Clonetics). The medium was supplemented with endothelial growth medium-2 (SingleQuots, Clonetics) containing FBS, human vascular endothelial growth factor-A, human fibroblast growth factor- B, human epidermal growth factor, insulin like growth factor-1, and ascorbic acid as indicated by the manufacturer. After 4 days of culture, non adherent cells were removed by washing the plates with PBS. The remaining adherent cells were trypsinated and reseeded 10^6 cells on fibronectin-coated 6-well plates. New media were applied, and the cell culture was maintained through to day 7. Fluorescent chemical detection was performed to determine the cell type of attached human peripheral blood mononuclear cells after 7 days in culture. To detect the uptake of 1,1(-dioctadecyl-3,3,3(,3(-tetramethylindocarbocyanine–labeled acetylated LDL (acLDL-DiI) (Molecular Probes), cells were incubated with acLDL-DiI (6 "g/ml) at 37°C for 2 hours. Cells were then fixed with 1% paraformaldehyde for 10 minutes, incubated with FITC-labeled Ulex europaeu agglutinin-1 (UEA-1, Sigma) for one hour** and viewed with an inverted fluorescence microscope. Double stained cells for both UEA-1 and acLDL-DiI were defined as EPCs. Two blinded investigators counted at least four randomly selected high-power fields.

We compared HPC and EPC numbers in patients with those of 64 age- and sex-matched healthy control subjects (median age of patients 62, range 20 - 78 years, healthy controls 59, range 22 - 78 years).

Circulating endothelial cells were isolated and enumerated as described previously (23). Briefly, samples of peripheral EDTA blood were drawn with non-traumatic venipuncture.

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CECs were isolated with M-450 Dynabeads (Dynal) coated with an anti-CD 146 antibody (Biocytex). After immunomagnetic isolation, the cells were incubated with UEA-1 for 1 hour in darkness. This staining step was included in order to augment the specificity of the technique and facilitate enumeration (23). The samples were washed and the cells finally suspended in buffer. Cells were counted in a Nageotte counting chamber with a fluorescence microscope.

Statistical analysis

The values are given as medians and ranges. The statistical significance was set at $p<0.05$. Cell numbers were compared using the Mann-Whitney U test, paired Wilcoxon test and the Friedman test for comparison of different time points. Correlation was assessed by Pearson´s test.

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Results

The demographic data and disease parameters of the patients with AAV are given in Table 1. Twenty-five patients had constitutional symptoms and/or arthralgias. **Kidney involvement was present in 19 patients, of those 14 had WG and 5 MPA. Extrarenal disease comprised manifestations in the upper airways in 11 patients, lung disease in 15, ocular involvement in 5, skin disease in 5, nervous system involvement in 3 and manifestations in further organs in 3 patients. Four WG patients had disease confined granulomatous manifestations of the upper and lower airways with or without constitutional symptoms**. All patients showed clinically and serologically active disease at study entry (Table 1). At that time point 18 patients were without immunosuppressive therapy.

Five patients were not available for follow-up: two patients died from uncontrolled disease activity shortly after the initiation of immunosuppressive therapy, one patient was lost to follow up and two patients required rHuEPO for correction of renal anemia, which has been shown to influence EPC numbers previously (8).

After initiation of treatment (high dose steroids in all together with cyclophosphamide in 28, methotrexate in two and azatioprine in one patients) Birmingham Vasculitis Activity Scores decreased significantly from 12 [4-30] to 4 [0-21] ($p<0.001$) after 1 month of therapy and to 0 [0-4] ($p<0.001$) after 3 and 6 months of treatment (Figure 1). Circulating endothelial cells were clearly elevated before treatment (Table 1) and decreased significantly after 3 (18 [4-72] cells/ml; $p<0.005$) and 6 months (10 [0-32] cells/ml; $p<0.001$) (Figure 2). The number of HPCs at study entry (1.5 [0.0 - 7.0]/µl]) did not differ significantly in patients with AAV from that of age- and sex-matched healthy control persons (1.8 [0.4 - 7.6]/µl). After initiation of the immunosuppressive treatment, HPCs increased significantly to 2.3 [0.01 - 9.2]/µl ($p=0.025$) after 1 month and to 2.6 [0.4 - 9.0]/µl ($p=0.041$) after 3 months (Figure 3). Since the individual time to remission is variable in AAV, we compared the highest HPC number at the time points 1 or 3 months after initiation of treatment with the baseline HPC number. This

