Rethinking Cell Therapy for Severe Limb Ischemia

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RETHINKING CELL THERAPY FOR SEVERE LIMB ISCHEMIA

NIEUWE INZICHTEN IN CELTHERAPIE VOOR ERNSTIG PERIFEER VAATLIJDEN

(met een samenvatting in het Nederlands)

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Hendrik Gremmels

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Promotor: Prof. dr. M.C. Verhaar

Copromotoren: Dr. J. O. Fledderus
Dr. M. Teraa

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CHAPTER 1

INTRODUCTION

Peripheral Artery Disease

Peripheral Artery Disease (PAD) arises from atherosclerosis of major arteries, with a predilection for the lower limbs. The current prevalence is estimated to exceed 25 million patients in Europe and North America(1). PAD is strongly associated with advanced age and shares risk factors with other presentations of atherosclerotic disease such as smoking, obesity and diabetes(1). Socio-economic costs of PAD are high, especially when revascularization or amputation are indicated(2). The symptomatic presentation of PAD can range from mild claudication to a very severe form, termed Critical or Severe Limb Ischemia (CLI or SLI). The latter occurs when blood supply to the leg has been compromised to such an extent that metabolic demands of even resting tissue can no longer be met(3). SLI is clinically defined as chronic ischemic rest pain or tissue loss, with evidence of arterial disease(1,3). SLI has an unusually untoward prognosis, with 20-40%(1) of patients having to undergo major amputation of the affected limb during the course of the disease (4).

Conventional treatment of SLI is aimed at restoring blood flow of the affected leg(5). Surgical and endovascular techniques have been developed to re-open the arterial lumen, including endarterectomy and stenting, or to bypass occlusions by the placement of a bypass graft. However, not all patients are eligible for either a surgical or endovascular intervention, especially when the crural arteries are affected(6). There is a great, unmet need for novel treatment strategies that promote neovascularization.

Angiogenesis and Stem Cells

The circulatory system has evolved in vertebrates to supply tissues with oxygen and nutrients at a high rate that meets the high energy expenditure associated with movement(7). Additionally, it allows for the rapid deployment of a centrally organized immune system, which protects the body against pathogens and aids in repair after tissue damage(8). In humans the primitive circulatory system develops in the third gestational week, when the embryo becomes too large to be supplied by simple diffusion(9). A common progenitor cell-type that gives rise to both blood and blood vessels, termed the hemangioblast, appears from the primitive mesoderm in clusters called blood islands(10,11). In each blood island, the cells located at the periphery differentiate into angioblasts and form intracellular vacuoles, which ultimately join together to form a primitive vascular lumen. This process is termed vasculogenesis and was long thought to occur only during embryonic development. In later stages of embryonic development and in the adult organism, extension of the vascular network occurs via angiogenesis, in which new vessels branch off form from existing vascular walls(7,11).

A publication by Asahara et al. in 1997(12) seemed to overthrow this paradigm as the authors proposed the existence of a cell type with angioblast-like features in adult human blood. Asahara et al. hypothesized that the markers CD34 and KDR, which in the

hematopoietic line are expressed only in primitive stem cells but are present on some forms of adult endothelium, would provide evidence for the existence of Endothelial Progenitor Cells (EPCs) in circulation(12). The authors isolated these putative EPCs and showed that they contribute to post-natal angiogenesis by homing to vascular structures(12). Subsequently it was demonstrated that EPCs are released from the bone marrow (BM) in response to ischemic stimuli(13). These findings were further extended by Kalka et al., who showed that injection of EPCs could enhance angiogenic repair beyond physiological levels(14), suggesting a potential therapeutic strategy for ischemic vascular disease. Vasa et al. showed that EPC numbers are reduced in number and function in the circulation of patients with cardiovascular risk factors(15). Taken together these findings suggest the existence of BM-derived EPCs that serve as an endogenous repair system for vascular damage(16). This repair system may be impaired in patients with cardiovascular disease, contributing to development and progression of the disease. Hence, isolation and targeted delivery of EPCs would be an attractive strategy to promote angiogenesis when endogenous vascular repair fails.

At the time of writing of this thesis, roughly a decade and a half after the publication of the first studies investigating EPCs, the nature and role of EPCs has become much more nuanced. One of the major complicating factors was the finding that the originally proposed cell surface markers which were used to identify EPCs, stain several ontogenetically distinct cell populations, all of which are related to cardiovascular disease(17,18). In addition it has been shown that circulating cells do not directly contribute to newly formed vessels(19,20), but only temporarily home to sites near vessel formation(21). These observations do not necessarily disqualify the various (progenitor) cell populations as either biomarker or therapeutic agent. A relationship between progenitor cell numbers in circulation and cardiovascular disease has been substantiated in many studies. While a direct contribution of circulating progenitor cells to recovery after ischemia has never been shown *in vivo*, *ex vivo* isolation and re-administration has shown a consistent treatment effect in pre-clinical studies.

Cell therapy trials in SLI patients

The translation from the initial preclinical observations to the first clinical study using progenitor cells occurred very quickly; within two years the first studies with cell therapy in patients with SLI(22) and myocardial infarction(23) were published. These and most subsequent studies used BM-derived mononuclear cells (BM-MNCs) as cell therapy product. The BM is considered to be the reservoir for (endothelial) progenitor cells, from which they are mobilized into circulation(24). BM aspirates contain roughly 20-fold higher numbers of CD34+ progenitor cells compared to peripheral blood(25), making it the preferred source for putative EPCs in clinical therapies.

The results of the first cell therapy trial in SLI, the Therapeutic Angiogenesis using Cell Transplantation (TACT) trial, showed that BM-MNCs were safe and potentially effective(22). In the following years, several case series and uncontrolled trials have reported(26) a positive effect on functional or surrogate outcome measures such as Ankle Brachial Index (ABI), pain-free walking distance and pain scores. However, studies were often small, suffered from lack of blinding or adequate controls(27). A meta-analysis by our group in 2013 showed that in the randomized placebo-controlled studies results remained inconclusive(27).

In order to obtain more definite proof of efficacy, our group initiated the JUVENTAS (reJUVenating ENdothelial progenitor cells via Transcutaneous intra-Arterial Supplementation) trial in 2006(28). The trial was designed as a double-blind, placebo-controlled, randomized trial, investigating repeated intra-arterial injection of BM-MNCs in patients with SLI. The primary outcome was amputation or death at 6 months after randomization; secondary outcomes were ABI, pain-free walking distance, Transcutaneous Oxygen pressure (TcO_2) and quality of life. The results of the trial did not show a treatment benefit of BM-MNCs with 15 and 10 amputations in the treatment and control group respectively after 6 months(29). Neither was there a significant treatment effect on secondary outcomes, although both BM-MNC- and placebo- treated groups improved compared to baseline.

The results of the JUVENTAS study indicate that treatment with unmodified BM-MNCs does not promote neovascularization or prevent amputation in patients with SLI. This does not exclude a potential effect of cell therapy in SLI, since the lack of effect might be due to disease related progenitor cell dysfunction, the mode of application, the cell type or dose, or the patient selection.

Re-thinking Cell therapy

In progenitor cell therapy with BM-MNCs, patients are treated with autologous cells in order to prevent rejection or graft-versus-host disease. Variability in treatment success is therefore determined by two factors: the (pro-angiogenic) potency of the cell isolate and the morbidity of the patient, in particular the affected limb. Studies have shown that BM-MNCs from patients with chronic cardiovascular disease, e.g. chronic ischemic cardiomyopathy, display an impaired ability to promote angiogenesis(30-33). This suggests an inherent limitation to progenitor cell therapy, as the same disease processes that cause atherosclerosis and SLI also apparently limit the treatment efficacy of BM-MNC isolates.

The underlying cause of the reduced neovascularization ability of BM-MNC isolates obtained from patients with cardiovascular disease remains to be identified. BM is a complex tissue containing many different cell types. It was originally thought that EPCs were the 'active angiogenic BM-component'. However, many cell types in BM have now been shown to actively participate in promoting neovascularization, including endothelial

cells (34), mesenchymal stromal cells (MSCs)(35), monocytes(36) and even lymphocytes(37,38). Conversely there may be inflammatory or pro-fibrotic cells that are deleterious to the treatment effect.(39). In addition to quantitative alterations in BM composition, progenitor cells obtained from both peripheral blood(15,40) and BM(41) of cardiovascular patients have been shown to be impaired in their function, in particular with regard to migration.

Improvement of cell therapy may therefore be achieved by enriching active primary cell populations(36,42) or expanding selected progenitor cells in culture.(43). The thus obtained cell populations are not necessarily homogenous, they are merely defined by the presence of a marker or their amenability to specific culture conditions(17,44). In cell therapy for SLI, the most commonly investigated cell populations are Circulating Angiogenic Cells (CACs), an *ex vivo* differentiated monocytic cell(17); Endothelial Colony Forming Cells (ECFCs)(17), proliferative endothelial cells obtained from peripheral or umbilical cord blood, and MSCs(45), a pericyte-like cell type, frequently obtained from BM.

It is at present not fully known if all culture-expanded cell populations suffer from similar disease mediated dysfunction as unmodified isolates, such as BM-MNCs. For CACs, which are easy to obtain, there is plenty of evidence that this is the case(15,40). Interestingly, it has been shown that both numbers of these cells in circulation(46), as well as the pro-angiogenic potential can be increased by pharmacological pre-treatment(47). Less is known about disease-mediated dysfunction in ECFCs or MSCs, and whether pre-treatment might enhance efficacy. Next to the intrinsic potential of the progenitor cells used for therapy, it may also be important to consider the tissue conditions the cells are exposed to upon application. A detrimental milieu may require pre-treatment to strengthen cellular resistance prior to injection.

In addition to selecting and improving the cell product, the success of cell-therapy may be improved by selecting patients who are most likely to benefit from the treatment. In studies investigating cell therapy for myocardial infarction (MI), it has been shown that patients with more severe infarction show greater benefit from cell treatment(48), whereas older patients show less response(49). Similar criteria may be identified for SLI, where selection of patients at high risk for amputation and specific subgroups may further improve cell therapy.

Outline of the thesis

The primary objective of this thesis was to further develop and optimize progenitor cell therapy for SLI. The first part of this thesis uses data from the JUVENTAS study to enhance insights on circulating and BM progenitor cells and to identify patients that are at greatest risk for major events, i.e. amputation or death, and hence in most urgent need for therapeutic intervention.

Few studies have previously investigated circulating progenitor cell populations in patients with SLI and, as BM harvesting is an invasive procedure, very little is known about the condition of BM progenitor cells in these patients. The JUVENTAS study provided us with the opportunity to study progenitor cells in the circulation as well as in the BM, the natural reservoir of progenitor cells from whence they are mobilized into circulation. In **Chapter 2** we examine the relationship between CD34+ and CD133+ progenitor cells in peripheral blood and BM and cardiovascular outcomes in SLI patients in the JUVENTAS study. Comparing these progenitor cell numbers allows the evaluation of the importance of progenitor cell mobilization in cardiovascular disease progression.
In **Chapter 3** we used the extended follow-up data from the JUVENTAS trial to identify potential biomarkers that are related to risk of limb amputation and death in SLI patients. Prospective identification of high-risk patients could prioritize treatment decisions, including the use of cell-therapy. In line, in **Chapter 4,** we examined the presence of diabetes mellitus (DM) as a risk factor for limb amputation and death in SLI, using both the JUVENTAS and PADI trial cohorts.
In **Chapter 5** we aimed to identify responders to cell therapy in a large clinical trial - the REPAIR AMI trial (48) - using BM-MNC therapy after myocardial infarction (MI). We perform this analysis in a different study for several reasons: Firstly, the REPAIR AMI trial showed an overall treatment benefit, indicating that the study population contained responders to treatment. Secondly, the study included 184 patients who completed follow-up, a sufficient number to provide power for subgroup analyses. Thirdly, heart function can be fairly reliably quantified by Left-Ventricular Ejection Fraction (LVEF). Using LVEF, improvement or deterioration can be established at the level of the individual patient, whereas this is more difficult using a binary outcome such as amputation or death. How heart function recovers after MI greatly differs per patient, depending i.a. on patient age, gender, body weight and size of the infarction. Here we used data from the REPAIR AMI trial to identify patient characteristics associated with a high treatment benefit.

The second part of the thesis focuses on the development of an improved cell therapy product to be applied in a future clinical trial in SLI. In **Chapter 6** we provide an overview of the different (endothelial) progenitor cell phenotypes that may be suitable for cell therapy**.** We describe how these cell populations are interrelated by gene expression profiling and examine how cultured cells relate to their substrates *in vivo*. We also study functional characteristics such as spontaneous vasculogenesis that may be used to select candidate cell populations for clinical studies.

One of the more promising cell candidates for cell therapy is the ECFC. This cell-type has been shown to be sensitive to detrimental environmental factors, in particular oxidative stress. Survival and intact function of ECFCs after injection *in vivo* is important

for their angiogenic effects. Nrf2 is a transcription factor that mediates the cellular response to oxidative stress, by inducing the expression of various anti-oxidant enzymes. In **Chapter 7** we examine the activation of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), as a pre-conditioning strategy for ECFCs.
An alternative option to enhance ECFC efficacy might be the application in combination with paracrine factors, as neovessel formation by ECFCs has been shown to be most extensive in combination with another cell-type that has strong paracrine effects, such as CACs(34) or MSCs(50). In **Chapter 8** we investigate the combination of ECFCs with a slow release system for growth factors. As a proof-of-concept we use gelatin micro-particles to supply VEGF as growth factor and examine the possibility to make tissue-engineered constructs via 3D printing.

In **Chapter 9** we investigate the application of Mesenchymal Stromal Cells (MSCs) as therapeutic option for SLI. MSCs have been shown to induce functional improvements in pre-clinical and clinical studies as therapy for myocardial infarction. In comparative studies of different cell types in pre-clinical models, MSCs generally show strong pro-angiogenic effects. MSCs are most often obtained by the *ex vivo* expansion of BM-MNCs. It is not known if disease-mediated dysfunction still persists after tissue culture. In this chapter we compare MSCs obtained from SLI patients to MSCs obtained from healthy controls. We investigate both their stem-cell characteristics such as differentiation ability, as well as their pro-angiogenic effects. The presence or absence of disease-mediated dysfunction is important in the decision to apply autologous or allogeneic MSCs.
In **Chapter 10** we discuss a recent phase I/II trial that used allogeneic MSCs for the treatment of SLI and discuss the advantages of allogeneic versus autologous application.

In **Chapter 11** we conclude the thesis by discussing current state of knowledge concerning cell therapy for SLI, with the view of setting the rationale for further clinical studies.

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Hendrik Gremmels M.D.[1] , Martin Teraa M.D.,Ph.D.[2,3], Femke van Rhijn-Brouwer M.D.[1],
Diana A. Papazova M.D.,Ph.D.[1], Joost O. Fledderus, Ph.D.,
Marianne C. Verhaar, M.D.,Ph.D.[1]

on behalf of the JUVENTAS study group

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, the Netherlands
[2] Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands
[3] Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands

CHAPTER 2

HIGHER CIRCULATING AND BONE MARROW PROGENITOR CELL NUMBERS ARE ASSOCIATED WITH INCREASED AMPUTATION-FREE SURVIVAL IN SEVERE LIMB ISCHEMIA

Submitted

ABSTRACT

Introduction

Lower numbers of progenitor cells in peripheral blood (PB PCs) have been associated with cardiovascular events in high-risk populations. It is currently unknown what causes the reduction in PB PC numbers: whether it is primary exhaustion of the BM niche, or a reduced mobilization of PCs from the bone marrow (BM). In this study we examine if PB and BM PC numbers predict Amputation-Free Survival (AFS) in patients with Severe Limb-Ischemia (SLI).

Methods and Results

PB and BM were obtained from 160 patients enrolled in a trial investigating BM cell-therapy for SLI. Samples were incubated with antibodies against CD34, KDR, CD133, CD184, CD14, CD105, CD140b, and CD31; cell populations were subsequently enumerated by flow cytometry. Median follow-up was 3 years. Higher PB CD34+ and CD133+ PC numbers were related to AFS (Hazard Ratio [HR_{event}]=0.56, p=0.003 and p=0.0007 respectively) but there was no association of AFS with any of the other cell populations in PB. BM PC numbers correlated with PB PC numbers and showed similar HRs for AFS. A further subdivision based on relative BM- and PB PC numbers showed that BM PC numbers, rather than mobilization, were primarily associated with AFS. Low PB and BM PC numbers proved to be an independent risk factor for AFS.

Discussion

Both PB and BM PC numbers are independently associated with AFS and show very similar risk profiles. Our data suggest that exhaustion of the BM-niche, rather than decreased PC mobilization underlies the association between PB PC numbers and cardiovascular risk.

INTRODUCTION

Patients with peripheral arterial disease (PAD) show faster functional decline and higher rates of cardiovascular events than patients without PAD (1,2). The most advanced stage of PAD, Severe or Critical Limb Ischemia (SLI or CLI), occurs when atherosclerotic occlusion of the arteries of the lower limb reaches a point where blood supply cannot meet metabolic demands of the tissue even in rest(3). In addition to risk factors associated with vascular damage, in particular smoking, increasing age and diabetes mellitus (DM), the deterioration of endogenous repair mechanisms contributes to progression of PAD and increased cardiovascular risk(4).

Progenitor Cells (PCs) in the circulation may represent such an endogenous repair mechanism and their numbers have been shown to be reduced in patients with cardiovascular disease(5,6), including SLI(7). Prospective studies show that numbers of PCs circulating in peripheral blood (PB PC) are inversely related to cardiovascular outcomes and may be used as a predictive biomarker(8-15). In these predictive studies, PB PCs are commonly identified by the progenitor cell markers CD34 and CD133(16), which partially overlap. In addition, PCs are often stained for the presence of KDR, also known as Vascular-Endothelial Growth Factor Receptor 2, in order to identify putative Endothelial Progenitor Cells (EPCs) (17,18).

Prior to mobilization into the circulation, CD34+ or CD133+ PCs reside in the bone marrow (BM) niche(19). The reduction in PB PC numbers is caused by a complex and multifactorial mechanism, wherein both a defect in PC mobilization and exhaustion of the BM reserve likely play a role. At present it is unclear, however, which of these two factors is predominant. Patients with diabetes, have been shown to display a 'mobilopathy', i.e. they respond less to exogenously administered mobilizing factors such as Granulocyte Stimulating Factor (G-CSF) or Stromal Derived Factor (SDF) 1α(20). In a previous cross-sectional study in SLI patients, on the other hand, we have shown that PC numbers are reduced in the BM niche itself(7). Moreover, other studies have shown that BM PC numbers are associated with recovery after myocardial infarction (21,22), implying alterations in the composition of the BM reservoir itself, in addition to a reduced sensitivity of PCs to mobilizing factors.

In the present study we examine the relationship between PB and BM PCs, and amputation or death in SLI patients. Furthermore, we attempt to separate the role of a potential BM reserve exhaustion from a mobilization defect in determining cardiovascular risk. To this end we use data obtained in the JUVENTAS trial, a randomized controlled trial investigating autologous BM cell therapy for the treatment of SLI(23), for which we have extended the initial follow-up. Amputation-free survival (AFS), a composite outcome of major amputation and death, was used as the primary outcome.

MATERIALS AND METHODS

Study population

The study population of this prospective cohort study consisted of patients included in the JUVENTAS trial; a single-center double-blind randomized placebo-controlled trial investigating repetitive intra-arterial infusion of autologous BM mono-nuclear cells (BMMNCs) for the treatment of SLI. Trial design(24) and results of the primary analysis (23) have been published elsewhere; study inclusion was from September 2006 until June 2012. Inclusion criteria for the trial were severe infra-popliteal atherosclerosis, Fontaine

grade IIB-IV, and ineligibility for surgical intervention. Exclusion criteria were a history of neoplasm or malignancy in the past 10 years, concomitant disease with life expectancy of less than one year, inability to obtain sufficient BM aspirate, known infection with human immunodeficiency virus, hepatitis B or C virus, and an inability to complete follow-up. Patients were randomized 1:1 to receive 3 intra-arterial infusions of autologous BMMNCs or placebo into the common femoral artery of the affected limb. The primary outcome was the incidence of major amputation, defined as amputation through- or above the ankle joint, at 6 months after inclusion. For the present study we have extended follow-up until the beginning of 2015; using AFS as primary outcome.
In addition to the study population 17 healthy controls were included, who underwent BM aspiration under full anesthesia prior to elective major surgery.

Ethics Statement

The study has been approved by the Medical Ethics Committee of the UMC Utrecht and was conducted in accordance with the declaration of Helsinki. All included patients provided their written, informed consent.

Sample preparation

Approximately 100 ml of BM was harvested from the right iliac crest by an experienced hematologist under local anesthesia and conscious sedation. Peripheral blood (PB) samples were obtained by venipuncture of the antecubital vein. Flow cytometry on PB and BM was performed using lyse-and-wash protocols. 100µl of PB or BM were incubated with the following antibody panels: 1: anti-CD34-FITC (BD Pharmingen, San Diego, CA, USA), anti-KDR-PE (R&D Systems, Minneapolis, MN, USA); 2: anti-CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany); 3: anti-CD184-PE (BD Pharmingen) and anti-CD14-ECD (Immunotech, Coulter, France), 4: anti CD-105-FITC (R&D) and anti-CD14-ECD; 5: anti-CD140b-PE (BD Pharmingen), for 45 min in the dark. Erythrocytes were lysed in an ammonium chloride buffer and remaining cells were washed with PBS and analyzed by flow cytometry (FC 500, Beckman Coulter, Fullerton, CA, USA). All samples were processed in duplicate. Spectral compensation was performed by using IgG isotype controls for the relevant fluorophores.

Flow Cytometry Data Analysis

Data were analyzed using FlowJo software version 10.0.8 (Treestar Inc., Ashland, OR); all files were analyzed by a blinded assessor. See supplementary methods and Supplementary Figure 1 for a description of the gating strategy. To account for variations in acquisition occurring due to inconsistency in erythrocyte lysis, all cell numbers are corrected for the number of granulocytes, as this population can be most reliably identified on FSC/SSC.

Statistical Analysis

Continuous variables are presented as means ± standard deviation (SD), or as medians with Interquartile Range (IQR). Differences between patients who reached an endpoint and who did not were assessed by chi square test for categorical variables, student's t-test for normally distributed continuous and a Mann-Whitney-U test for non-normally distributed variables. Cell counts were non-normally distributed and were transformed by taking the natural logarithm (*e*) of the (cell number +1). Correlations between cell-types were quantified by spearman rank coefficient. To assess the relative contribution of mobilization, patients were divided into quadrants of either high and low numbers of BM and PB PCs, based on the median values of PC numbers.

Survival analysis was performed using Cox proportional hazards regression models; throughout the manuscript Hazard Rates (HR) are given as exp(β), where β is the slope of the regression model for one *e* fold increase in PCs. Additional covariates were added as described in the relevant tables. For multivariable models, automated backward exclusion of model factors based on an AIC criterion was performed in order to derive an optimal model. The proportional hazards assumption was evaluated by plotting Schoenfeld residuals. All analyses were conducted in R, version 3.1.0(25). P-values < 0.05 were considered statistically significant. The Benjamini Hochberg method was used to limit the False Discovery Rate resulting from multiple testing.

RESULTS

Trial and Patients

160 patients were included in the JUVENTAS trial and were followed for a median of 3.0 years at the point of the present analysis, totaling 509 patient-years of follow-up. Table 1 presents characteristics of the trial population, divided into patients with and without a major event (defined here as amputation or death) during follow-up. Patients who underwent amputation or died were more likely to be male, older, and to have had a history of cerebrovascular accident (CVA) or myocardial infarction. Moreover, they were more likely to have a more advanced disease stage and larger ulcer size. Consistent with the results of the trial, there was no effect of BMMNC treatment on AFS.

Blood and Bone Marrow Progenitor Cell Associations with Amputation-Free Survival

The univariate association of PB and BM subpopulations with AFS is presented in Table 2. Higher numbers of CD34+ and CD133+ PCs in PB were associated with longer AFS, both showing a Hazard Ratio (HR) of 0.56 (p=0.003 and p=0.0007, respectively) per *e* fold increase in number of PCs. Division into tertiles showed a HR of 2.02 of the lowest compared to the highest tertile for PB CD34+ PCs (Figure 1).

Table 1. Baseline

Juventas Baseline	Total Cohort (n=160)	Event (n=67)	No Event (n=93)	P-Value
Sex (M/F)	108/52 (68%)	52/15 (78%)	56/37 (58%)	0.03
Age (yrs)	67 [56-76]	71 [62-79]	62 [52-72]	0.001
BMI (kg/m^2)	26.4 (4.52)	26.6 (4.96)	26.3 (4.20)	0.74
Smoking (Current/Past/Never)	42/95/23	13/44/10	29/51/13	0.24
Systolic BP (mm Hg)	131.0 (19.5)	132.5 (20.57)	130.0 (18.6)	0.42
Diastolic BP (mm Hg)	72.9 (9.9)	72.1 (10.22)	73.5 (9.7)	0.39
Creatinine (umol/l)	90.0 [75-115]	105 [75-147]	87 [75-107]	0.03
GFR (MDRD, ml/min/1.73cm^2)	69.7 (27.6)	66.1 (32.0)	72.3 (23.8)	0.18
Cholesterol (mmol/l)	4.26 (1.14)	4.11 (1.13)	4.37 (1.13)	0.15
HDL (mmol/l)	1.20 (0.42)	1.11 [0.47)	1.26 (0.39)	0.022
Triglycerides (mmol/l)	1.66 (1.01)	1.71 (1.12)	1.62 (0.94)	0.58
DM (IDDM/NIDDM/None)	33/27/100	16/14/37	17/13/63	0.27
History of CVA	23 (14%)	19 (28%)	4 (4%)	0.000
History of MI or Angina	66 (41%)	36 (46%)	30 (32%)	0.01
History of Dialysis	5 (3.1%)	2 (3.0%)	3 (3.2%)	0.99
Anti-platelet Drugs	112 (70%)	49 (73%)	63 (68%)	0.58
Oral Anti-Coagulants	61 (38%)	26 (39%)	35 (38%)	0.99
Lipid-lowering Drugs	135 (84%)	55 (82%)	80 (86%)	0.65
ACE Inhibitor	62 (39%)	29 (43%)	33 (35%)	0.4
ß-Blocker	71 (44%)	35 (52%)	36 (39%)	0.12
Diuretics	72 (45%)	36 (54%)	36 (39%)	0.08
Rutherford class (3/4/5/6)	8/51/92/9	0/16/45/6	8/35/47/3	0.007
Fontaine class (IIB,III,IV)	8/51/101	0/16/51	8/35/50	0.004
Ulcer	101 (63%)	51 (76%)	50 (54%)	0.006
Ulcer Area (cm^2)	1.63 [1.0-4.3)	2.13 [1.0-5.0]	1.50 [0.75-2.75]	0.04
BMMNC Treatment	81 (51%)	33 (49%)	48 (52%)	0.89

Table displaying baseline characteristics of the full cohort, patients who underwent an event and patients who did not undergo an event. Numbers between parentheses indicate standard deviation (SD) and numbers between square brackets indicate interquartile range [IQR].

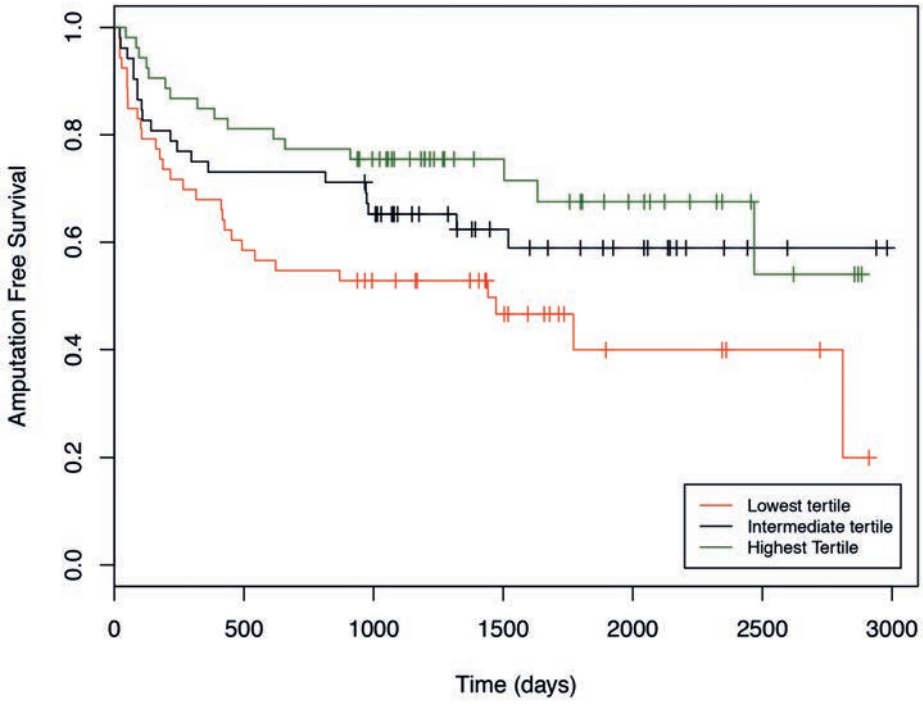

Figure 1. Kaplan-Meier curve for amputation free survial as divided by tertiles of circulating CD34+ progenitor cells in peripheral blood.

PB CD34+ and CD133+ showed modest potential as biomarkers in this study, with C-statistics on the ROC curve of 62.1% and 63.8% respectively (Supplemental Figure 3). We observed no association between CD34+/KDR+ cells and AFS (HR 0.93, p=0.55). The other cell populations assayed, including CD14+ monocytes, CD140b (PDGFRb)+ cells, CD105+ mesenchymal cells, and CXCR4+ cells were not associated with either death or limb loss. There appeared to be a trend towards an association between CXCR4+ cells and major outcomes (HR 0.77, p=0.049), but this may be due to chance as is evident by the correction for multiple testing.

BM PC populations showed a highly similar risk profile compared to PB PCs. Higher numbers of CD34+ and CD133+ were associated with longer AFS (HR of 0.58, p=0.008 and HR of 0.53, p=0.028 respectively). In analogy to PB, other BM subpopulations were not associated with AFS (Table 2).

Table 2. Cell populations and Amputation Free Survival

Cell Population	Blood				Bone Marrow			
	HR	95% CI	P-Value	Adj p-val	HR	95% CI	P-Value	Adj p-val
CD34+	0.56	0.38-0.83	0.003	0.012	0.58	0.39-0.87	0.008	0.03
CD34+/KDR+	0.93	0.74-1.17	0.55	0.99	0.85	0.63-1.16	0.3	0.99
CD133+	0.56	0.40-0.80	0.001	0.003	0.53	0.30 -0.93	0.028	0.12
CD14+	0.81	0.52-1.24	0.33	0.99	1.27	0.70-2.28	0.433	0.99
CD140b+ (PDGFRb)	0.98	0.82-1.19	0.89	0.99	0.87	0.6-1.27	0.48	0.99
CD105+	N/A	N/A	N/A	N/A	0.91	0.66-1.26	0.58	0.99
CXCR4+	0.77	0.60-0.99	0.049	0.21	0.78	0.54-1.12	0.18	0.76
CFU-GM	N/A	N/A	N/A	N/A	0.55	0.36-0.87	0.01	0.04
BFU-E	N/A	N/A	N/A	N/A	0.62	0.37-1.04	0.07	0.27

Table showing Hazard Ratios (HR) for amputation or death associated with various (progenitor) cell populations in PB and BM as measured by flow cytometry. For each cell population, HR and 95% confidence interval is given, as well as associated p-value and a p-value adjusted for multiple testing.

Correlations between Blood and Bone Marrow PC Numbers

The numbers of PCs identified by the two markers CD34 and CD133 in PB and BM were highly correlated (r=0.72 in PB and r=0.80 in BM, Supplemental Figure 2). On the other hand, correlations of the numbers of cells displaying either marker between PB and BM were intermediate (r= 0.40 and 0.31 for CD34+ and CD133+ cells respectively), with absolute numbers of PCs being approximately 20-fold higher in BM aspirates. CD34+/KDR+ double-positive cells correlated poorly with other PCs and there was no correlation between PB and BM. Coefficients of variance (CV) were also higher for CD34+/KDR+ cells (21%) compared to other PC cell types (~10%), indicating an increased amount of random fluctuation in the assay.

Relative Contribution of Blood and Bone Marrow Progenitor Cell numbers to Amptutation-Free Survival

To attain insight in the relative contribution of BM exhaustion and a mobilization defect towards risk of amputation and death, we examined the variation around the identity line for PB versus BM PCs. In analogy to a study by Fadini et al(26) , we divided patients into four quadrants, on the basis of high- and low numbers in PB versus BM (Figure 2A and B). Patients who showed low numbers of PCs in both PB and BM, were defined as patients with an exhausted BM reserve and termed 'Exhausted'. Patients with high numbers of PCs in BM, but not in PB were considered to possess a relatively isolated mobilization defect and were termed 'Poor Mobilizer'. Patients who have low numbers of PCs in BM, but relatively high numbers of PCs in PB were thought to display active signs of compensation and were designated "Compensators". Patients with high number

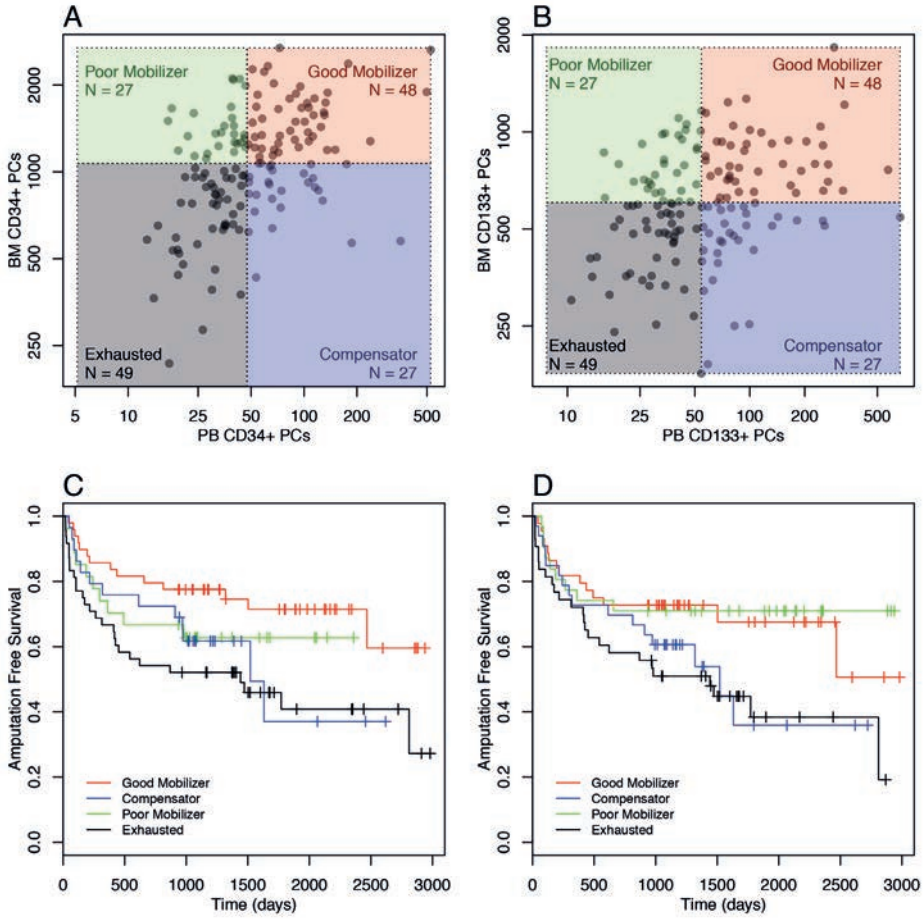

Figure 2. Relative Contribution of PB and BM PCs to Cardiovascular Risk. Patients were divided into quadrants based on whether PB or BM values were higher or lower as compared to the median for either cell population. Division into quadrants is shown for CD34+ cells in Panel A and for CD133+ cells in Panel B. The quadrant of patient with both BM and PB PCs numbers below the median was designated "Exhausted"; the quadrant with PB PC numbers below the median, but BM PC numbers above the median was designated "Poor Mobilizers", the quadrant with PB PC numbers above the median was designated "Compensators" and the quadrant with both high PB and BM PC numbers was called "Good Mobilizers". Panels C and D show the Kaplan Meier Survival curves for the four quandrants of CD34+ PCs and CD133+ PCs respectively.

of PCs in both PB and BM were termed "Good Mobilizers". Kaplan Meier survival curves for the four quadrants are shown in Figure 2C and D. For both CD34+ and CD133+ PCs the "Exhausted" group displayed the worst prognosis (HR 2.5, p=0.005 and HR 2.1, p=0.03 for CD34+ and CD133+ PCs, compared to "Good Mobilizers"). The "Poor Mobilizers" showed a lower risk for AFS than the "Compensators" (HR 1.6 vs 1.9 for CD34+PCs and HR 0.84 vs 1.7 for CD133+ PCs).

In a comparison of the JUVENTAS cohort to a small cohort of 17 healthy BM donors, we observed that healthy controls displayed and 1.88 fold increase in PB CD34+ PCs (p=0.003) but an 2.25 fold increase in BM CD34+ PCs (p=0.00002). In 6/17 cases the number of BM CD34+ PCs exceeded the range of observed values in SLI patients (Supplementary Figure 4). Numbers of PB CD133+ PCs were increased 1.13 fold in healthy controls (p=0.6), but BM CD133+ PCs were increased 1.7 fold (p=0.003).

Association of Progenitor Cell Numbers with Traditional Risk Factors

Associations between the number of PB and BM PCs and traditional cardiovascular risk factors are presented in Table 3. Most PC populations were slightly lower in males, except for CD133+ cells in PB, which were nearly 9% lower. PC numbers in both blood and BM were negatively correlated with age, although the effect was greater in blood (r=-0.24 and -0.19 for CD34+ and CD133+ PCs compared to r=-0.14). There was a significant association of PC numbers with Glomerular Filtration Rate (GFR), which was of similar magnitude (r~0.2) in both blood and BM. Total cholesterol and triglyceride levels were both positively correlated with PB PC numbers. There was an inverse association between more advanced stages of SLI (Fontaine IV vs IIb and III) and PC numbers.

Adjustment for Traditional Risk Factors

Adjusted models, correcting for Sex, Age, GFR, history of CVA, history of MI or Angina, presence of ulcers, triglyceride levels and cholesterol levels show higher HRs for PB PC numbers than unadjusted models (CD34+: HR=0.71, p=0.07, CD133+: HR=0.64, p=0.012). In BM the adjusted HR was 0.49 (p=0.03) for CD34+ PCs, and 0.65 (p=0.16) for CD133+ PCs. Prior history of CVA was most strongly associated with amputation or death, followed by disease stage (presence of ischemic ulcers). In all models both PB and BM PCs proved to be independent predictors of major outcomes. For further details regarding adjusted and reduced models (Supplemental Table 1).

DISCUSSION

The present study shows that CD34+ and CD133+ PCs in both PB and BM were independently associated with AFS in patients with SLI. The respective BM and PB PC populations displayed similar risk profiles and were moderately interrelated. Subdivision of patients based on their relative PB and BM PC values, separating patients with BM exhaustion from those with a mobilization defect, showed that BM PC numbers more strongly determined outcome. This suggests that the impairment in endogenous vascular repair mechanisms in SLI is partly due to a lower availability of PCs in the BM, rather than solely due to a defective mobilization to the PB.