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maximum increment was highly significant, i.e. to 3.2 [0.76-9.2] /µl ($p<0.001$). Thereafter, HPCs decreased significantly to month 6 (1.7 [0.5 - 4.3]/µl ($p<0.01$). At study entry, patients with active disease had EPC numbers (261 [171-643]/high-power field) comparable to those in the age- and sex-matched healthy controls (224 [72-518]/high power field). One month after the initiation of immunosuppressive treatment, EPCs were 352 [127 – 522]/high power field, and after 3 months they had peaked to 489 [103 – 996]/high-power field. Thereafter their numbers declined to 400 [197 – 896]/high-power field at 6 months. The differences were not statistically significant due to the high time variability in peak numbers. However, when we analyzed the maximum EPC peak at months 1 to 3 in individual patients (470 [168 - 996]/high-power field) the increase in EPCs was significant ($p<0.021$, Figure 4). For eight patients in long-term remission follow-up measurements after at least 12 months did not show differences for EPCs and CECs (median: EPC 399/high power field, CEC 12/ml) compared to values at month 6.

There was no difference in numbers of HPCs, EPCs or CECs between AAV patients with and without renal involvement or patients with WG or MPA at any time point, although patients with WG showed a trend towards higher maximum HPC (3.91 numbers/µl versus 2.74 /µl in patients with MPA; p=0.143).

There was no correlation of leukocyte and thrombocyte counts, and creatinine clearance (Cockroft-Gault) with EPCs or HPCs. However, we found a significant correlation between the maximal individual increase of HPCs and the initial disease activity assessed with the BVAS ($r=0.430$; $p<0.025$).

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Discussion

Microvascular endothelial injury is a major feature of ANCA-associated vasculitis (24-26), and an important factor for chronic morbidity. Despite the achievement of remission in the majority of patients (27, 28) permanent morbidity develops in about 90 % of the patients with AAV; this includes chronic renal insufficiency, hearing or visual loss, pulmonary fibrosis, peripheral motor or sensory neuropathy and treatment-related problems like diabetes, bone marrow failure and premature infertility. Most of the damage occurs early in the course of the disease (29), i.e. mainly during the first 6 months. Therefore, beside the early induction of remission, a swift healing process of the endothelial damage is important.

In our study, EPCs were normal in patients with active AAV and increased with the achievement of remission. The time kinetics of HPCs and EPCs were in an inverse relationship to CECs (Figure 5), the latter being a sensitive marker of endothelial damage. These results permit the conclusion that EPCs could be involved in the repair of vascular defects in patients with AAV. A similar time course of EPCs has been reported in patients with Kawasaki disease (30). Nakatani et al. found that EPCs were highest in the subacute phase, i.e. significantly higher than in the acute disease or in the remission phase (30). The authors suggested that EPCs might be involved in both the repair of endothelial damage and in the neovascularization that can be observed in patients with Kawasaki disease. In contrast to our findings, Holmen et al. (31) reported reduced numbers of EPCs in patients with active vasculitis. However, their comparatively small control group was considerably younger than their patients with AAV, and this fact could explain the conflicting results, at least in part. It is known that the EPC numbers decrease with senescence (32). In addition, we presented a time course of HPC and EPC numbers during the induction of remission, whereas Holmen et al. studied only one time point in their patients. This may also confound the results because we could show that the induction of remission and, in parallel, EPC numbers have a highly variable time course. The former is known from other studies in patients with AAV (28), the latter is a new finding.

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With respect to repair of endothelial damage, the rapid endothelisation of denuded injured vessels is essential to avoid severe complications, such as bleeding and thrombosis. Individual susceptibility to vasculitic damage and scarring may therefore depend on the ability to mobilize EPCs. This concept is supported by data in patients with systemic sclerosis and patients after pneumonia. Endothelial progenitor cells were reduced in systemic sclerosis and patients with extremely low cell numbers were likely to have profound vascular scars and ulcers (33). In patients with bacterial pneumonia, the number of circulating EPCs significantly increased after the resolution of pneumonia; EPC levels seem to predict the ability for lung repair as patients with low numbers developed persistent fibrotic changes after recovery from pneumonia (34). Beside the positive effects of early endothelial healing with regard to preservation of organ function, rapid reconstitution of vessels by EPCs may lead to the restoration of endothelial function and thus prevent accelerated arteriosclerosis by inhibiting neointimal formation (35). These positive effects of EPC-induced vascular regeneration have been documented in animal models with the use of statins (36). Moreover, it has been hypothesized that, in patients with SLE, reduced circulating EPCs contribute to increased cardiovascular risk (37). Therefore, mobilisation of EPCs may have therapeutic implications in patients with AAV. Statins, rHuEPO and various growth factors such as VEGF and GM-CSF have been shown to increase EPC numbers in vivo (8, 12, 14). Unfortunately, GM-CSF, as used in experimental models by Takahashi et al. (15) in order to mobilize stem cells for re-endothelialization of the damaged vessel wall, cannot be used in patients with AAV, because the resulting neutrophilia may trigger a relapse (38). Nevertheless, in future treatment of ANCA-associated vasculitis may not only include adequate immunosuppressive medication, but also strategies to increase EPC numbers or function in order to facilitate endothelial repair mechanisms. Further studies are warranted with respect to the influence of immunosuppressive treatment and the course of disease activity on EPCs, since it is conceivable that immunosuppressive treatment per se affects EPC proliferation. In line with this assumption, recently we could demonstrate that the supplementation of cell culture media with prednisolone or cyclosporin A led to a clear and statistically significant reduction

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in EPC numbers (39). The relation between EPCs and CECs and the residual organ damage after treatment is another interesting question and should be taken into consideration in further studies with a more homogenous patient cohort regarding organ involvement.