Table 3. PCs and Risk Factors

Risk Factor	PB CD34		PB CD133		BM CD34		BM CD133	
	Effect	P-value	Effect	P-value	Effect	P-value	Effect	P-value
Sex (Male)	-2.2%	0.47	-8.8%	0.005	-1.9%	0.13	-1.2%	0.24
Age (yrs)	ρ -0.24	0.002	ρ -0.19	0.015	ρ -0.14	0.07	ρ -0.14	0.08
BMI (kg/m^2)	ρ 0.05	0.51	ρ -0.01	0.89	ρ 0.04	0.65	ρ -0.06	0.45
Smoking (Ever)	5.9%	0.17	1.3%	0.77	1.3%	0.46	1.2%	0.41
Systolic BP (mm Hg)	ρ -0.01	0.90	ρ -0.01	0.88	ρ 0.06	0.46	ρ 0.03	0.72
Diastolic BP (mm Hg)	ρ 0.10	0.20	ρ 0.07	0.41	ρ 0.05	0.53	ρ 0.05	0.54
GFR (MDRD, ml/min/1.73cm^2)	ρ 0.23	0.003	ρ 0.18	0.022	ρ 0.23	0.004	ρ 0.2	0.012
Cholesterol (mmol/l)	ρ 0.22	0.005	ρ 0.16	0.046	ρ 0.17	0.03	ρ 0.14	0.08
HDL (mmol/l)	ρ 0.10	0.23	ρ 0.08	0.29	ρ 0.14	0.08	ρ 0.12	0.14
Triglycerides (mmol/l)	ρ 0.20	0.011	ρ 0.17	0.033	ρ 0.03	0.67	ρ 0.01	0.92
Diabetes Mellitus	-2.3%	0.42	-4.3%	0.18	-2.0%	0.11	-1.3%	0.23
History of CVA	-1.4%	0.72	-2.0%	0.65	-0.3%	0.85	0.2%	0.86
History of MI or Angina	-4.3%	0.14	-4.3%	0.17	-3.1%	0.009	-2.6%	0.013
History of Dialysis	-20.0%	0.04	-17%	0.10	-10%	0.006	-7.6%	0.01
Anti-Platelet Drugs	1.1%	0.73	0.1%	0.98	-0.3%	0.80	-0.6%	0.62
Oral Anti-Coagulants	-2.3%	0.44	1.7%	0.61	-0.8%	0.51	0.0%	0.97
Lipid-lowering Drugs	-1.3%	0.73	-2.3%	0.59	-0.7%	0.68	-0.2%	0.92
ACE Inhibitor	0.9%	0.77	-1.0%	0.75	2.1%	0.09	-1.4%	0.21
ß-Blocker	0.8%	0.78	1.6%	0.63	-0.2%	0.89	-0.3%	0.81
Diuretics	-3.1%	0.27	-6.7%	0.03	-2.0%	0.09	-1.2%	0.26
Fontaine IV (Ulcer or Necrosis)	-10.0%	0.000	-8.0%	0.009	-2.1%	0.09	-3.0%	0.004
Ulcer Area (cm^2)	ρ -0.15	0.13	ρ -0.17	0.09	ρ -0.15	0.14	ρ -0.09	0.40

Association of cardiovascular risk factors and numbers of progenitor cells in PB and BM. For each cell population, and estimate of effect size is given: % increase or decline for binary variables and Spearman's ρ for continuous variables.

There are few prospective studies(8-12) that have examined the link between PB PCs and cardiovascular outcomes. To our knowledge this is the first study that investigates the relationship between BM PCs and cardiovascular outcomes. Schmidt-Lucke et al.(9) and Werner et al.(10) were the first to show that CD34+/KDR+ PB EPCs were associated with cardiovascular outcomes in patients with coronary artery disease. More recent studies also showed that CD34+ PB PCs were associated with myocardial infarction and death in patients undergoing coronary angiography(8) and patients with Type II Diabetes(11-13). HRs for major outcomes with regard to CD34+ PCs observed in those studies were generally of very similar magnitude as the ones observed in the present study, with roughly a halving of risk per *e*-fold increase or 1 SD increase, depending on reporting of results.

In the present study, we did not observe an association between CD34+/KDR+ cells and AFS. This is in agreement with the study of Patel et al.(8), who also did not find an association of CD34+/KDR+ cells with cardiovascular events in two large cohorts comprising a total of 905 patients. The difference with the earlier conducted studies might lie in the anti-KDR antibody used, as currently available monoclonal anti-KDR antibodies show poor reproducibility in staining(27).

Data on the relationship between the BM niche and risk of future cardiovascular events is sparse. In a recent study Schutt et al.(21) found no association between the percentage of CD34+ or CD133+ BM PCs and infarct size after six months in a cohort of patients with acute myocardial infarction. However, the same group showed that in patients with chronic ischemic heart disease(22) (IHD), which may have more similarity to our chronic ischemic SLI patients, very high numbers of CD34+ PCs in BM were associated with favorable left ventricular ejection fraction (LVEF).

Apart from PB and BM CD34+ and CD133+ PCs, other main cell populations were not associated with AFS in our study. Notably, we did not find an association between CD14+ cells and AFS, even though previous studies have shown that various monocyte subsets are associated with cardiovascular events (28-30). However, we did not differentiate between classical and non-classical or intermediate monocytes, which may explain the negative finding(29). In addition, we did not observe associations with AFS and numbers of BM cells positive for CD105+, a marker for Mesenchymal Stromal Cells (MSCs)(31). MSCs constitute the perivascular part of the hematopoietic niche and are not mobilized into the circulation(32); congruently we did not observe CD105+ cells in PB. The lack of association between CD105+ cell numbers in BM and AFS or disease severity is in agreement with a previous study with culture expanded MSCs in this cohort(33), in which we found SLI MSCs did not differ from controls.

One of the most interesting aspects of the present study is that both BM and PB PC numbers were quantified, which may provide some insight in the underlying mechanism behind the reduction of PC numbers. It is classically thought that lower numbers of PB PCs mostly reflect a defect in mobilization from the BM, due to desensitization to or reduced availability of signaling molecules such as nitric oxide(34). Indeed, patients suffering from cardiovascular disease, particularly diabetes, are resistant to exogenous PC mobilizing factors such as G-CSF(20) (35,36). On the other hand we have previously observed a reduction in BM PC numbers in a subset of the JUVENTAS population as compared to healthy controls(7). In the present study we show that there is a moderate correlation between PB and BM numbers ($R \sim 0.4$ for CD34+ cells), which is in agreement with previous studies (Fadini et al. $R \sim 0.43$(26) and Cogle et al. $R \sim 0.34$(22)). This correlation implies that there are common factors that affect both BM and PB PC numbers: in this study we observed that disease history, age and renal function were associated with PC numbers regardless of compartment. As the correlation mentioned

above only explains a small proportion of the variance, we have used the reasoning as proposed by Fadini et al.(26), and subdivided patients based on relative PB-to-BM numbers. Here we show that patients who appear to have an isolated mobilization defect, i.e. high numbers of BM PCs but low numbers of PB PCs ("Poor Mobilizers") have a fairly benign risk profile, at least as good as patients who have high PB PC numbers but low BM PC numbers ("Compensators"), and nearly as good as the "Good Mobilizers". Furthermore we have observed that CD34+ and CD133+ are relatively more increased in BM of healthy donors compared to controls than the respective PB PC subpopulations. In the two competing visions for the etiology of the reduction in PB PC numbers observed in cardiovascular disease, mobilization defect versus BM exhaustion, it seems that the latter has an important if not dominant role.

The partial exhaustion of the PC pool in BM may have bearing on the rationale of BM MNC therapy in cardiovascular disease, and SLI in particular. Autologous cell-therapy using BMMNCs or BM PCs is increasingly proposed as a treatment for SLI patients(37), from the reasoning that a mobilization defect can be circumvented by BM aspiration. If the BM PC population is also reduced, however, this limits some of the efficacy of autologous therapy, and may contribute to the finding that BMMNC isolates from patients with cardiovascular disease have reduced pro-angiogenic efficacy (38). MSCs, which can be administered allogeneically or autologously, would at the present state of development be a possible alternative(39).

It is at present unclear whether the findings of the present study are generalizable to other populations with cardiovascular disease. Notably, we did not observe a significant effect of Diabetes Mellitus (DM) on PB PC numbers, despite strong evidence that it is particularly associated with a mobilization defect(36).

In summary, we have demonstrated that lower numbers of BM PCs are associated with a worse prognosis in SLI patients. Moreover, BM PC numbers correlated with PB PCs and show a similar risk profile with regard to cardiovascular outcomes. Our findings imply that an exhaustion of the BM niche, rather than a defect in PC mobilization underlies the association between PB PC numbers and cardiovascular risk.

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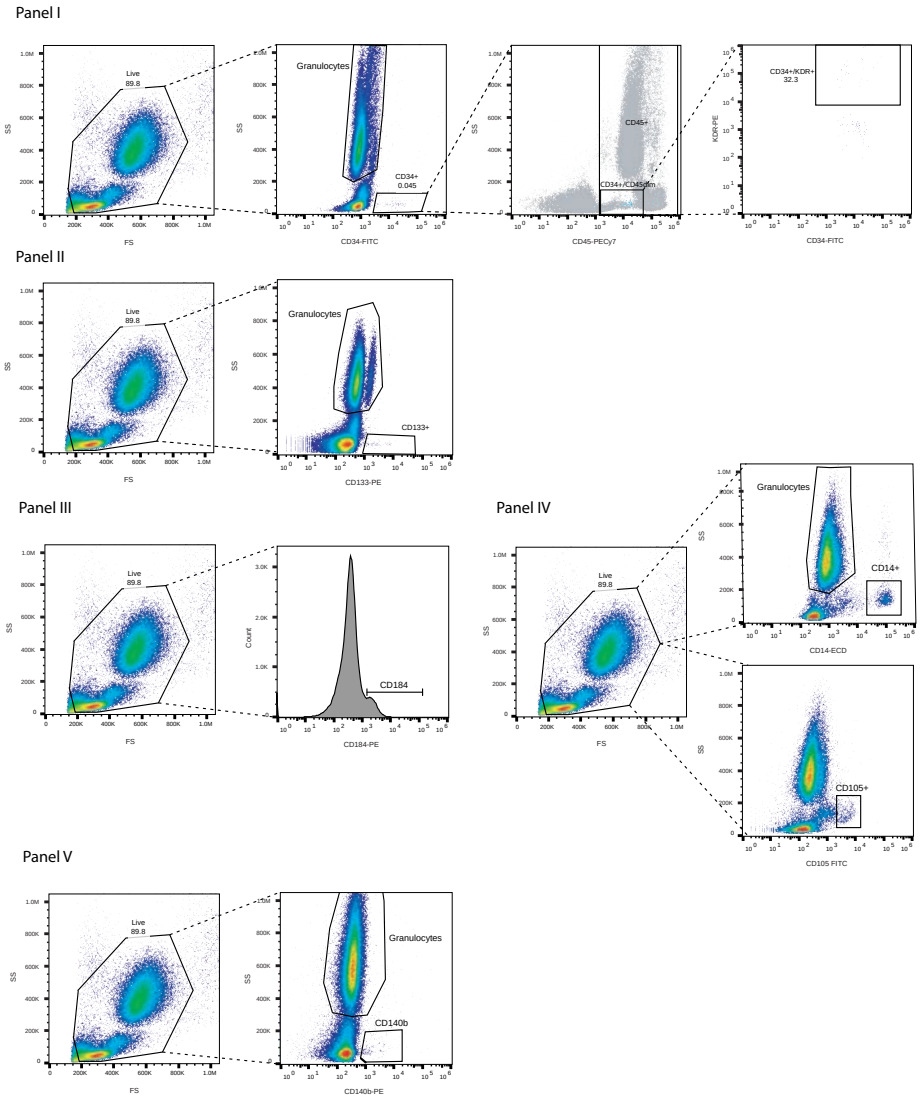
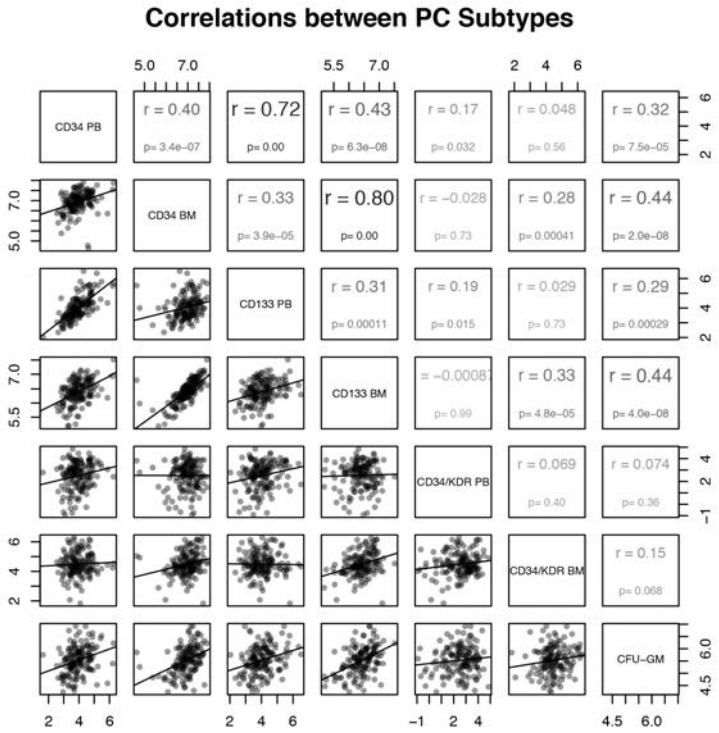
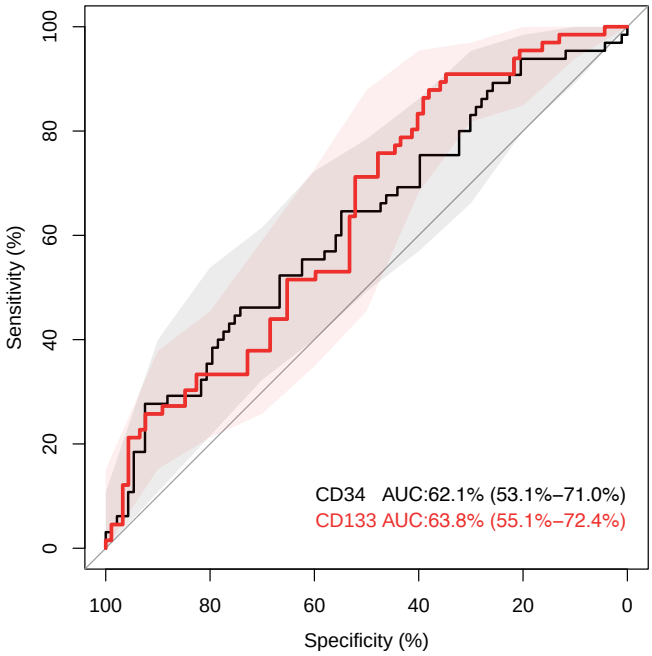
SUPPLEMENTAL DATA



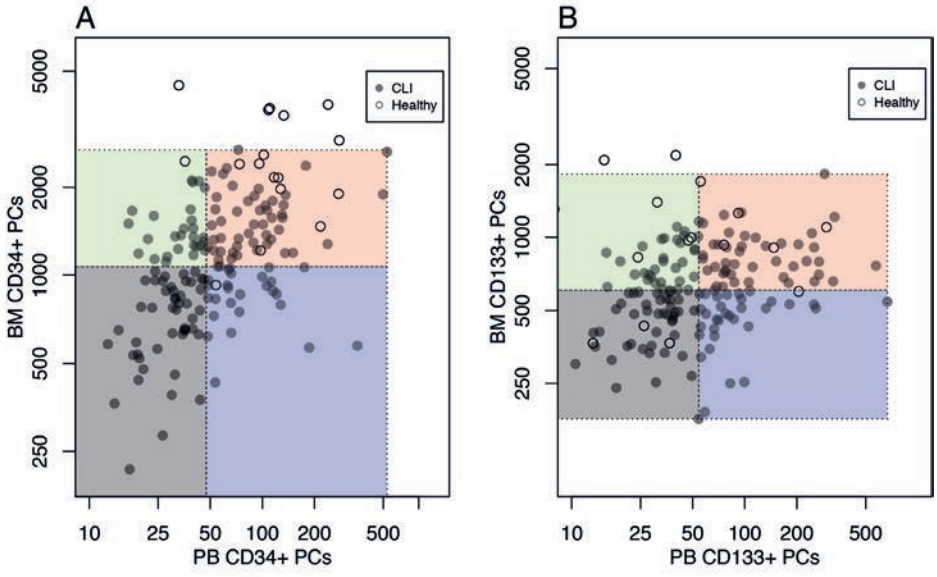
Supplemental figure S1. **Gating Strategy.** Figure showing the gating strategies for the 6 flow cytometry panels as described in the *Methods* section. Data analysis was performed by first creating "Live"-gate around the three populations of lymphocytes, monocytes and granulocytes on the Forward/Sideward-Scatter plot (see suppl fig 1). For *panel I*: **CD34+** were first gated and the population was further refined by selecting the **CD45dim** subpopulation. Within the latter population the **KDR+** cells were subsequently enumerated (see suppl fig 1). In *panel II*, **CD133+** cells were selected by plotting side-scatter versus PE and gating CD133+ cells in the lymphocytic region. In *panel III* the the **CXCR4/CD184+** cells were gated on the histogram plot. In *panel IV* **CD14+** and **CD105+** cells were gated as shown, no double positive cells were observed. In *panel V* **CD140b/ PDGFRb+** Cells were gated in the lymphocytic region.



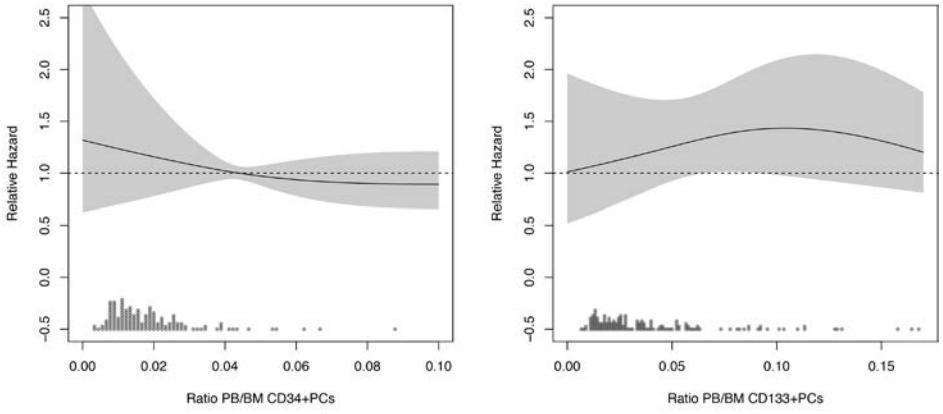
Supplemental figure S2. **Correlationmatrix.** Correlations between PC populations in peripheral blood and bone marrow. In the left/lower half correlation plots are given, in the right/upper half the Spearman's rank correlation coefficent for an pairwise comparison and the associated p-value are given.



Supplemental figure S3. **ROC Curve.** Receiver Operated Curve (ROC) displaying the sensitivity and specificity of various cutoffs in PC numbers in discrimnating patients who will undergo an event and patients who will not experience a major event. Areas Under Curve (AUCs) + 95% CI for CD34+ (in black) and CD133+ (in red) cells in peripheral blood are given in the lower right corner. Shaded areas indicate 95% confidence intervals of the curves, as derived by bootstrapping (2000 iterations).



Supplemental figure S4. **Relative BM and PB PC numbers in CLI patients compared to Healthy Controls.** Figure shows BM PC number on y-axis and PB PC numbers on x-axis for 34+ PCs in panel **A** and CD133+ PCs in panel **B**. Closed circles denote CLI patients and open circles indicate healthy controls. The colored quandrants are based on the median values of the CLI patients for the respective cell populations as in main Figure 2. Healthy controls show relatively higher BM PC values (p = 0.00002 for CD34+ and p = 0.003 for CD133+), than PB PC numbers (p = 0.003 for CD34+ and p = 0.61 for CD133+). In addition, healthy control BM PC numbers exceed the range of CLI numbers in 6/17 cased for CD34+ PCs (p = 0.018) and 2/17 cases (ns) for CD133+ PCs; PB PC numbers showed a complete overlap in range.



Supplemental figure S5. **Ratio of PB/BM and Hazard Ratio:** A restricted cubic spline was fit in cox regression analysis using the ratio between PB and BM progenitor cells as regressor and AFS as outcome. Relative hazard of undergoing a major event was unaffected by relative number of progenitor cells.

Supplemental Table 1. Adjusted Models. Cox proportional hazards models corrected for Age, Sex, GFR, history of CVA, history of MI, ulcers, triglycerides and cholesterol are presented in the upper half of the table. In the lower half the results of automated backward exclusion of model factors based on AIC are presented. In each case the optimal model is given with associated AIC value, below the penalty of further exclusion of model factors are presented.