We could not find significant differences in numbers and kinetics of all three cell types neither between patients with WG or MPA, nor between patients with or without renal involvement. However, the number of patients was too small to allow meaningful subgroup analysis. An aspect that deserves future attention is the precise characterization of EPCs, which is still a field of ongoing research. Up to today, there is no "standard" in vitro protocol for EPC isolation, and there are also no surface markers that identify circulating EPCs unambiguously, possibly different subsets of EPCs exist. In fact, the protocol used to isolate EPCs in the present study is currently a widely applied method by the most experienced groups in the field (40-42), and the thus generated EPCs have been shown to have functional properties of endothelial cells.

In conclusion, in patients with AAV circulating CD34+ HPCs and EPCs increase significantly after the institution of immunosuppressive therapy and disease remission. This finding points to a potential role of circulating EPCs in endothelial repair in vasculitis patients. The targeted (pharmacological) stimulation of these regenerative cells might represent a new possibility of improving vascular healing in AAV.

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References

1. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small- vessel vasculitis. Lancet 2003;361(9353):206-10.
2. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85(3):221-8.
3. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275(5302):964-7.
4. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95(3):952-8.
5. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, et al. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 2000;87(9):728-30.
6. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97(7):3422-7.
7. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;89(1):E1-7.
8. Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, et al. Erythropoietin regulates endothelial progenitor cells. Blood 2004;103(3):921-6.
9. Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103(5):634-7.

For Peer Review

10. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation 2002;106(24):3009-17.

11. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 2004;364(9429):141-8.

12. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. Embo J 1999;18(14):3964-72.

13. Kalka C, Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T, et al. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg 2000;70(3):829-34.

14. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 2001;108(3):391-7.

15. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5(4):434-8.

16. Bahlmann FH, de Groot K, Duckert T, Niemczyk E, Bahlmann E, Böhm S, et al. Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. Kidney International 2003;64(5):1648-1652.

17. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum. 1990;33:1101 - 1107.

18. Jennette JC, Falk RJ, Andrassy K, Bacon P, Churg J, Gross WL, et al. Nomenclature of systemic vasculitides: The proposal of an International Consensus Conference. Arthritis Rheum. 1994;37:187-192.

For Peer Review

19. Masi AT, Hunder GG, Lie JT, Michel BA, Bloch DA, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis). Arthritis Rheum 1990;33(8):1094-100.

20. Luqmani RA, Bacon PA, Moots RJ, Janssen BA, Pall A, Emery P, et al. Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. Q J Med 1994;87:671 - 678.

21. de Groot K, Bahlmann FHB, Sowa J, Koenig J, Menne J, Haller H, et al. Uremia causes endothelaial progenitor cell deficiency. Kidney Int 2004;66:641-646.

22. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 1996;5(3):213-26.

23. Woywodt A, Goldberg C, Scheer J, Regelsberger H, Haller H, Haubitz M. An improved assay for enumeration of circulating endothelial cells. Ann Hematol 2004;83(8):491-4.

24. Raza K, Thambyrajah J, Townend JN, Exley AR, Hortas C, Filer A, et al. Suppression of inflammation in primary systemic vasculitis restores vascular endothelial function: lessons for atherosclerotic disease? Circulation 2000;102(13):1470-2.

25. Filer AD, Gardner-Medwin JM, Thambyrajah J, Raza K, Carruthers DM, Stevens RJ, et al. Diffuse endothelial dysfunction is common to ANCA associated systemic vasculitis and polyarteritis nodosa. Ann Rheum Dis 2003;62(2):162-7.

26. Bacon PA. Endothelial cell dysfunction in systemic vasculitis: new developments and therapeutic prospects. Curr Opin Rheumatol 2005;17(1):49-55.

27. Hoffman GS, Kerr GS, Leavitt RY, Hallahan CW, Lebovics RS, Travis WD, et al. Wegener's granulomatosis: an analysis of 158 patients. Ann. Intern. Med. 1992;116(6):488 - 498.