Full Model								
Risk Factor	**PB CD34+ PCs**		**PB CD133+ PCs**		**BM CD34+ PCs**		**BM CD133+ PCs**	
	HR	**P-value**	**HR**	**P-value**	**HR**	**P-value**	**HR**	**P-value**
PCs	0.71	0.07	0.62	0.012	0.49	0.03	0.65	0.16
Age	1.02	0.12	1.02	0.15	1.02	0.07	1.02	0.06
Sex (Male)	1.37	0.34	1.12	0.74	1.34	0.38	1.31	0.40
GFR	1.00	0.66	1.00	0.51	1.00	0.37	1.00	0.61
History of CVA	2.28	0.008	2.47	0.004	2.76	0.002	2.34	0.007
Histor of MI	1.41	0.22	1.48	0.16	1.33	0.31	1.35	0.28
Ulcers	1.92	0.04	1.99	0.03	1.95	0.038	1.98	0.032
Triglycerides	1.05	0.73	1.06	0.66	1.03	0.82	1.05	0.75
Cholesterol	1.01	0.92	1.01	0.92	1.05	0.72	1.01	0.94
Likelihood Ratio test		0,000		0,000		0,000		0,000

Most efficient models by backward factor Reduction

PB CD34+ PCs	**Model factors: CD34+ PCs, Age, History of CVA, History of MI, Ulcers**		**PB CD133+PCs**	**Model factors: CD133+ PCs, History of CVA, History of MI, Ulcers**	
Optimal Model Reduction	AIC: 603.8		Optimal model Reduction	AIC 599.8	
Model - CD34+PCs	AIC: 605.1	p = 0.07	Model - CD133+ PCs	AIC: 606.7	p = 0.003
Model - Age	AIC: 604	p = 0.14	Model - CVA	AIC: 608.9	p = 0.0009
Model - CVA	AIC: 609.5	p = 0.006	Model - MI	AIC: 601.1	p = 0.06
Model - MI	AIC: 604.6	p = 0.09	Model - Ulcers	AIC: 603.9	p = 0.014
Model - Ulcers	AIC: 607	p = 0.02			

BM CD34+ PCs	**Model Factors: CD34+ BMPCs, Age, History of CVA, Ulcers**		**BM CD133+ PCs**	**Model factors: CD133+ BMPCs, Age, History of CVA, Ulcers**	
Optimal Model Reduction	AIC: 602.8		Optimal Model Reduction	AIC: 605.1	
Model - CD34+ PCs	AIC: 606.0	p = 0.023	Model - CD133+ PCs	AIC: 606.0	p = 0.09
Model - Age	AIC: 604.7	p = 0.048	Model - Age	AIC: 607.2	p = 0.04
Model - CVA	AIC: 611.7	p = 0.001	Model - CVA	AIC: 612.1	p = 0.003
Model - Ulcers	AIC: 605.2	p = 0.036	Model - Ulcers	AIC: 608.0	p = 0.03

Hendrik Gremmels M.D.[1], Martin Teraa M.D., Ph.D[2,3], Joost O. Fledderus Ph.D[1], Frans L. Moll M.D. Ph.D[2,3], Marianne C. Verhaar M.D., PhD[1]

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, the Netherlands
[2] Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands
[3] Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands

CHAPTER 3

THE INFLAMMATORY BIOMARKERS IL-6 AND IP-10 PREDICT AMPUTATION AND MORTALITY IN PATIENTS WITH SEVERE LIMB ISCHEMIA

Manuscript in Preparation

Background
Patients with Severe Limb Ischemia (SLI) have a high risk of amputations and mortality. Inflammation contributes to atherosclerotic disease progression. In this study we investigate whether levels of inflammatory plasma cytokines are associated with major outcomes in SLI.

Methods
Data were collected from a randomized controlled trial cohort investigating cell therapy for SLI (the JUVENTAS study) from 2006 to 2012. The primary outcome was major events, defined as amputation or death, at 1 year. A panel of cytokines consisting of GROα, HGF, LIF, SCF, SCGFb, SDF1α, TRAIL, IL-6, IL-8, bFGF, GCSF, GMCSF, IP10, MCP1, PDGFbb, RANTES, TNFα, VEGF, sICAM, sVCAM, TM, and E-selectin was measured in baseline serum samples of a subset of patients.

Results
108 patients were followed for a median of 3.6 years, during this period 53 events were recorded; 34 patients had experienced an event at 1 year after inclusion. Four cytokines: GROa, IL-6, IL-8 and IP-10 were more abundant in patients who experienced an event compared to patients who remained event-free. In multivariate analysis a clinical prediction model consisting of IL-6, IP-10, Ankle-Brachial Index and Hemoglobin was found to best predict outcomes, with a C-statistic of 86.6%. In survival analysis, patients in the highest tertile had a Hazard Rate of 13.3 (95% CI 5.4 – 32.7, $p = 1.65 \times 10^{-8}$) for major events compared to the lowest tertile of model scores.

Conclusion
We identified two inflammatory biomarkers, IL-6 and IP-10 that predict major events, defined as major amputation or death, in a cohort of patients with SLI. In longitudinal follow-up levels of IL-6 and IP-10 were strongly related to disease-outcome, consistent with a role for inflammation in disease progression.

INTRODUCTION

Peripheral Artery Disease (PAD) is one of the most prevalent manifestations of atherosclerosis, affecting over 27 million individuals in Europe and North America(1). The most severe manifestation of PAD is termed Severe Limb Ischemia (SLI), which occurs when atherosclerotic lesions impede blood supply below the metabolic demands of the tissue even in rest. Patients with SLI present with symptoms of chronic rest pain and/or gangrene or ulcerations of the lower limb. SLI is associated with an unfavorable

prognosis, with up to 20-40% of patients requiring a major amputation within one year of diagnosis(2). The disease imposes a high socio-economic burden, particularly after amputation(3).

It is thought that atherosclerotic disease progression in PAD is partially incited by an inflammatory response in the vascular wall(4,5). Potential inflammatory triggers include traditional risk factors for cardiovascular disease such as smoking and diabetes mellitus(6,7). Some patients with PAD display a combination of non-traditional risk factors that are characterized by a persistent systemic inflammatory response. Such risk factors include a history of infectious disease(8), autoimmune disease(9,10), or a genetic risk(11) with polymorphisms in genes associated with inflammation(12).

Circulating markers of systemic inflammation have been shown to be associated with future cardiovascular events and can serve as predictors for myocardial infarction, stroke and PAD(13). Several plasma biomarkers have been linked with incident PAD in the general population, including CRP(14), ICAM-1(15) and IL-6(16). Little is known about factors associated with progression of established PAD, although levels of pro-inflammatory molecules seem to be associated with functional decline in terms of walking distance(17). There is a great unmet need for better biomarkers and models for predicting outcomes and risk stratification in PAD patients(18).

In this study we use baseline samples of patients included in the JUVENTAS cohort(19) to identify predictors for major amputation or death. The JUVENTAS study is a randomized placebo-controlled trial that investigated Bone-Marrow Mononuclear Cell (BM-MNC) infusions as potential treatment for no-option SLI. This study showed no treatment effects on either primary or secondary outcomes(19). For the present study we have extended follow-up until 2014, using major amputation and death as a combined primary outcome.

MATERIALS AND METHODS

The JUVENTAS Cohort and controls

The JUVENTAS study is a double-blind randomized placebo-controlled trial investigating BM-MNC therapy for no-option SLI; details of the trial design(20) and results (19) have been described elsewhere. A substudy on 108 patients from the JUVENTAS study was performed. Included patients had severe infra-popliteal PAD, defined as severe intermittent claudication (Fontaine IIB), ischemic rest pain (Fontaine III) or non-healing ischemic ulcers (Fontaine IV). Additional inclusion criteria were an ankle-brachial index (ABI) of ≤ 0.6 or unreliable measurement and ineligibility for conventional revascularization.

Patient history and clinical characteristics were recorded at baseline by means of a standardized questionnaire and physical exam. ABI and pain-free walking distance were

further assessed at baseline, 2- and 6 months follow-up. Death and major amputation, defined as amputation through- or above the ankle joint, at 1 year after inclusion were recorded as primary endpoints. Amputation-free survival (AFS) was used as combined endpoint. Follow-up was extended until December 2014. Control subjects (n=34) of similar age (median age 65 years) and gender were recruited from hospital personnel. The institutional review board of the University Medical Center Utrecht approved the study protocol, the study was conducted according to the Declaration of Helsinki, and all patients provided written informed consent prior to the study interventions.

Multiplex Analysis and Quantification of Cytokines, Chemokines and CAMs

Biochemical parameters (i.a. liver enzymes, kidney function, lipid spectrum, glucose and homocysteine level) and complete cell counts were measured using standard clinical laboratory procedures. A multiple cytokine assay (Bio-rad, Hercules, CA) was used to determine a panel of cytokines and growth factors consisting of basic Fibroblastic Growth Factor (bFGF), Granulocyte-colony stimulating factor (G-CSF), Growth regulated oncogene-alpha (GRO-α), Hepatocyte Growth Factor (HGF), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interferon gamma-Induced Protein 10 (IP-10), Monocyte Chemotactic Protein 1 (MCP-1), Platelet-Derived Growth Factor-bb (PDGF-bb), Regulated upon Activation Normal T-cell Expressed, and presumably Secreted (RANTES), Stem Cell Factor (SCF), Stem Cell Growth Factor-beta (SCGF-b), Tumor Necrosis Factor-alpha (TNF- a), Tumor Necrosis factor related Apoptosis Inducing Ligand (TRAIL), and Vascular Endothelial Growth Factor (VEGF), soluble E-selectin (sE-selectin), Thrombomodulin (TM), soluble Intercellular Cell-Adhesion Molecule 1 (sICAM) and soluble Vascular Cell Adhesion Molecule 1 (sVCAM). Assays were performed using Bio-plex protein array with manufacturer-supplied software.

Statistical Analysis

Statistical analyses were performed using the 'R' statistical programming environment (21). Continuous data are reported as mean ± standard deviation (S.D.) when normally distributed, or as median and interquartile range (I.Q.R.) when skewed. An independent samples Student's t-test was performed for normally distributed data, a Mann-Whitney-U test for non-normally distributed continuous data and a Fisher's exact test for categorical data.

Time-to-event analysis was performed by the Kaplan Meier method or Cox regression; point-wise 95% confidence intervals are given. Prediction models were made using multivariable logistic regression, using an AIC criterion for comparing different models. Discriminative potential was assessed by Receiver Operating Characteristic (ROC) curves. P-values < 0.05 were considered to be statistically significant.

RESULTS

Baseline Characteristics and follow-up

At 1 year after inclusion 28 patients had undergone a major amputation and 11 patients had died (see Table 1 for baseline characteristics). 74 patients did not reach a primary endpoint. Median Age was 66 years, the majority of the patients was male and had a high burden of concomitant cardiovascular disease. Patients who suffered amputation or death were more likely to be older and had more advanced PAD (see Table 1).

Table 1.

Juventas Training Cohort	Total Cohort (n=108)	Primary Endpoint (n=34)	No Primary Endpoint (n=74)	P-Value	Control
Sex (M/F)	74/34	24/10	50/24	0.93	21/13
Age (yrs)	66 [58-74]	71 [61-76]	65 [54-72]	**0.03**	65 [60-72]
BMI (kg/m^2)	26.7 (4.58)	25.8 (5.08)	27.13 (4.31)	0.19	23.2 (2.32)
Smoking (Current/Past/Never)	26/69/13	7/21/6	19/48/7	0.45	0/7/27
Systolic BP (mm Hg)	129.91 (19.7)	128.22 (21.5)	130.7 (18.9)	0.57	128 (23.3)
Diastolic BP (mm Hg)	72.7 (9.7)	70.8 (10.4)	73.6 (9.3)	0.17	72.5 (8.8)
Urea (mmol/l)	6.0 [4.1-8.3]	7.2 [3.8-11.2]	5.8 [4.3-7.4]	0.28	-
Creatinine (umol/l)	91 [76-112]	92 [69-137]	91 [79-112]	0.78	80 [76-90]
GFR (MDRD, ml/min/1.73cm^2)	66 [57-85]	72 [46-94]	66 [58-84]	0.82	78 [72-87]
Cholesterol (mmol/l)	4.30 (1.11)	4.16 (1.06)	4.20 (1.10)	0.53	4.91 (0.96)
HDL (mmol/l)	1.21 (0.45)	1.22 [0.54)	1.20 (0.41)	0.92	1.41 (0.53)
Triglycerides (mmol/l)	1.45 [0.9-2.0]	1.2 [0.9-1.7]	1.6 [0.9-2.0]	0.38	0.6 [0.6-0.8]
Hemoglobin (mmol/l)	8.16 (1.09)	7.67 (0.83)	8.38 (1.12)	0.0004	8.9 (0.81)
CRP (mg/ml)	5.34 [2.1-11.9]	7.94 [3.0-22.4]	4.28 [1.8-9.4]	0.02	-
History of CVA	8 (7.4%)	5 (14.7%)	3 (4.0%)	0.11	0 (0%)
History of MI or Angina	42 (38.9%)	17 (50%)	25 (29.4%)	0.14	0 (0%)
History of Major Amputation	9 (8.3%)	5 (14.7%)	4 (4.7%)	0.24	0 (0%)
History of Dialysis	3 (2.8%)	0 (0.0%)	3 (4.1%)	0.55	0 (0%)
Diabetes (No, NIDDM, IDDM)	66/20/22	19/8/7	47/12/15	0.62	34/0/0
ABI	0.53 (0.31)	0.42 (0.37)	0.58 (0.27)	**0.019**	-
Rutherford class (3/4/5/6)	7/36/61/4	0/8/23/3	7/28/38/1	**0.024**	0
Fontaine class (IIB,III,IV)	7/36/65	0/8/26	7/28/39	**0.026**	0
Ulcer	65 (60.0%)	26 (76%)	39 (53%)	**0.021**	0 (0%)
Ulcer Area (cm2)	1.73 [1.0-4.3]	2.25 [0.88-5.0]	1.50 [1.0-3.3]	0.57	0
BMMNC Treatment (No/Yes)	53/55	16/18	37/37	0.99	0 (0%)
Anti-Platelet Drugs	74 (69%)	24 (71%)	50 (68%)	0.32	0 (0%)
ACEi or ARB	62 (57%)	22 (65%)	40 (54%)	0.41	0 (0%)
Diuretic	53 (49%)	19 (56%)	34 (46%)	0.45	0 (0%)
Beta blocker	48 (44%)	17 (50%)	31 (42%)	0.56	0 (0%)

Differences in Biomarker Abundance

Levels of GROα, HGF, SCF, IL-6, IL-8, G-CSF, IP-10, VEGF, sICAM-1, sVCAM-1 and TM were higher in SLI patients compared to controls (see Supplementary Figure 1). Of these, GROα, IL-6, IL-8 and IP-10 were higher in patients who experienced an event, compared to patients who remained event-free (Figure 1).

Combined Models

We created logistic regression models to predict the occurrence of major events within 1 year. Step-wise in- and exclusion of model factors led to the exclusion of IL-8, as it was highly correlated to IL-6 (Supplementary Figure 2). Adjusted odds ratios (OR) were 2.12 (95% CI 1.22 – 4.32, p=0.005) for IL-6, 1.72 (95% CI 1.05 – 3.66, p=0.030) for GROα and

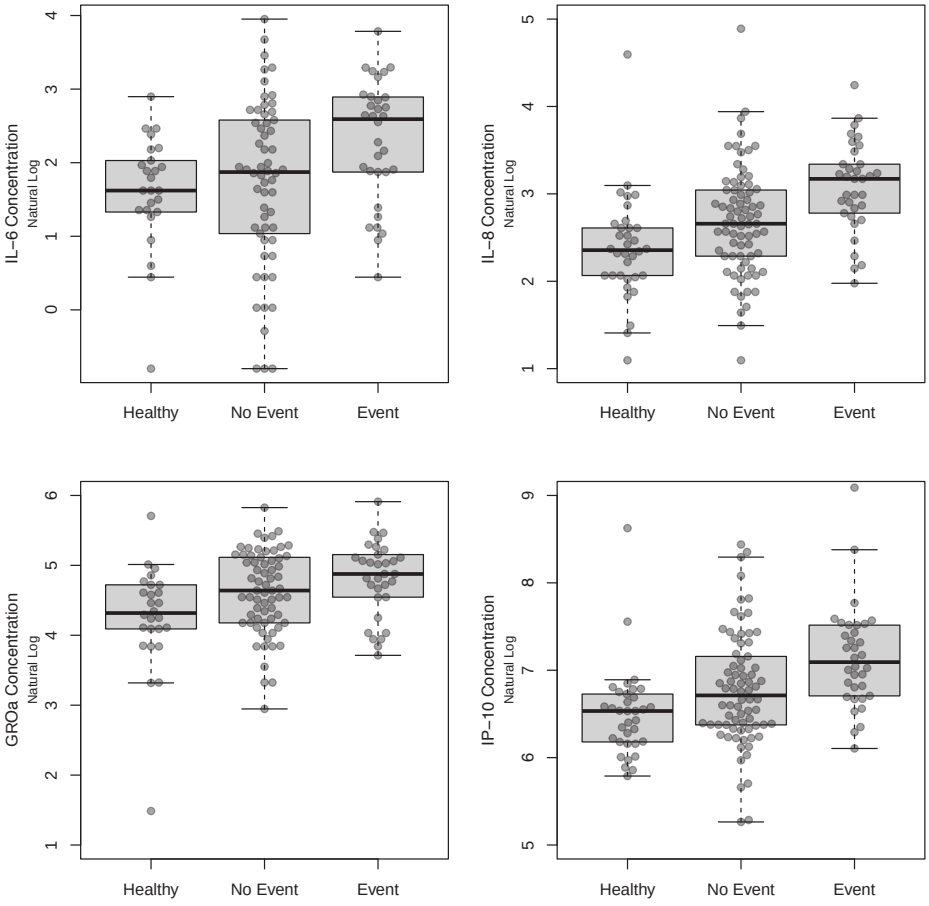


Figure 1. Cytokine Measurements. Boxplots showing levels of biomarkers that discriminated between patients with- and without major outcome. Healthy Controls are shown as reference.

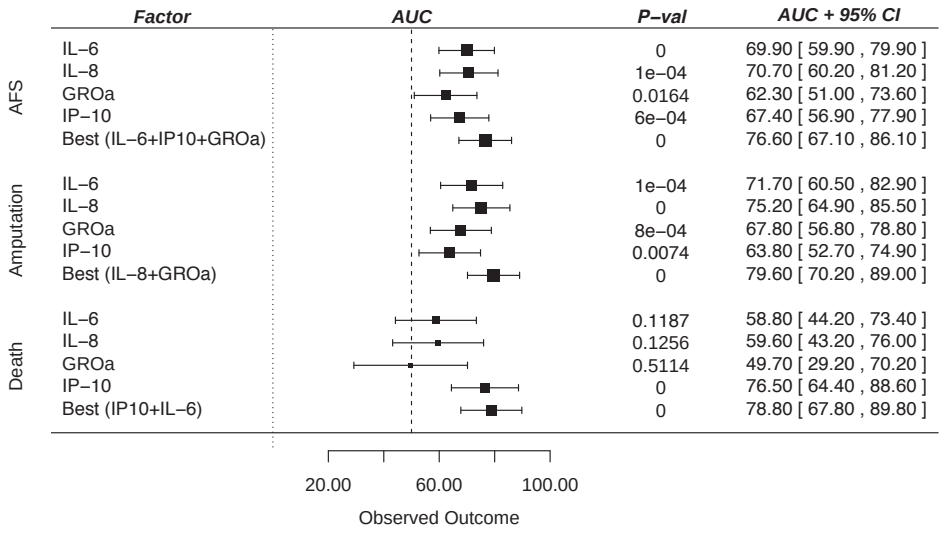


Figure 2. Biomarker Prediction Performance. Areas-under-curve of receiver operated characteristic curves based on logistic regression models based on biomarkers to predict major outcomes at 12 months follow-up.

1.83 (95% CI 1.15 – 3.09, p=0.010) for IP-10. The ROC of the combined model had an Area-Under-Curve (AUC) of 76.6%. Separately the individual biomarkers performed poorer, with AUCs of ca. 70%.

A separate analysis on amputation and mortality suggests that IL-6, IL-8 and GROa were more closely associated with amputation at 1 year (AUCs 71.7%, 75.2% and 67.8%) than death (AUCs 58.8%, 59.6% and 49.7%). IP-10 was more closely associated with mortality (AUC 76.5%) than amputation (AUC 63.8%).

Clinical Parameters and Scores

We assessed whether the biomarkers investigated in this study provide additional discriminative value in combination with clinical parameters. Blood Hemoglobin (Hb), ABI, C-reactive Protein (CRP) and the presence of ulcers were associated with a major event at 1 year (Table 1). In multivariate logistic regression analysis, IL-6 (OR 2.6, 95% CI 1.4 - 5.8, p=0.0015), IP-10 (OR 1.92, 95% CI 1.13 - 3.53, p=0.015), Hb (OR 0.34, 95% CI 0.17 – 0.60, p=0.0001) and ABI (OR 0.27, 95% CI 0.12 – 0.5, $p = 6 \times 10^{-6}$) proved independent predictors for major events at 1 year. The AUC of the ROC was 86.6% (78.5 – 94.6%; Figure 3).

Next, we compared the biomarker-based models to existing clinical models: the Prevent 3(22), Finnvasc (23) and Basil (24) in prediction of major events. The AUCs for the clinical models ranged from 62.3% for the Finnvasc, to 75.0% for the Basil model (see Figure 4).

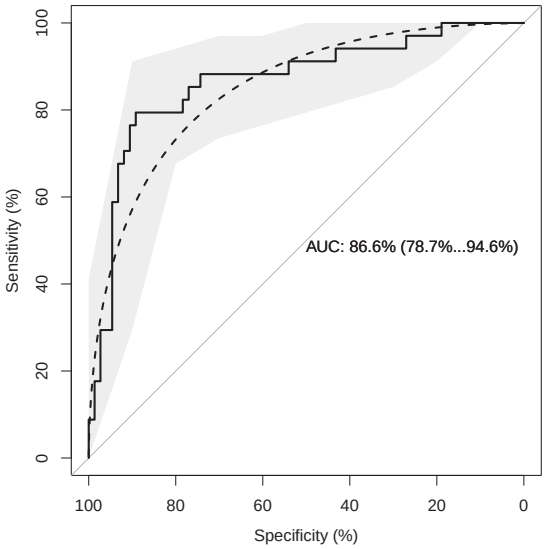


Figure3. Combined Model with Clinical Factors. Receiver operated characteristic (ROC) curve of a 4-factor model, using serum IL-6 and IP-10 levels as well as patient Hb and ABI. Shaded region indicates 95% CI based on bootstrapping (2000 iterations).

Longitudinal Follow-up

To assess the validity of the combined prediction model across different follow-up times we divided patients into tertiles based on probability from the combined model and used Kaplan-Meier analysis and Cox regression to assess longitudinal follow-up (Figure 5). Patients in the middle tertile showed a Hazard Ratio (HR) of 2.87 (95% CI 1.12 – 7.35, p=0.028) compared to the lowest tertile (p =0.028), patients in the highest tertile showed a HR of 13.3 (95% CI 5.4 – 32.7, p = 1.65 x 10^{-8}).

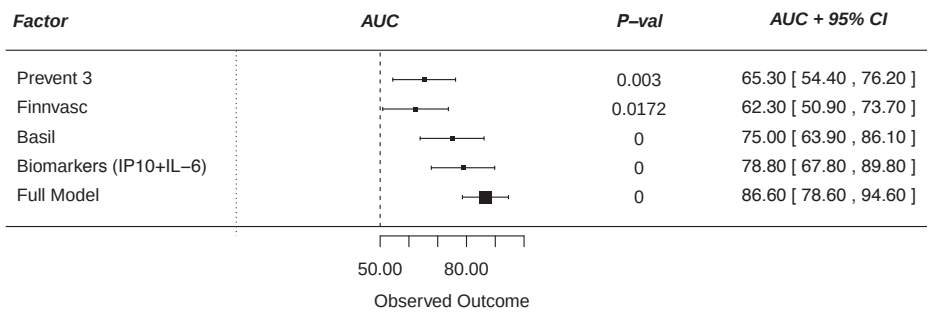


Figure 4. Biomarker Models *versus* Pre-existing clinical models. Areas-under curve of pre-existing prediction models and the model proposed in the present study.

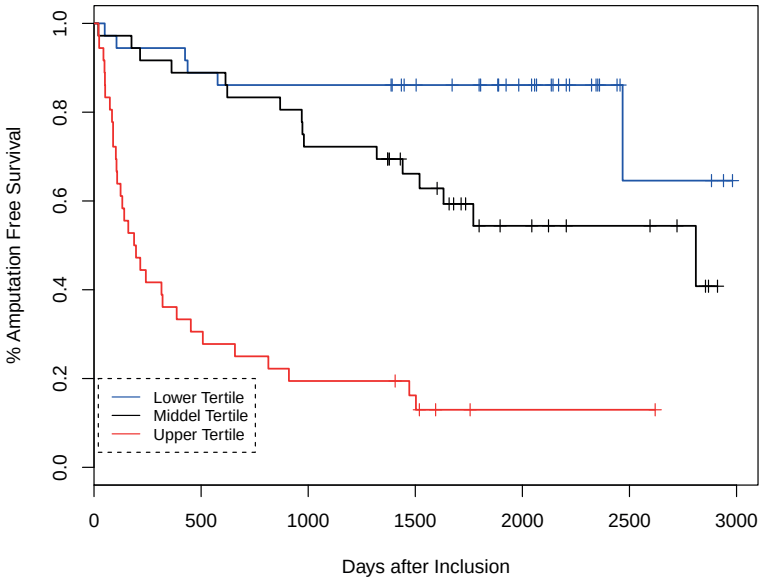


Figure 5. Longitudinal Follow-up. Patients were divided into tertiles based on model scores and the occurence of major outcomes was registered over time. Patients in the middle tertile showed a Hazard Ratio (HR) of 2.87 (95% CI 1.12 - 7.35, p = 0.028) compared to the lowest tertile, but patients in the highest tertile showed a HR of 13.3 (95% CI 5.4 - 32.7, p = 1.65 x 10^{-8})

DISCUSSION

In the present study we identified two inflammatory biomarkers, IL-6 and IP-10 that predict major events, defined as major amputation or death, in a cohort of patients with SLI. A prediction-model using these two biomarkers in combination with ABI and blood Hb, predicted major outcomes with a C-statistic of 86.6%, which is considerably better than existing prediction models for outcomes in SLI patients.

We identified IL-6 as an independent predictor of major events, in particular major amputation. IL-6 is a soluble polypeptide that acts as one of the principle humoral regulators of the inflammatory response(25). In particular IL-6 induces the acute phase response in the liver, leading to secretion of CRP, Serum Amyloid A and Fibrinogen (26). Several studies have implicated IL-6 in the development of cardiovascular disease(27), and polymorphisms in the IL-6 receptor have been associated with development of cardiovascular disease(28). IL-6 has been shown to be an independent predictor of myocardial infarction in the Physicians' Health study(29), a result which has been replicated in over 25 patient cohorts, with a total of nearly 8000 patients(30).

Literature on IL-6 in PAD has been comparatively scarce; IL-6 has been shown to be associated with decreases in ABI and the development of PAD in the Edinburgh Artery

Study(16). Furthermore, a cross-sectional study has found elevated IL-6 levels in patients with severe claudication (31). This is the first study to show that IL-6 predicts major clinical events in SLI.

Downstream targets of IL-6, in particular CRP have been previously associated with major events in SLI patients(32,33). We found a similar association in the present study, but IL-6 proved a better predictor of major outcomes. A more direct causal involvement of IL-6 in the pathogenesis of cardiovascular disease may explain the better predictive value of IL-6(34). Elevated levels of CRP have been linked to the presence of wound infection(33,35), which has recently been taken up in the Wound, Ischemia, and foot Infection (WIfI) classification as risk factor for adverse outcomes(36). We did not observe a statistical interaction between IL-6 levels and the presence of ulcers in the present study, and IL-6 predicted outcomes equally well in patients without ulcers.

The other independent prognostic biomarker identified in this study, IP-10, has not previously been reported to be associated with major cardiovascular outcomes. Pre-clinical studies have shown that IP-10 is secreted by several cell types, including monocytes, endothelial cells and fibroblasts, and can be induced by Th-17–associated cytokines(37,38). It is implicated in smooth muscle proliferation and expressed in relationship to arterial damage(39) and within atheromatous plaques(40). It is thought that IP-10 mediates an influx of perivascular CXCR3+ macrophages(41) and regulatory T-cells(42) that are involved in arterial remodeling. IP-10 is mainly involved in intimal and medial hyperplasia and IP-10 knock-out mice are protected from atherogenesis(42). Fewer studies have investigated IP-10 in human subjects. IP-10 has been shown to be elevated in patients with recurrent coronary artery disease (43,44) and associated with poor collateral development within these patients(45). Recently, a study by Ko et al.(46) identified IP-10 as a biomarker for Kawasaki disease, an inflammatory vasculitis that preferentially affects coronary arteries. These studies suggest that IP-10 plays an important role in arterial inflammation, especially of the coronary arteries and is associated with defective arteriogenesis in the heart. Interestingly, we observed a closer association of IP-10 levels with mortality compared to amputation, which may point to cardiac involvement, although we did not classify cause of death. We recommend further investigation of this biomarker in other patient groups at risk for cardiovascular events.

Assessment of disease severity in patients with SLI remains difficult as physiological parameters such ABI are moderately reproducible and patient-centered functional outcomes such as ambulation, pain and quality of life are subjective. Treatment decisions, including the decision to amputate the affected limb, would benefit from improved risk-stratification(18). The biomarkers identified in this study allowed the creation of a model that predicts major clinical events with reasonable accuracy, showing an AUC of the ROC curve of 78.8% for biomarkers alone and 86.6% for biomarkers in combination with clinical parameters. This is considerably better than the existing prediction models

Finnvasc(23) and Prevent 3(22) , which perform poorly to moderately, with AUCs of ca. 60% in our study, in accordance with previous studies(18). The more elaborate Basil model performed better in our study, with an AUC of 75%. This is a higher AUC than was reported in the original study, despite the fact that we did not have access to Bollinger angiography scores, one of the model parameters(47).

In summary, we identified several inflammatory biomarkers that predict major outcomes in SLI. Two independent biomarkers, IL-6 and IP-10, allowed the creation of a model that could predict the occurrence of major outcomes with high accuracy. Prediction models using inflammatory biomarkers could be instrumental in improving risk-stratification in SLI patients. The findings of the present study should be validated in different, ideally prospective, cohorts.

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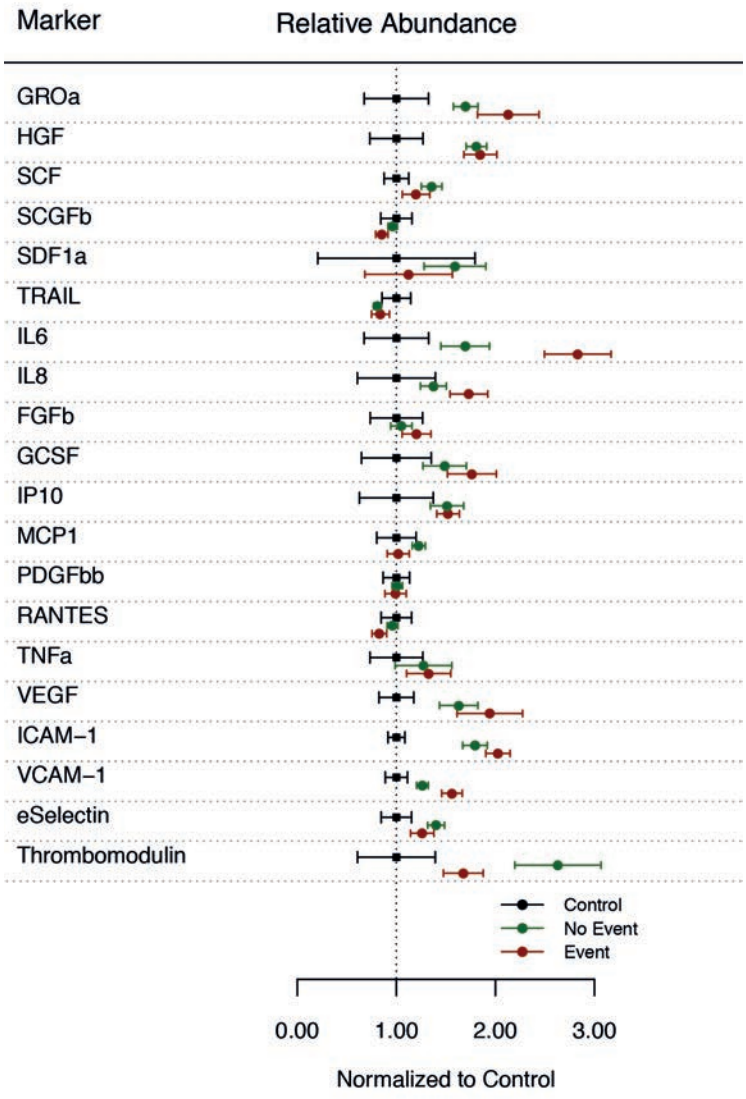
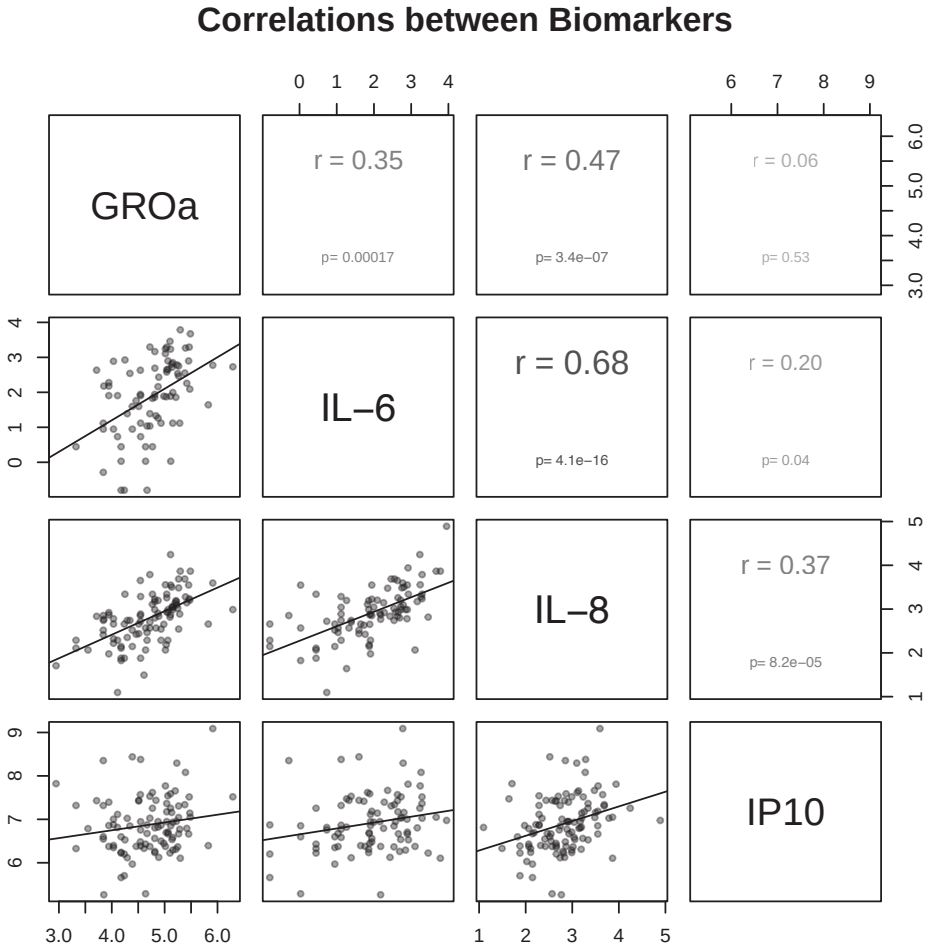
SUPPLEMENTARY INFORMATION



Supplemental Figure 1. Relative Abundance of Cytokines, Growth Factors or Adhesion Molecules. Values for controls are normalized to 1 and denoted in black. Patients who underwent an event are indicated in red and patients with event-free survival are indicated in green. Error bars indicate Standard Error of the Mean (SEM)



Supplemental Figure 2. Correlation of Measured Cytokines.

Marlon I. Spreen MD[1], Hendrik Gremmels MD[2], Martin Teraa MD, PhD[3],
Ralf W. Sprengers MD PhD[4], Marianne C. Verhaar MD PhD[2],
Randolph G. Statius van Eps MD PhD[5], Jean-Paul P.M. de Vries MD PhD[6],
Willem P.Th.M. Mali, MD PhD[4], Hans van Overhagen MD PhD[1]

On behalf of PADI and JUVENTAS Study Groups

[1] Department of Radiology, Haga Teaching Hospital, The Hague, The Netherlands
[2] Department of Nephrology & Hypertension, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands
[4] Department of Radiology, University Medical Center Utrecht, Utrecht, The Netherlands
[5] Department of Vascular Surgery, Haga Teaching Hospital, The Hague, The Netherlands
6 Department of Vascular Surgery, Sint Antonius Hospital, Nieuwegein, The Netherlands

CHAPTER 4

DIABETES MELLITUS IS ASSOCIATED WITH DECREASED LIMB SURVIVAL IN PATIENTS WITH CRITICAL LIMB ISCHEMIA

POOLED DATA FROM TWO RANDOMIZED CONTROLLED TRIALS

Submitted

ABSTRACT

Objective

Although never tested prospectively, diabetes mellitus (DM) is assumed to negatively affect the outcomes of critical limb ischemia (CLI). DM was highly prevalent in two recently conducted randomized controlled trials in CLI patients, the PADI and JUVENTAS trials. To determine the implications of DM in a true CLI population, clinical outcomes were compared in patients with and without DM.

Research Design and Methods

Individual data from patients with CLI (Rutherford category ≥ 4) from the two trials were pooled. Patients were considered to suffer from DM when treated with oral antidiabetic and/or insulin therapy at baseline. Major amputation (above ankle level) and major event (major amputation or death) rates were compared between CLI patients with and without DM. Hazard ratios (HRs) were calculated.

Results

Of a total of 281 patients, 49.1% suffered from DM. The major amputation rate at five years follow-up was higher in patients with DM than in patients without DM (34.1% versus 20.4%, p=0.015). The major event and death rate did not differ. Unadjusted HR of diabetes for the major amputation risk was 1.87 (95%CI 1.12-3.12). Model factors with significant HRs in the multivariate analysis were baseline Rutherford category (HR 1.95; 95%CI 1.24-3.06) and ankle-brachial index (ABI) >1.4 (HR 2.78; 95%CI 1.37-5.64).

Conclusions

CLI patients with DM are at substantially higher risk of major amputation when compared with CLI patients without DM. This increased risk is associated with a higher prevalence of baseline ABI>1.4 and more severe ischemia at initial presentation in patients with DM.

INTRODUCTION

Critical limb ischemia (CLI) is the most severe form of peripheral artery disease (PAD) and imposes an increasing burden on health care. The current incidence is substantial with 500-1000 new cases per 1 million inhabitants every year in Western Europe and North America.(1, 2) Moreover, with a six-month major amputation rate in non-revascularizable CLI patients ranging from 10 to 40% and a one-year mortality rate of 25%, its poor prognosis is striking.(1-3)

One of the main goals in the treatment of CLI is to prevent major amputation, as a lower leg amputation in these patients is a high-risk procedure with a 30-day mortality of ±10% and less than 30% of surviving patients ambulatory outdoors at 17 months follow-up.(4) It is important to identify those patients who are at particular high risk of major amputation, in order to treat this group more aggressively at an earlier stage, which may reduce the amputation rate.
PAD progresses more rapidly in patients with diabetes mellitus (DM).(5) The risk of developing CLI is four times higher in patients with DM than in patients without DM.(1) PAD in patients with DM is often accompanied by peripheral neuropathy with sensory dysfunction, which is thought to contribute to the development of foot ulcers and progressive tissue loss in patients with CLI.(6) Although it is often assumed that CLI in patients with diabetes has a worse prognosis, this has not been proven prospectively in populations consisting exclusively of CLI patients. In studies that focus on CLI, patients with and without DM are usually reported as one group.(7) Because CLI patients are typically elderly, vulnerable, and fragile patients who are at high risk for cardiovascular events(1), CLI study populations are often small, which limits subgroup analyses and separate reporting of results for patients with and without DM.

The PADI and JUVENTAS trials investigated different treatment strategies in patients with CLI.(8, 9) In both studies, diabetes was a common comorbidity. Aim of our study was to determine whether, based on pooled data from these aforementioned studies, the prognosis regarding major amputation and major events differs between CLI patients with and without DM.

RESEARCH DESIGN AND METHODS

PADI trial

Study design, population and procedures

Between October 2007 and February 2013, 137 patients with 144 limbs were enrolled in the PADI trial (Percutaneous transluminal Angioplasty (PTA) versus Drug eluting stents (DES) for Infrapopliteal lesions), an investigator-initiated, multi-center, randomized controlled, non-blinded, double-arm study.

Adult patients were eligible for enrolment if they suffered from CLI (defined as Rutherford category ≥ 4(10)) due to infrapopliteal lesions. Major exclusion criteria were (sub)acute limb ischemia, increased risk of bleeding, estimated glomerular filtration rate (eGFR) <20 mL/min/1.73 m^2. Treatment with DES (paclitaxel-eluting coronary stents; TAXUS Liberté; Boston Scientific, Natick, MA) was compared with PTA with optional bail-out bare metal stents (BMS). The rationale of this study, detailed in- and exclusion criteria, and study procedures have been reported previously.(11)

Follow-up and endpoints

Patient assessments were planned before intervention, at discharge, after three, six, and 12 months and annually until five years or until a major endpoint (major amputation or death) was reached. Long-term follow-up is still ongoing. Follow-up of patients who underwent a major amputation was obtained by means of assessments by phone, or using data from patient medical records.