For Peer Review

28. Reinhold-Keller E, Beuge N, Latza U, de Groot K, Rudert H, Nolle B, et al. An interdisciplinary approach to the care of patients with Wegener's granulomatosis: long-term outcome in 155 patients. Arthritis Rheum 2000;43(5):1021-32.

29. Exley AR, Carruthers DM, Luqmani RA, Kitas GD, Gordon C, Janssen BA, et al. Damage occurs early in systemic vasculitis and is an index of outcome. Qjm 1997;90(6):391-9.

30. Nakatani K, Takeshita S, Tsujimoto H, Kawamura Y, Tokutomi T, Sekine I. Circulating endothelial cells in Kawasaki disease. Clin Exp Immunol 2003;131(3):536-40.

31. Holmen C, Elsheikh E, Stenvinkel P, Qureshi AR, Pettersson E, Jalkanen S, et al. Circulating inflammatory endothelial cells contribute to endothelial progenitor cell dysfunction in patients with vasculitis and kidney involvement. J Am Soc Nephrol 2005;16(10):3110-20.

32. Imanishi T, Moriwaki C, Hano T, Nishio I. Endothelial progenitor cell senescence is accelerated in both experimental hypertensive rats and patients with essential hypertension. J Hypertens 2005;23(10):1831-7.

33. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. Lancet 2004;364(9434):603-10.

34. Yamada M, Kubo H, Ishizawa K, Kobayashi S, Shinkawa M, Sasaki H. Increased circulating endothelial progenitor cells in patients with bacterial pneumonia: evidence that bone marrow derived cells contribute to lung repair. Thorax 2005;60(5):410-3.

35. Kong D, Melo LG, Mangi AA, Zhang L, Lopez-Ilasaca M, Perrella MA, et al. Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells. Circulation 2004;109(14):1769-75.

36. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002;105(25):3017-24.

For Peer Review

37. Luijten RK, Hoefer IE, Koomans HA, Derksen RH, Verhaar MC. Endothelial progenitor cell availability is impaired in subjects with systemic lupus erythematosus. J Am Soc Nephrol 2005;16:527A.

38. Hellmich B, Csernok E, Trabandt A, Gross WL, Ernst M. Granulocyte-macrophage colony-stimulating factor (GM-CSF) but not granulocyte colony-stimulating factor (G-CSF) induces plasma membrane expression of proteinase 3 (PR3) on neutrophils in vitro. Clin Exp Immunol 2000;120(2):392-8.

39. de Groot K, Bahlmann FH, Bahlmann E, Menne J, Haller H, Fliser D. Kidney graft function determines endothelial progenitor cell number in renal transplant recipients. Transplantation 2005;79(8):941-5.

40. Aicher A, Heeschen C, Sasaki K, Urbich C, Zeiher AM, Dimmeler S. Low-energy shock wave for enhancing recruitment of endothelial progenitor cells: a new modality to increase efficacy of cell therapy in chronic hind limb ischemia. Circulation. 2006;114(25):2823-30. Epub 2006 Dec 4.

41. Galasso G, Schiekofer S, Sato K, Shibata R, Handy DE, Ouchi N, Leopold JA, Loscalzo J, Walsh K. Impaired angiogenesis in glutathione peroxidase-1-deficient mice is associated with endothelial progenitor cell dysfunction. Circ Res. 2006;98(2):254-61. Epub 2005 Dec 22.

42. Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T, Dimmeler S, Zeiher AM. Transcoronary transplantation of progenitor cells after myocardial infarction. N Engl J Med. 2006;355(12):1222-32.

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Table 1: Epidemiological data and disease specific parameters of 31 patients with active ANCA-associated vasculitides.

		Range
No. of patients	31	
No. of episodes of active vasculitis New diagnosis of AAV Relapse	32 14 18	
WG/ MPA/CSS	24/6/1	
Median age [years]	62	20-78
Median BVAS at start	13	4-30
Median circulating CD34+ cells (HPCs)/µl at start	1.5	0-7.0
Median circulating endothelial cells (CEC) /ml at start	44	8-724
Median CRP at start [mg/l]	42	1-290
Median EPCs/high-power field at start (n=11)	261	171-643
Median creatinine at start [µmol/l]	117	57 - 720

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Figure legends

Figure 1: Disease activity as assessed the Birmingham vasculitis activity score in 32 episodes of active disease in 31 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile

Figure 2: Circulating endothelial cells (CECs) in 32 episodes of active disease in 31 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile.

Figure 3: CD34+ hematopoietic progenitor cells (HPCs) in 32 episodes of active disease in 31 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile.

Figure 4: Endothelial progenitor cells (EPCs) in 11 episodes of active disease in 11 patients before and after initiation of treatment. Whiskers signify the 25th and 75th percentile.

Figure 5: Exemplary course of CECs, HPCs, EPCs in one patient with AAV.

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