Primary endpoint of the PADI trial was six-month primary binary patency of treated lesions, defined as ≤ 50% stenosis on Computed Tomography Angiography (CTA). Secondary endpoints were Rutherford classification(10), minor and major amputation (below and above ankle level, respectively) of the trial leg, and peri-procedural (within 30 days) complications, serious adverse events, and death. Short-term results have been published previously.(12)

The trial was registered at ClinicalTrials.gov with the identifier NCT00471289.

JUVENTAS trial

Study design, population and procedures

The JUVENTAS trial is a single-center, double-blind placebo-controlled randomized controlled trial, in which the effects of repetitive infusion of bone marrow mono-nuclear cells (BMMNCs) into the common femoral artery were investigated.(9) One hundred sixty patients (160 limbs) were included. Inclusion criteria consisted of severe infra-popliteal PAD, defined as class IIB to IV in the Fontaine Classification(1), that was not amenable for conventional revascularization. Major exclusion criteria were factors that diminished life expectancy and/or precluded follow-up.

The intervention consisted of three intra-arterial infusions of autologous BMMNCs. Placebo patients received an autologous peripheral blood infusion, designed to mimic the cell therapy product.

Follow-up and endpoints

Primary outcome was major amputation (amputation through or above the ankle joint) or death at six months. Secondary outcomes were amputation at two months and during the entire observation period, as well as changes in Fontaine/Rutherford classification, minor amputations, ulcer size, ankle-brachial index (ABI), and Quality of Life.(8) Results have been published elsewhere.(8) Follow-up was extended until maximum five years for this additional analysis, using patient medical records or by contacting patients by phone.

The trial was registered at ClinicalTrials.gov under number NCT00371371.

Patient selection

Data of the PADI and JUVENTAS trials were pooled on a patient level. We selected patients who suffered from CLI (i.e. Rutherford category 4 / Fontaine stage III, or Rutherford category 5 or 6 / Fontaine IV). Four patients were included in both trials; they were included in the analysis only once, with the longest available follow-up period. Selected PADI and JUVENTAS patients were analyzed according to the presence of DM. Patients were considered to suffer from DM when they used anti-diabetic medication at baseline, either oral antidiabetic and/or insulin therapy.

Outcomes

Baseline characteristics, presence of ulcers at baseline and after six months follow-up, major amputation and major event rates until five years post-treatment were compared between patients with and without DM. Major amputation was defined as amputation above ankle level. A major event was defined as a major amputation or death. In addition, survival rates were analyzed separately for patients with and without a major amputation.

Statistical analysis

Categorical variables were compared with the use of the 2-sided chi-square test, ordinal variables with the Mann Whitney test, and continuous variables were compared with the use of the 2-sided Student's t test. A 2-sided p-value ≤0.05 was considered to indicate statistical significance. Missing data at inclusion were <5% for any parameter. In case of missing data, data points were imputed by multiple regression.

The observed amputation and major event rates were estimated with the Kaplan-Meier method. To correct for the effects of treatment, the analysis was stratified by randomization. Patients were censored at end of follow-up.

Hazard ratios (HRs) of DM for the risk of major amputation were calculated with Cox proportional hazards regression models that were stratified by randomization, in order to correct for effects of treatment. A full model adjusted for age, smoking, history of stroke, history of coronary artery disease, previous treatment for PAD, impaired renal function (estimated glomerular filtration rate (eGFR) < 30 ml/min/1.73m^2), Rutherford category at baseline, and categorized ABI at baseline (<0.7; 0.7-1.4; >1.4 (including immeasurable ABI due to incompressible vessels)) was created for the multivariate analysis. Missing ABIs as covariate for this model were imputed. Additionally, we performed backward reduction of model factors. The best performing model was based on lowest Akaike information criterion. Potential interactions between variables were analyzed. The proportional hazards assumptions for all presented Cox models was evaluated by plotting Schoenfeld residuals.

Analyses were performed in SPSS version 22 for Mac and R version 3.1.0.

RESULTS

Patient characteristics

Of the PADI trial, 133 patients fulfilled all inclusion criteria and were selected for this pooled analysis.(12) Eighty-four patients (63.2%) of the PADI population suffered from DM. Of the JUVENTAS cohort, 152 patients were selected, of whom 57 patients (37.5%) had DM. Four patients were included in both trials, thus in total 281 subjects were selected. Table 1 shows the baseline characteristics. Overall, 138 patients (49.1%) suffered from DM. Compared to the patients without DM, significantly more patients with DM had a history of stroke or transient ischemic attack, and coronary artery disease. Significantly more patients without DM were current smokers or had smoked in the past.

Patients with DM showed a significantly higher Rutherford category at baseline. More patients without DM had an ABI<0.7, whereas a larger proportion of patients with DM showed an ABI between 0.7 and 1.4, or an ABI>1.4 (p=0.000).

Table 1. Baseline characteristics, according to diabetes state

	Patients without DM	**Patients with DM**	**P ***
	N=143 patients	N=138 patients	
Mean age in years (SD)	67.9 (15.0)	70.9 (11.3)	NS†
Male sex	94 (65.7)	99 (71.7)	NS
Smoking status			0.000
Ex-smoker	62 (43.4)	54 (39.1)	
Current smoker	48 (33.6)	24 (17.4)	
Previous stroke or TIA	14 (9.8)	33 (23.9)	0.002
Coronary disease	47 (32.9)	62 (44.9)	0.038
Impaired renal function (eGFR<30)	15 (10.5)	19 (13.8)	NS
Renal disease requiring dialysis	8 (5.6)	9 (6.5)	NS
On anticoagulation medication	136 (95.1)	129 (93.5)	NS
History of PAD	107 (74.8)	102 (73.9)	NS
Rutherford category			
mean (SD)	4.8 (0.6)	5.0 (0.6)	0.002‡
median (min-max)	5 (4-6)	5 (4-6)	
Ankle-brachial index			
Mean (SD)	0.57 (0.30)	0.70 (0.35)	0.003†
<0.7	97 (67.8)	55 (39.9)	0.000
0.7-1.4	36 (25.2)	60 (43.5)	
>1.4/ immeasurable	10 (7.0)	23 (16.7)	

Data are number (%) unless stated otherwise. * Chi square test, unless stated otherwise. † T-test. ‡ Mann Whitney test. Missing ankle-brachial indices were imputed. DM, diabetes mellitus. SD, standard deviation. NS, non significant. TIA, transient ischemic attack. eGFR, estimated glomerular filtration rate (in mL/min/1.73 m^2). PAD, peripheral arterial disease.

Supplemental Table S1 shows the baseline characteristics separately for patients with and without diabetes in the PADI and JUVENTAS cohorts.

Endpoints

Patients were followed for a median duration of 142.5 weeks, equivalent to 767 patient-years of observation. The mean follow-up time of surviving patients with DM was 184.5 weeks (standard deviation (SD) 92.8) and of surviving patients without DM 197.6 weeks (SD 112.6).

Table 2 shows the significantly higher rate of major amputations in patients with diabetes compared with patients without diabetes, with an estimated rate of 34.1% of the former undergoing a major amputation during five years follow-up versus 20.4% of the latter

(p=0.015). This is also graphically shown by the Kaplan Meier curves of the estimated cumulative incidence rates of major amputation (Figure 1A). In both groups, the majority of major amputations were performed in the first six months after randomization.
The major event rate (either major amputation or death) and death rate did not differ significantly between patients with or without DM (Table 2, Figure 1B). Figure 1C shows that survival is significantly decreased in patients following amputation (p=0.006). This poor survival after major amputation is comparable in patients with and without DM (supplemental Figure S1, p=0.63). Neither did the survival of patients who did not experience a major amputation differ between patients with and without DM (Supplemental Figure S2, p=0.99).
Both at baseline and at six months follow-up, a significantly larger percentage of patients with diabetes suffered from ulcers (p=0.043 and p=0.002, respectively) (supplemental Table S2).

Table 2. Cumulative proportion experiencing amputation/death categorized by diabetes

	Patients without DM		**Patients with DM**		**P value***
	N= 143 patients		N=138 patients		
Major Amputation	**N**	**% (95% CI)**	**N**	**% (95% CI)**	
0-6 months	16	11.4 (6.1-16.7)	26	19.7 (12.8-26.6)	
0-12 months	22	16.0 (9.9-22.1)	31	23.8 (16.5-31.1)	
0-24 months	24	17.8 (11.3-24.3)	36	28.3 (20.5-36.1)	
0-36 months	25	18.7 (12.0-25.4)	37	29.2 (21.2-37.2)	
0-48 months	26	20.4 (13.1-27.7)	38	30.8 (22.4-39.2)	
0-60 months	26	20.4 (13.1-27.7)	39	34.1 (23.9-44.3)	.015
Death	**N**	**% (95% CI)**	**N**	**% (95% CI)**	
0-6 months	11	8.0 (3.5-12.5)	17	12.8 (7.1-18.5)	
0-12 months	24	17.9 (11.4-24.4)	25	19.2 (12.3-26.1)	
0-24 months	34	25.6 (18.2-33.0)	41	32.0 (24.0-40.0)	
0-36 months	41	31.3 (23.3-39.3)	48	38.1 (29.5-46.7)	
0-48 months	45	36.1 (27.3-44.9)	52	43.0 (33.8-52.2)	
0-60 months	53	48.0 (37.6-58.4)	59	55.7 (44.7-66.7)	0.78
Amputation/Death	**N**	**% (95% CI)**	**N**	**% (95% CI)**	
0-6 months	25	17.5 (11.2-23.8)	38	27.5 (20.1-34.9)	
0-12 months	42	29.4 (22.0-36.8)	47	34.1 (26.3-41.9)	
0-24 months	51	35.7 (27.9-43.5)	62	45.0 (36.8-53.2)	
0-36 months	59	41.7 (33.5-49.9)	70	51.2 (42.8-59.6)	
0-48 months	63	46.3 (37.7-54.9)	74	55.6 (47.0-64.2)	
0-60 months	69	54.4 (44.8-64.0)	82	68.7 (58.7-78.7)	.19

*Overall log rank test, stratified by randomization. DM, diabetes mellitus. CI, confidential interval.

The unadjusted hazard ratio (HR) of DM for the risk of major amputation was 1.87 (95% confidential interval (CI) 1.12-3.12; p=0.017) (Table 3). The multivariate analysis with all factors included and with stratification by randomization showed a hazard ratio of DM of 1.59 (95% CI 0.91-2.78; p=0.11). The model factors with significant hazard ratios in the multivariate analysis were Rutherford category at baseline (HR 2.03; 95% CI 1.28-3.21; p=0.003) and a baseline ABI>1.4 (HR 2.62; 95% CI 1.23-5.57; p=0.012).
Multivariate analysis with the inclusion of DM, baseline Rutherford category, and baseline ABI category yielded the best performing model with the lowest Akaike information criterion. In this model the hazard ratio of DM was 1.56 (95% CI 0.92-2.65; p=0.10), of baseline Rutherford category 1.95 (95% CI 1.24-3.06; p=0.004) and of baseline ABI>1.4 2.78 (95% CI 1.37-5.64; p=0.005).

Table 3. Results of Cox proportional hazards regression analysis of variables for prediction of major amputation

Variables at baseline	Hazard Ratio	95% CI	P value*
Univariate analysis			
Diabetes mellitus	1.87	1.12-3.12	0.017
Multivariate analysis			
Age	1.01	0.99-1.03	0.29
Diabetes mellitus	1.59	0.91-2.78	0.11
Stroke	0.88	0.45-1.70	0.70
Coronary disease	1.02	0.59-1.75	0.95
PAD	1.47	0.73-2.95	0.28
Former smoker	0.91	0.45-1.81	0.78
Current smoker	1.39	0.65-2.98	0.40
eGFR < 30	1.59	0.81-3.13	0.18
Rutherford category	2.03	1.28-3.21	0.003
ABI<0.7	1.26	0.68-2.32	0.46
ABI>1.4	2.62	1.23-5.57	0.012
Multivariate analysis, best performing model			
Diabetes mellitus	1.56	0.92-2.65	0.10
Rutherford category	1.95	1.24-3.06	0.004
ABI<0.7	1.32	0.73-2.41	0.36
ABI>1.4	2.78	1.37-5.64	0.005

*Stratified by randomization. CI, confidential interval. eGFR, estimated glomerular filtration rate (in mL/min/1.73 m^2). PAD, peripheral artery disease. ABI, ankle-brachial index.

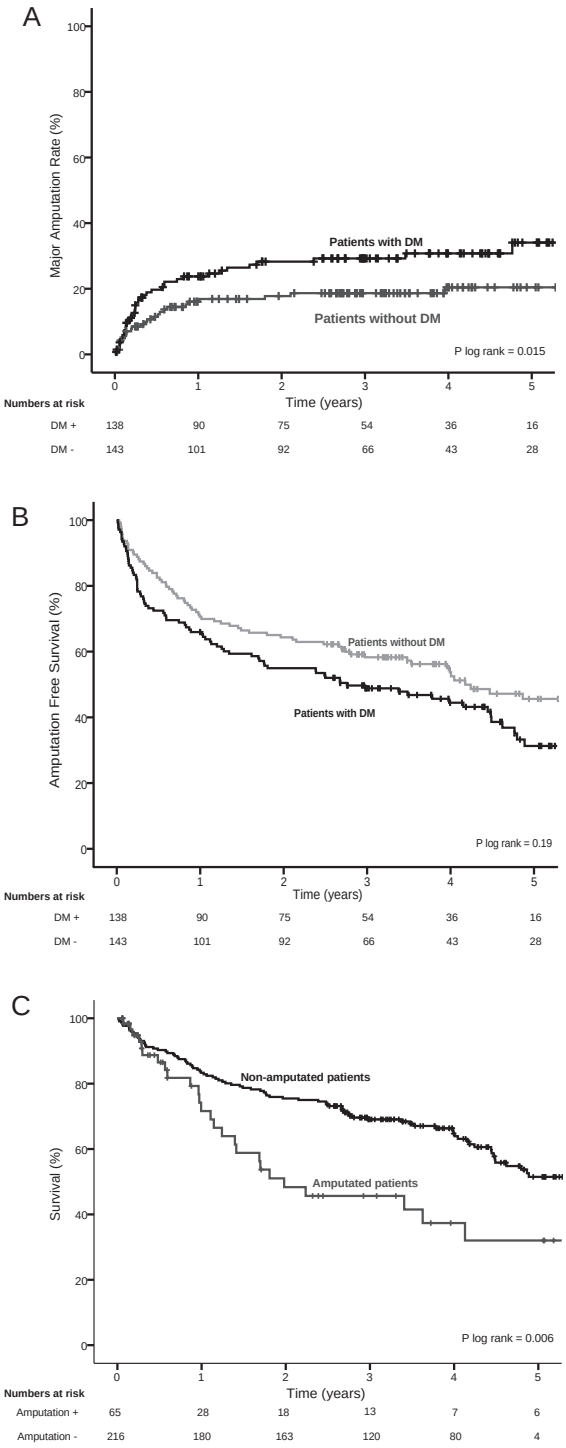


Figure 1. Kaplan-Meier curves representing the estimated cumulative incidence rates of major amputation per patient for patients with and without DM (A); the estimated cumulative incidence rates of amputation-free survival per patient for patients with and without DM (B); and the estimated cumulative incidence rates of survival in amputated and non-amputated patients (C) DM, diabetes mellitus.

CONCLUSIONS

Our data show that a significantly larger percentage of patients with CLI and diabetes suffer from a major amputation within five years compared with CLI patients without DM. Patients with CLI and DM of our cohorts had an almost 20% risk to undergo major amputation in the first six months after inclusion, versus 10% in patients without diabetes. Within five years the estimated major amputation rate in patients with DM is one out of three versus one out of five in patients without DM. After major amputation survival is poor, both for patients with and without DM.

Higher baseline Rutherford category and ABI>1.4 were significant predictors of major amputation. Both factors are more common in patients with DM. In addition, compared to non-diabetic patients significantly more diabetic patients had a history of stroke, transient ischemic attack, and coronary artery disease indicating more extensive vascular disease.

The association between DM and the development of peripheral arterial disease (PAD) has been described previously but is not well known.(1) The course of PAD in patients with DM is more aggressive compared with patients without DM, with the former group being at higher risk of developing CLI.(1, 13) The fate of the ischemic leg in a patient related to the presence or absence of concomitant DM is less well studied.(7) To our knowledge this study is the first that has proven prospectively in a true CLI population that DM is associated with lower limb survival in patients with CLI, by comparing 138 CLI patients with DM and 143 CLI patients without DM from two prospective randomized trials. Our results are supported by a previous retrospective population-based cohort study showing a lower amputation-free survival after leg bypass surgery in CLI patients with DM than in CLI patients without DM. (14)

The higher mean Rutherford category at baseline in the diabetic subgroup in the present study may relate to the high prevalence of concomitant peripheral neuropathy in these patients. Since pain perception is blunted in case of neuropathy, patients are not aware of the development of an ischemic ulcer or gangrene. Consequently, the presentation of CLI in patients with diabetes is usually at a later stage with more severe lesions.(6) This is supported by our finding that the presence of ulcers at baseline and at six months follow-up is more prevalent in patients with DM.

An ABI>1.4 is probably related to medial artery calcification (MAC), leading to poorly compressible, stiffened arteries. MAC is more often seen in patients with DM and end-stage renal disease.(15-18) Several studies have reported an association between an elevated ABI and amputation.(18-20) Our data confirm that an ABI>1.4 is strongly associated with a higher risk of amputation in patients with DM. Arterial wall stiffness due to MAC is associated with reduced arterial flow volume in the lower extremities of

patients with DM.(21) In these patients, besides of recanalization, treatment should be considered for the stiff and calcified vessel wall, although options in this field thus far are limited.

It is recommended that in case of a high or immeasurable ABI, additional non-invasive diagnostic testing such as toe systolic pressures, pulse volume recordings, transcutaneous oxygen measurements or vascular imaging (e.g. duplex ultrasound) should be performed in order to detect coexisting stenotic or occlusive arterial disease.(1, 15) A high or immeasurable ABI in a diabetic population with a clinical suspicion of CLI requires a careful diagnostic process and treatment strategy in order to avoid amputation. Our study underscores the limited value of the ABI in the assessment of PAD in DM, as almost half of our patients with DM and CLI had baseline ABI values between 0.7 and 1.4.(22)

Amputation-free survival was lower in patients with DM compared with patients without DM at all times at follow-up, although this difference did not reach statistical significance. The difference in amputation-free survival rate is mostly attributable to the higher amputation rate in patients with DM, since the death rate in these patients is comparable with that in patients without DM.

Survival was significantly lower in patients following a major amputation during follow-up. This is analogous to a previously conducted study, which reported a survival rate after major amputation of only 55% at 3 years follow-up(4), and illustrates the poor prognosis of patients after major amputation. The diminished survival following major amputation did not differ between patients with and without DM, but these subgroups were considered too small for further subanalysis.

A unique and major strength of our study is that all patients, both with and without DM, suffered from extensive, infrapopliteal arterial disease. Therefore, our results are not confounded by the more extensive and more distal nature of peripheral arterial disease in patients with DM.(23)

Some limitations of this study need to be considered. As the study population consisted of two patient cohorts from randomized controlled trials designed to test interventions in CLI, potential heterogeneity exists because of treatment effects. To forestall this limitation, we have stratified by randomization in the Kaplan-Meier survival and Cox regression analyses in order to correct for effects of different treatment strategies. It was also ascertained that there were no statistical interactions between treatment arm and the presence of DM.

Furthermore, data regarding the presence of concomitant infection in some patients with ulcers and necrosis are lacking, while infection is known to increase the risk of major amputation.(1, 24)

In conclusion, CLI patients with DM are at a substantially higher risk of major amputation compared with patients suffering from CLI without DM. The higher amputation risk is associated with a higher proportion of patients with DM with ABI>1.4 at baseline and a

more advanced clinical stage at presentation. In addition, the diabetic subgroup shows significantly more prevalent cardiovascular comorbidity. After major amputation survival significantly declines.

Author Contributions

M.I.S. participated in data collection, researched data, wrote the manuscript, and takes overall responsibility. H.G. participated in data collection, researched data, and wrote the manuscript. M.T. reviewed/edited the manuscript. R.W.S. participated in data collection and researched data. M.C.V. reviewed/edited the manuscript. R.G.S.E. participated in data collection. J.P.P.M. participated in data collection and reviewed/edited the manuscript. W.P.Th.M.M. participated in data analysis, wrote/edited the manuscript, and takes overall responsibility. H.O. participated in data collection, data analysis, wrote/edited the manuscript, and takes overall responsibility.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

PADI Study Group

Members of the PADI Study Group are M.I. Spreen, MD; J.M. Martens, MD; B.E. Hansen, PhD; B. Knippenberg, MD PhD; E. Verhey; L.C. van Dijk, MD PhD; J.P.P.M. de Vries, MD PhD; J.A. Vos, MD PhD; G.J. de Borst, MD PhD; E.J.P.A. Vonken, MD PhD; J.J. Wever, MD PhD; R.G. Statius van Eps, MD PhD; W.P.Th.M. Mali, MD PhD; H. van Overhagen, MD PhD.

JUVENTAS Study Group

Members of the JUVENTAS Study Group are R.W. Sprengers, MD; M. Teraa, MD; M.C. Verhaar, MD, PhD; F.L. Moll, MD, PhD; R.E.G. Schutgens, MD, PhD; I.C.M. Slaper-Cortenbach, PhD; Y. van der Graaf, MD, PhD; A. Algra, MD PhD; I. van der Tweel, PhD; P.A. Doevendans, MD, PhD; and W.P.Th.M. Mali, MD, PhD.

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ONLINE-ONLY SUPPLEMENTAL MATERIAL

Supplemental Table S1. Baseline characteristics, according to cohort and separately for patients with and without DM

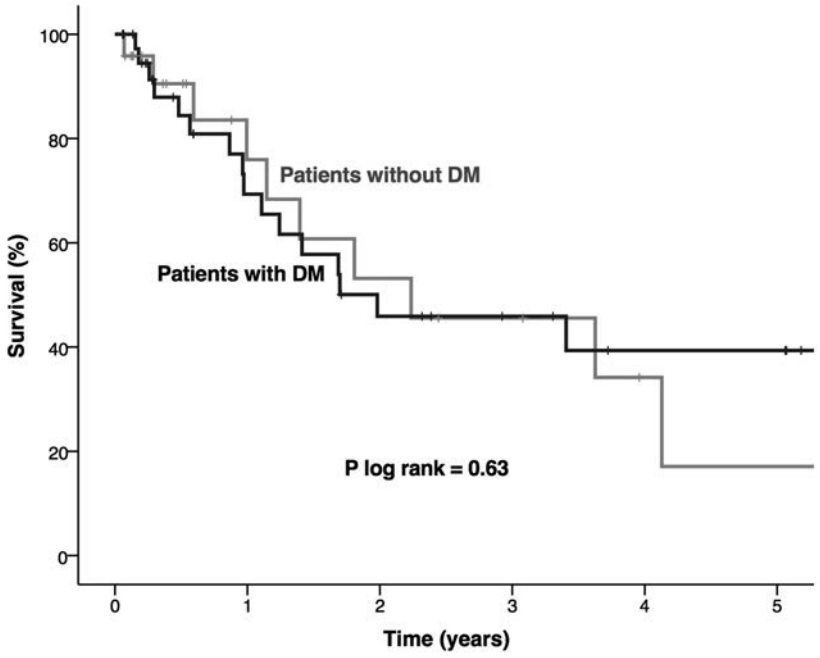
	PADI		JUVENTAS	
	Patients without DM	**Patients with DM**	**Patients without DM**	**Patients with DM**
	N=49 patients	N=82 patients	N=94 patients	N=56 patients
Mean age in years (SD)	76.4 (11.8)	73.0 (11.3)	63.5 (14.7)	67.7 (10.8)
Male sex	32 (65.3)	59 (72.0)	62 (66.0)	40 (71.4)
Smoking status				
Ex-smoker	12 (24.5)	17 (20.7)	50 (53.2)	37 (66.1)
Current smoker	13 (26.5)	18 (22.0)	35 (37.2)	6 (10.7)
Previous stroke or TIA	7 (14.3)	17 (20.7)	7 (7.4)	16 (28.6)
Coronary disease	16 (32.7)	33 (40.2)	31 (33.0)	29 (51.8)
Impaired renal function (eGFR<30)	7 (14.3)	12 (14.6)	8 (8.5)	7 (12.5)
On dialysis	5 (10.2)	8 (9.8)	3 (3.2)	1 (1.8)
Anticoagulation medication	44 (89.9)	75 (91.5)	92 (97.9)	54 (96.4)
History of PAD	26 (53.1)	55 (67.1)	81 (86.2)	47 (83.9)
Rutherford category				
mean (SD)	5.1 (0.6)	5.1 (0.6)	4.6 (0.6)	4.9 (0.5)
median (min-max)	5 (4-6)	5 (4-6)	5 (4-6)	5 (4-6)
Ankle brachial index*				
<0.7	29 (59.2)	25 (30.5)	68 (72.3)	30 (53.6)
0.7-1.4	17 (34.7)	41 (50.0)	19 (20.2)	19 (33.9)
>1.4/ immeasurable	3 (6.1)	16 (19.5)	7 (7.4)	7 (12.5)

Data are number (%) unless stated otherwise. *Missing ankle brachial indices were imputed. DM, diabetes mellitus. SD, standard deviation. TIA, transient ischemic attack. eGFR, estimated glomerular filtration rate (in mL/min/1.73 m2). PAD, peripheral artery disease.

Supplemental Table S2. Ulcers in patients with and without DM

Ulcers	Patients without DM	Patients with DM	P value*
Baseline	N=143 limbs	N=138 limbs	
	84 (58.7)	97 (70.3)	0.043
6 months	N=112 limbs	N=95 limbs	
	31 (27.7)	46 (48.4)	0.002

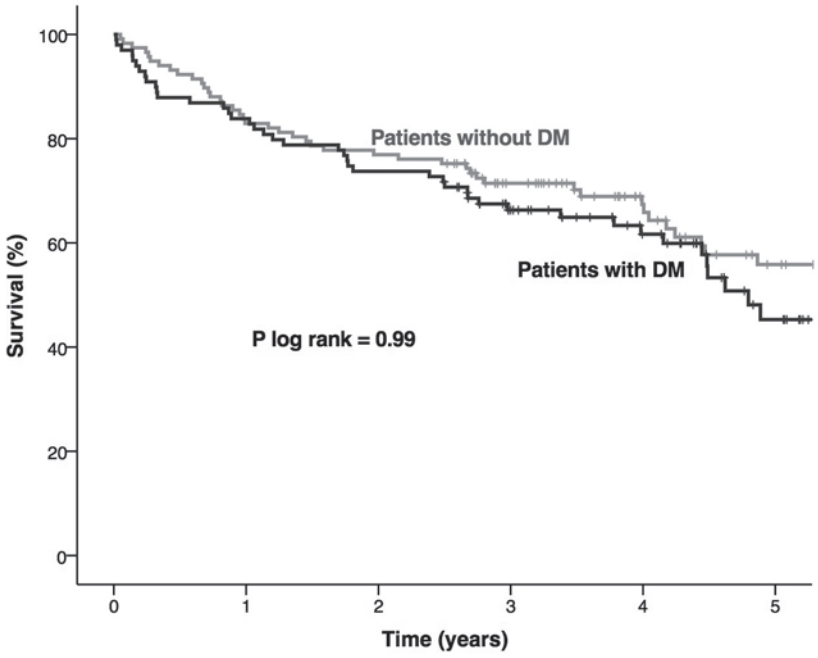
Values are n (%). *Chi-square test. DM, diabetes mellitus.



Numbers at risk

Years	0	1	2	3	4	5
Patients with DM	39	18	11	8	5	5
Patients without DM	26	10	7	5	2	1

Supplemental figure S1. Kaplan-Meier curves representing the estimated cumulative incidence rates of survival following major amputation in patients with and without DM. DM, diabetes mellitus.



Numbers at risk

Years	0	1	2	3	4	5
Patients with DM	99	83	73	54	36	16
Patients without DM	117	97	90	66	44	29

Supplemental figure S2. Kaplan-Meier curves representing the estimated cumulative incidence rates of survival in non-amputated patients with and without DM. DM, diabetes mellitus.

P.P. Zwetsloot[1], H. Gremmels[2*], B. Assmus[3*], S. Koudstaal[1], J.P. Sluijter[1], A.M. Zeiher[2], S.A.J. Chamuleau[1]

[1] Department of Cardiology, Experimental Cardiology Laboratory, University Medical Center Utrecht, the Netherlands
[2] Department of Nephrology & Hypertension, University Medical Center Utrecht, the Netherlands
[3] Division of Cardiology, Department of Medicine III, Goethe University Frankfurt, Germany

CHAPTER 5

RESPONDER DEFINITION IN CLINICAL STEM CELL TRIALS IN CARDIOLOGY; WILL THE REAL RESPONDER PLEASE STAND UP?

Submitted

INTRODUCTION

Cell-based treatment to augment functional recovery after myocardial infarction (MI) has shown highly promising results, but high variability between and within studies has limited wider adoption in a clinical setting. Defining responders to cell treatment based on functional measurements in cardiac stem cell trials has been troublesome, and it may be considered as the Holy Grail. The functional recovery after myocardial infarction (MI) can range from only mild impairments and recovery to progression into heart failure at the next clinical visit regardless of the therapy given. In a clinical trial with adequate randomization, this will not pose an issue on the overall outcome of the trial. However, subgroup analyses become difficult, as the variable course of the disease influences the end-result on top of the effect of the cell treatment. In other words, even patients who have suffered significant loss of functional cardiac capacity may still have benefited from cell therapy compared to the potential 'reference point' of the same patient in the placebo group. Furthermore, the power needed to show specific responding groups is beyond the number of participants included in hitherto conducted cell therapy trials.

Meta-analyses including all randomized controlled studies have consistently shown significant positive effects of treatment with bone marrow mononuclear cells (BMMNCs) after MI. Stratified subgroup meta-analyses hint towards different effects with increasing age and/or specific functional parameters.[1] Researchers have questioned the availability and quality of autologous cells harvested from patients with multiple risk factors.[2] The negative effects of endogenous risk factors on bone marrow- and circulating progenitor cells have been confirmed with regards to age, smoking, heart failure, diabetes and general risk factor profiles.[2] To date, it is not known if the negative effect of clinical risk factors on BMMNC function and the recipient heart is also reflected in the outcomes of clinical studies. Furthermore, the invasiveness and cost of BMMNC therapy call for better prediction of treatment response after MI. In the present study, we demonstrate a method based on multivariable statistical interactions, which is able to identify potential treatment responders, while simultaneously correcting for relevant factors that affect general disease outcome. With the identification of components that positively influence the (probability of a) functional gain after cell therapy, it might be possible to predict who the real responders are.

METHODS AND RESULTS

As a proof of concept, we used the data from the REPAIR-AMI trial; a multicenter randomized controlled trial, conducted from April 2004 till October 2005.[3] In the REPAIR-AMI trial, difference in ejection fraction after 4 months compared to baseline

(ΔEF) was used as the initial primary outcome. Patient characteristics, baseline imaging and cell characteristics were recorded (Table 1, left columns). 204 patients were randomized, of which 186 had complete data after 4 months for functional outcome and characteristics.

This post-hoc analysis was not prospectively declared, but initiated and executed by independent researchers not affiliated with the primary study team. On the basis of an *a priori* power analysis, we defined 18 variables as possible predictors, based on previous literature and clinical expertise[4]. We applied linear regression analyses with ΔEF as outcome and the statistical interaction of cell therapy with the single possible predictors as variable of interest. These interaction terms resemble the difference between the cell treated and placebo group, regardless of the effect of the variable on the functional outcome itself. A significant interaction therefore identifies predictors in which the effect of cell therapy compared to the placebo is different within groups (Table 1, middle columns).

Next, we performed multivariable linear regression for these interactions with subsequent step-wise backward selection (cut-off value used is the AIC (p=0.157)) to identify a combination of independent factors that most accurately predicts the outcome in this dataset (Table 1, right columns).[4] The analysis was performed using R version 3.1.2[5] with the additional rms package.

The randomization of the REPAIR-AMI study generated comparable groups for our analysis with minor baseline differences (Table 1). The combination of independent predictors for treatment response to cell therapy through interaction was patient age (-0.18%/yr, p = 0.05), weight (+0.17%/kg, p=0.02), $EF_{baseline}$ (0.42%/% p=0.002) and $ESV_{baseline}$ (-0.09%/ml, p=0.08) (Table 1). β-values are expressed as EF change per unit of assessment. These outcomes suggest, that advanced age is associated with poor response to BM-MNC therapy, whereas higher weight, and high initial functional loss are associated with greater treatment benefit in this dataset (Suppl. Figure 1 A-D).

DISCUSSION

Here we show the concept of multivariably assessing the benefit of cell therapy by comparing outcomes to a patient's 'reference point' instead of the patient's baseline measurement. Distinguishing responders from non-responders could be a next step for clinical cell therapy, ultimately tailoring cell therapy to patients who will most likely benefit. Statistically correcting for the whimsical nature of the disease is an insightful step in this process. When doing so, it appears that in the REPAIR-AMI trial, younger patients with larger infarcts and risk factors such as smoking and obesity derive more benefit from BMMNC therapy compared to the patients with a negligible risk factor

profile. Our findings are partially in line with results from previous meta-analyses, showing more effects of cell therapy in patients with lower baseline EF and age.[1] For the effect of baseline cardiac function and cell therapy, results have been conflicting in both single studies and meta-analyses, of which a comprehensive overview was recently published.[18] In these papers the imaging method is also discussed, in which studies using MRI showed reduced effects compared to those using LV angiography, such as the REPAIR-AMI. A recent individual patient meta-analysis could not confirm the findings with regards to stratified variables for age and functional parameters, nor find other associated risk factors with any outcome.[19] Weight as an effect modifier on functional response after cell therapy has never been described before to our knowledge.

Table 1. Baseline characteristics of the REPAIR-AMI trial and interaction modeling of ΔEF with univariable regression analysis and multivariable regression analysis with backwards selection.

Variable	**Placebo**	**BMC**	**Main Effect**	**Univ. Interaction**		**Multiv. Interaction**	
	(n=91)	(n=95)	p-val	p-val	β	p-val	β
Delta EF (4 months)	3.2	5.5	0.02				
Age (years)	56.6	55.4	0.55	**0.04**	-0.18	**0.05**	-0.18
Gender (% male)	84.6	81.2	0.52	0.52	1.69		
Weight (kg)	84.3	80.4	0.07	**0.07**	0.12	**0.02**	0.17
BMI	27.6	26.8	0.13	0.26	0.29		
Hypertension (%)	59.3	52.6	0.36	0.51	1.32		
Hyperlip (%)	60.4	51.6	0.23	0.79	-0.53		
Diabetes (%)	23.1	10.5	0.02	0.64	-1.33		
Fam. Hist. CAD (%)	36.3	34.7	0.83	0.08	3.6		
Previous MI (%)	6.5	5.2	0.95	0.29	4.7		
Smoking Hist. (%)	68.1	74.7	0.32	0.06	4.1		
Active smoker (%)	42.9	47.4	0.54	0.11	3.2		
Baseline imaging							
EF (%)	47	48.3	0.36	**0.02**	-0.25	**0.002**	-0.42
ESV (ml)	74	67.4	0.12	**0.11**	0.06	**0.08**	-0.09
EDV (ml)	138.2	128.5	0.12	0.5	0.02		
Intervention							
Days MI-therapy	4.3	4.3	0.66	**0.05**	1.4		
Days MI-BM asp	3.9	3.8	0.6	0.09	1.6		
Basal migr	91.5	103.7	0.22	0.16	-0.02		
SDF stim migr	161.8	170.9	0.49	0.46	-0.008		

From a mechanistic standpoint, one explanation for results of the present analysis would be that cell therapy predominantly affects adverse remodeling after MI. An increased effect of cell therapy with increasing weight is in line with this hypothesis, as waist circumference and BMI are associated with increased incidence of heart failure after MI[6] and increase in bodyweight/BMI is associated with an increased risk of developing heart failure in general.[7] The same holds true for lower baseline EF and developing heart failure after MI.[8] BMMNC therapy might be predominantly counteracting this process through its paracrine mechanisms.

An alternative hypothesis would be that patients with a high risk-factor burden benefit more from cell treatment, as they have a reduced endogenous repair capacity. Decreased numbers of circulating progenitor cells have been observed in for example smoking[9], diabetes[10] and obesity[11] and this decrease in circulating cells ultimately is associated with a worse cardiovascular prognosis.[12] Interestingly, the acute increase in circulating progenitor cells after MI[13] is also diminished with risk factors like diabetes[14] and history of MI.[15] In BMMNC therapy after MI, this defect in progenitor cell mobilization might be partially circumvented by mechanical BMMNC aspiration and subsequent direct administration. It is conceivable however, that patients with few risk factors, and therefore an intact mobilization response, gain little additional benefit from BMMNC treatment. This is also in line with the findings from the CCTRN trials that personal bone marrow characteristics could explain infarct size reduction irrespective of cell therapy in both MI and heart failure.[16, 17]

Variability in treatment success in clinical autologous stem cell trails is determined by two factors: the potency of the cell isolate and the disease state of an affected patient. This is in contrast to for instance medicinal therapy, where the variability in treatment response is theoretically solely dependent on the patient, as potency of different drug batches should ideally be nearly identical. Interestingly, the direction of effects from the identified risk factors, almost all besides age, pointed to a greater treatment response with an adverse risk-factor profile. This finding is contrary to results from preclinical studies studying the cell product, which show that cardiovascular risk-factors are associated with poorer pro-angiogenic capacity of human bone marrow cells in preclinical models.[21, 22] Pre-clinical studies have heretofore only been able to demonstrate a reduction in the pro-angiogenic potential of the BMMNC graft, but supply little information on the recipient risk factor-fed hearts.

An important limitation of the present study is that the size of the REPAIR-AMI is insufficient to investigate all potential interactions that one might expect, as interaction analyses need at least 4 times more participants to obtain adequate power than analyses of main effects in linear regression models.[20] It is likely that this analysis is incomplete in identifying the effects of biological interactions that predict response in the general population of MI patients and that the interactions identified here will vary in effect size

in other populations. This analysis should be seen as a proof-of-concept, and primarily hypothesis generating; the observed (combination of) independent predictors should be confirmed in other datasets and ideally be prospectively declared in larger trials (like the currently recruiting BAMI trial (NCT01569178, www.bami-fp7.eu) to generate the responder characteristics within the included population.
Myocardial infarction and its aftermath can have a variable course, obscuring any effect of therapy to specific subgroups. Identifying responding populations through additional analyses might however be the next step towards optimal cell therapy in clinical care. In this paper we show a first step in identifying these subgroups using interaction models in a multivariable fashion. Future steps include prediction models for responder identification based on more retrospective and prospective data, to ultimately treat the patients that will benefit most from cell therapy.

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Non-standard Abbreviations and Acronyms

BMMNCs	Bone Marrow Mononuclear Cells
MI	Myocardial Infarction
EF	Ejection fraction
ΔEF	Difference in ejection fraction between baseline and 4 months follow-up
EDV	End diastolic volume
ESV	End systolic volume

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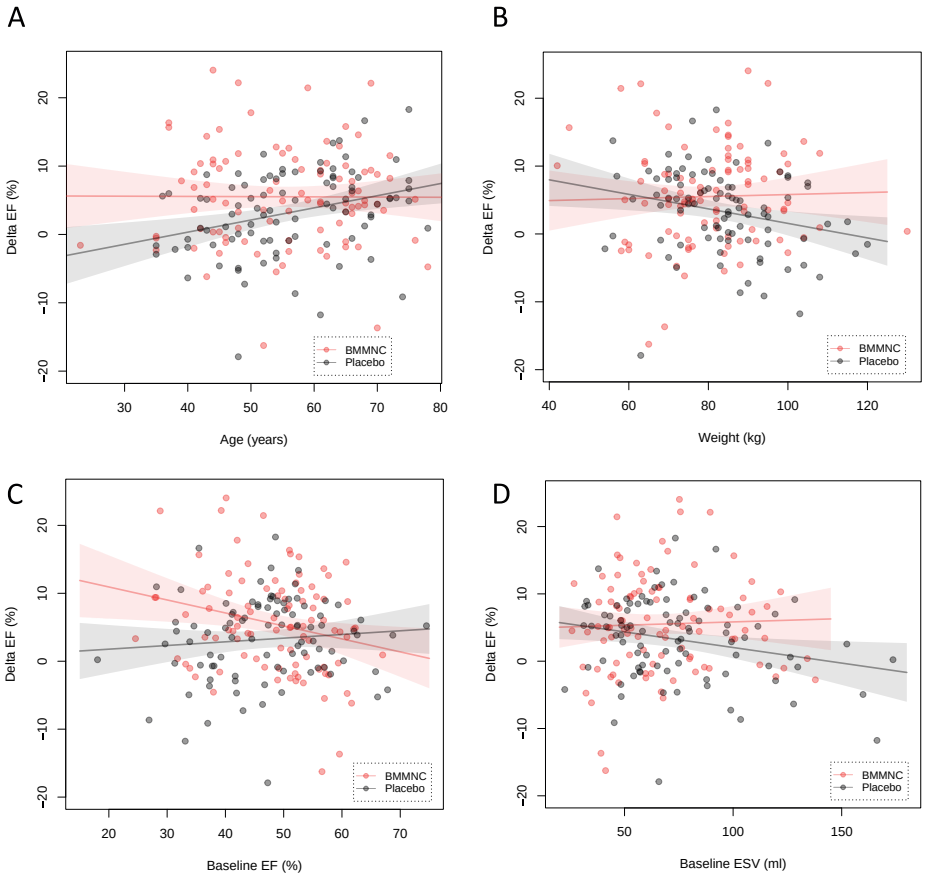
SUPPLEMENTARY INFORMATION



Supplemental Figure 1 A-D. Visualization of the interactions for (A) age, (B) weight, (C) baseline ejection fraction, and (D) baseline end systolic volume.

Hendrik Gremmels M.D.[1], Joost O. Fledderus Ph.D.[1], Bas W.M. van Balkom Ph.D.[1], Marianne C. Verhaar M.D., Ph.D[1]

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, The Netherlands

CHAPTER 6

TRANSCRIPTOME ANALYSIS IN ENDOTHELIAL PROGENITOR CELL BIOLOGY

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"Antioxidants and Redox Signaling"

ABSTRACT

The use of endothelial progenitor cells (EPCs) is a promising new treatment option for cardiovascular diseases. Many of the underlying mechanisms that result in an improvement of endothelial function *in vivo* remain poorly elucidated to this date, however. We summarize the current positions and potential applications of gene-expression profiling in the field of EPC biology. Based on our own and published gene-expression data, we demonstrate that gene-expression profiling can efficiently be used to characterize different EPC types. Furthermore, we highlight the potential of gene-expression profiling for the analysis of changes that EPCs undergo during culture and examine changes in gene transcription in diseased patients. Transcriptome profiling is a powerful tool for the characterization and functional analysis of EPCs in health and disease.

INTRODUCTION

Cardiovascular diseases are the leading cause of morbidity and mortality in the Western world and impose an ever-increasing socioeconomic burden (78, 140). The underlying pathological mechanism of these diseases is atherosclerosis; a multifactorial chronic inflammatory disorder characterized by progressive endothelial dysfunction and formation of lipid-rich plaques containing macrophages and smooth muscle cells within the vascular wall (70). Current therapies consist of slowing the progression of atherosclerosis through risk-factor reduction by changes in lifestyle and pharmaceutical intervention. When focal plaques occlude major arteries, circulation can be restored through surgical or endovascular interventions (86). These therapies have limitations, however, and many patients reach a point at which treatment options are exhausted (43, 86). Through the rapidly developing field of regenerative medicine, a third treatment modality may be available in the form of progenitor cell therapy. This therapy aims at restoring tissue perfusion and preserving end-organ function by using progenitor cells to improve arterial dysfunction or to stimulate neovascularization.

The concept of regenerative medicine in cardiovascular disease dates back to a study by Asahara et al. in 1997 (5). The authors demonstrated that bone-marrow (BM)-derived cells with properties resembling primitive vascular cells could be isolated from peripheral blood and that these cells contribute to the formation of new vessels (4, 6). The cells were termed endothelial progenitor cells (EPCs), and the following decade saw a large number of publications confirming and extending the findings of Asahara et al. Later, it was shown that numbers of EPCs are reduced in the blood of patients with arterial disease or diabetes mellitus (DM) (21, 30?32, 45, 48, 66, 75, 83, 119, 129, 135) and that circulating EPC

numbers can serve as a biomarker to predict future cardiovascular events (108, 135). These observations suggest a role for EPCs in vascular homeostasis (15) and the potential use of progenitor cells as a treatment for cardiovascular disease. Several therapies have been devised in the form of autologous transplantation of BM (47, 114, 118) or mobilized progenitor cells (49, 51, 58), or alternatively, by increasing progenitor cell numbers and function in the circulation through the administration of growth factors (3, 29, 81, 127). However, much debate exists about which cell types actually contribute to the functional improvement and increased perfusion observed in clinical trials thus far. Until recently, the term EPC was used rather promiscuously, referring to circulating and cultured cells alike, although different phenotypes arise when different isolation techniques and culturing methods are applied (55, 57, 93, 120, 142). Furthermore, seemingly minor differences in experimental procedures produce widely differing results (23, 42, 109). Therefore, principal questions remain unanswered: What exactly constitutes the EPC? From which lineage do EPCs originate? Are cultured EPCs analogous to their counterparts *in vivo*? Until now, EPC biology has been very much an empiric science, in which several therapies have been tried *in vivo* and even in a clinical setting, without necessarily a complete or accurate understanding of mechanistic underpinnings. A more integrated view of EPC biology may be necessary to reconcile findings and to develop promising strategies for forthcoming therapies. Other fields, such as oncology, have benefited greatly from gene-expression profiling to identify cellular markers and targets for treatment. Here we review the implementation of transcriptional profiling in the field of EPC biology.

Transcriptome Analysis

The Human Genome Project (68, 130) and the subsequent development of technologies that enable large-scale genomic analyses (26a, 36, 72) have widened the scope of molecular biologic analyses to an unprecedented degree. At present, a whole range of "omics" has been developed, each dedicated to giving a comprehensive view of one aspect of cellular functioning (104, 105). Among the "omics", transcriptomics, or gene-expression profiling, is currently the most widely implemented. As methods and guidelines for reporting experiments have been largely standardized (11), reproducibility has reached a level at which direct comparisons of multiple experiments have become possible (76).

The most common method for transcriptional profiling is the hybridization of target nucleotides to oligonucleotide DNA microarrays. These arrays consist of a large number of gene-specific short nucleotide sequences, called probes, immobilized as millions of identical copies at a unique location on a solid support (54, 72, 73). Usually, each gene is represented by one or more probes, and each probe is present in duplicate or multiplicate on the microarray. Labeled target nucleotide sequences, derived from mRNA, are hybridized to their complementary probes to give a quantitative estimate of the abundance of the target mRNA sequence. As microarrays are constructed to probe the transcriptome

as comprehensively as possible, it is not necessary to know beforehand which genes are involved in the process under investigation (73). This makes gene-expression profiling particularly suited to generate hypotheses about processes of which underlying mechanisms are poorly understood. In addition, it makes analyses relatively unbiased toward expected or hypothesized effects.

Transcriptome analysis using microarrays has been used to answer questions that are not unlike the questions that currently dominate the field of EPC biology. Gene-expression profiling may provide insight into the characterization of EPCs, alterations of the EPC phenotype during differentiation, and comparison of EPCs in healthy and diseased states (Figure 1). One potential application of gene-expression profiling is the characterization of closely related cell types. Microarrays have, for instance, been extensively used to distinguish different tumor phenotypes among morphologically very similar tumors (2, 40, 98, 133, 139). The advantage over methods investigating only a few markers, such as flow cytometry, immunohistochemistry or polymerase chain reaction (PCR), lies not only in number of markers investigated. As microarrays probe the transcriptome

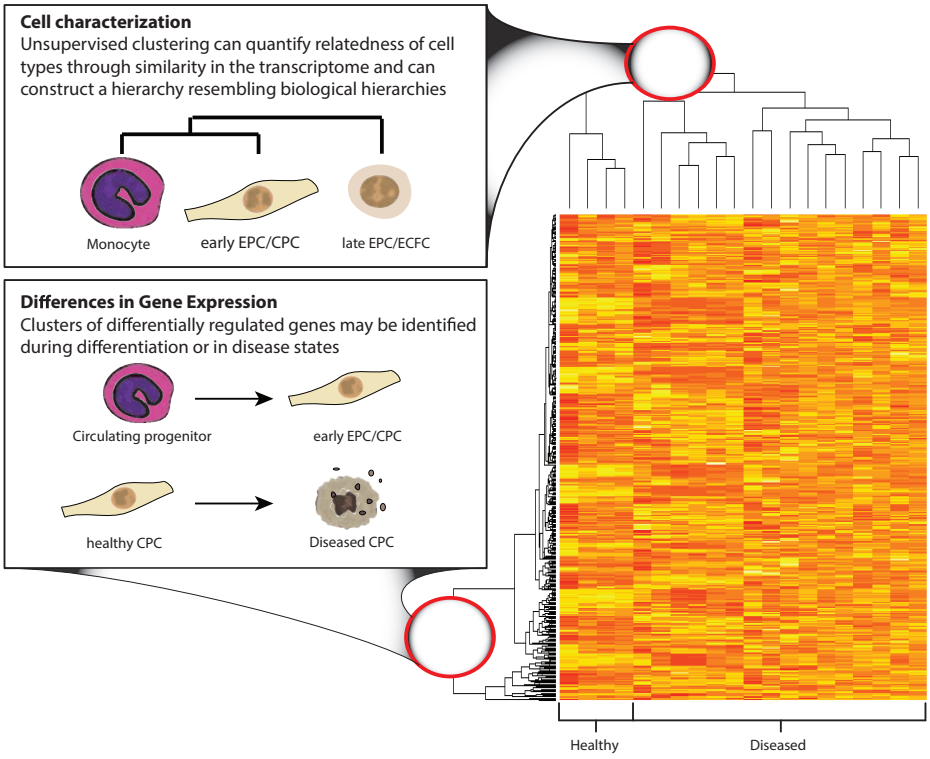

Figure 1. Potential applications of gene expression profiling in EPC biology.

Table 1. Overview of Published EPC Transcriptome Data

First Author	Year	EPC Type	Microarray Platform	Comparison
Dernbach, E. (24)	2004	CACs	-	CACs vs $CD14^+$ monocytes, HMECs and HUVECs
Urbich, C. (122)	2005	CACs	HG -U95Av2	CACs vs $CD14^+$ monocytes, HMECs and HUVECs
Urbich, C. (124)	2005	CACs	HG -U95Av2	CACs vs $CD14^+$ monocytes, HMECs and HUVECs
Fiorito, C. (33)	2008	CACs	HG-U133 Plus 2.0	Effects of Vitamin C and E on resting and TNFα treated CACs
Igreja, C. (54)	2008	CACs	HG-U133A	CACs at day 1 and day 13 in culture
Desai, A. (25)	2009	CFU-Hill	HG-U133 Plus 2.0	Changes in gene-expression after 3 months of exercise, comparison of CFU-Hill to other cell types in database
Maeng, Y.S.	2009	CB-CAC, CB-ECFC	HG-U133 Plus 2.0	Differences in gene-expression between the two progenitor types
Van Oostrom, O. (127)	2009	CACs	Illu Human Ref-8	Differences in gene expression between patients with DM I and healthy controls
Medina, R.J. (84)	2010	CACs, ECFCs	Illu WG-6 v3.0	Comparison between CACs and ECFCs, as well as HDMEC and $CD14^+$ Monocytes
Zeisberger, S.M. (144)	2010	ECFCs	HG-U133 Plus 2.0	Gene expression in EGM-2 medium with FCS versus SF-1
Tan, K. (118)	2010	ECFCs	HG-U133 Plus 2.0	Cells from patients with proliferative diabetic retinopathy versus healthy controls

comprehensively, transcriptional profiling allows the attachment of a meaningful quantitative measure to the relatedness of different transcriptomes (59, 63). This allows the construction of a hierarchy of relatedness through clustering algorithms (63) that may reflect the taxonomy in biologic hierarchies, such as the hematopoietic hierarchy (13, 132). Cells of uncertain ontogeny may be compared in this fashion with existing hierarchies to attain a better understanding about their origins. Ideally, BM- resident and circulating EPCs could be integrated into an expression profile-based hierarchy ranging from the hemangioblast to the terminally differentiated endothelial cell. Moreover, transcriptomes of different cultured EPC pheno-types could be compared with the endothelial and the hematopoietic hierarchy for a proper transcriptome-based classification of these cells (54). The changes that isolated EPCs undergo during the selection and differentiation process of culture can be elucidated, as it seems that isolation and culture alter the characteristics of a cell type to some degree, allowing cells both to acquire markers and perhaps to induce functional alterations (94, 120).

One of the pillars of regenerative cellular therapies is the use of autologous cells in the treatment of disease. In the case of EPCs, however, the function of these progenitor cells may be impaired in diseased subjects (45, 119, 128). Identifying which pathways and mechanisms are affected by disease processes may provide leads for interventions or adaptation of culture protocols that improve progenitor cell function. Taken together, transcriptome analysis has the potential to answer principal questions and to enhance our understanding of EPC identity, differentiation, and function in health and disease. In this review, we address these issues and provide an overview of published EPC transcriptome data (Table 1).

EPC Identity

During embryonic development, endothelial cells and hematopoietic cells are thought to arise from a common progenitor, termed the hemangioblast (99, 100), although the exact sequence of intermediates is still unknown (67) (Figure 2). On endothelial differentiation, the newly formed angioblasts acquire a set of early endothelial markers, including vascular endothelial growth factor receptor 2 (VEGFR-2/KDR), CD31, and TEK tyrosine kinase, forming primitive blood islands (15). Vascular networks sprout from these primitive blood islands in a process termed vasculogenesis. These networks extend, providing an integrated vasculature throughout the body, in a process called angiogenesis. Until the 1990s, the prevailing paradigm was that, although endothelium has the capacity to proliferate locally (37) in the adult, as was demonstrated in grafts (35, 79, 92) or in transplanted organs (62), or even by shedding circulating endothelial cells (115), no vasculogenesis occurs in adult life. In 1997, Asahara et al. (5), however, demonstrated the existence of circulating BM-derived cells that are able to form colonies that have a high resemblance to primitive vascular structures, *in vitro*. These cells had characteristics of endothelial cells, such as CD31, KDR, and endothelial nitric oxide synthase (eNOS) expression, but also displayed markers associated with early progenitor cells, such as CD34 (5, 120) and CD133 (89), and were consequently termed EPCs. It was demonstrated that these EPCs also integrate into damaged vessels in a hindlimb-ischemia model (4, 5, 116). Kalka et al. (61) demonstrated that these cells could have therapeutic applications by injecting human EPCs into an athymic nude mouse hindlimb-ischemia model. Mice that were injected with EPCs showed increased restoration of blood flow compared with untreated controls or mice treated with mature endothelial cells (61). A translation toward a clinically viable therapy was devised shortly thereafter, by using local infusion of autologous BM-derived cells in patients with coronary or peripheral arterial disease (9, 47, 114, 118). Further evidence for the importance of EPCs in cardiovascular disease came from a number of studies investigating the number of EPCs in patients with arterial disease. The amount of both circulating EPCs (cEPCs, defined as CD34+/KDR+ cells) and EPCs arising in culture was found to be reduced in patients with coronary

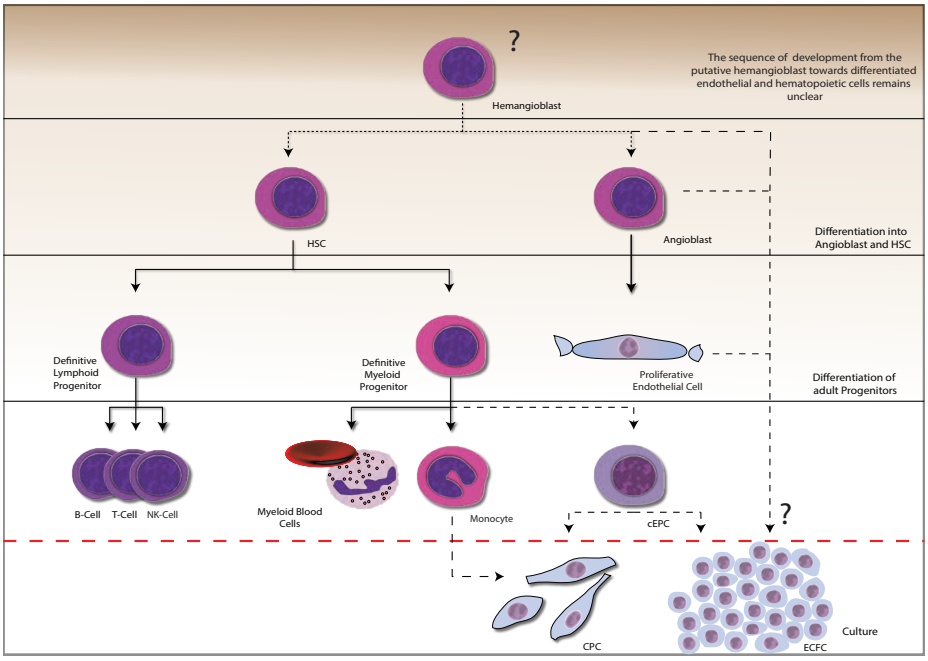

Figure 2. Putative origin of different EPC types observed in circulation and in culture. The exact sequence of differentiation steps from the hemangioblast to the hematopoietic and endothelial lineages still remains a topic of intense investigation. CACs and CFU-Hills are thought to arise from myeloid leukocytes, whereas ECFCs are true endothelial cells. *Solid lines* indicate well-established routes of differentiation; *dashed lines*, current uncertainties.

artery disease, as was the migratory capacity of the EPCs cultured from these patients (128). A study by Hill et al. (48) introduced a new culture assay using peripheral blood mononuclear cells (PBMCs) that involved a preplating step to remove circulating differentiated endothelial cells, as endothelial shedding in cardiovascular disease was considered a possible "contamination" in EPC culture. The authors then counted the colonies, now commonly called CFU-Hills, which arose in the second plating step. The number of colonies was found to be inversely related to the Framingham cardiovascular risk score and robustly related to endothelial function, as measured with flow-mediated brachial dilatation.

Evidence began to accumulate that a large heterogeneity exists in cultured EPC phenotypes. In 2000, Lin et al. (71) had shown that another, distinct phenotype of EPCs, which is highly proliferative and closely resembles endothelium, arises in long-term culture. This contrasted with the previously studied spindle-shaped EPCs that formed after culturing PBMCs for 7 days, including a washing step at day 4 to remove nonadhering cells (128, 129). Hur et al. (55) closely characterized the two phenotypes that arise after

short- and long-term culture and coined the terms early- and late-outgrowth EPCs. Later these cells were renamed circulating angiogenic cell (CAC) and endothelial colony-forming cells (ECFCs), respectively, although at present, no guidelines exist for a consistent nomenclature of EPC subtypes. It was discovered that CACs have little or no proliferative capacity (55, 146) and are unable to form tubular networks on basement membrane matrix (Matrigel) (85). Interestingly, they do colocalize to nodes in vascular networks (55) formed by endothelial cell lines *in vitro*, but do not fully integrate into the networks (110). CACs do secrete large amounts of growth factors (55, 110) and likely participate in extracellular matrix (ECM) remodeling (41), thereby promoting angiogenesis in a paracrine fashion (110, 143). Furthermore, it was shown that CACs express myeloid lineage markers, such as CD14 and CD45 (33, 122) and exhibit phagocytic ability (96, 146). ECFCs, conversely, are characterized by the absence of myeloid markers and appear more closely to resemble endothelial cells. For instance, they form tubular structures when plated on Matrigel and form cobblestone-shaped monolayers with smooth cytoplasm in culture (55, 71, 142). Also, they have been shown to be highly expandable (38) and do not incur genomic aberrations during expansion (97).

Table 2. EPC Glossary

Name	cEPC	CAC	CFU-Hill	ECFC
Synonyms	EPC, circulating EPC	EPC, early outgrowth, CPC, EPC, CE-EPCs, CMMCs, ELC, PAC, APC, EAC	EPC, CFU	EPC, late outgrowth EPCs, ECs, BOECs, OECs, EPDCs
Definition	By flow-cytometry, usually as CD34+/KDR+ or CD34+/KDR+/CD133+	Cells derived after plating of PBMCs for 4-7 days on fibronectin coated-wells, in EGM-2 medium. Bind lectin and take up LDL.	Cells in colonies generated from re-plated PBMCs in the Hill assay	Cells derived from late appearing colonies with a cobble-stone appearance.
Origin	Depends on exact marker definition, early hematopoietic line	Myeloid, closely related to monocytes	Mixture of T-cells and monocytes	Unclear, appears not to be derived from hematopoietic line, likely endothelial

EPC: Endothelial Progenitor Cell; CPC: Circulating progenitor cell CD34: Cluster of Differentiation 34; KDR: Kinase insert Domain Receptor (VEGFR2); APC: Angiogenic Progenitor Cell; CAC: Circulating Angiogenic Cell; CE-EPCs: Culture Expanded EPCs; CMMCs: Culture Modified Monocytic Cells; CFU-Hill: Colony Forming Unit, Hill-type; EAC: Early Outgrowth Angiogenic Cell; ELC: Endothelial Like Cell; ECs: Endothelial Cells; BOECs: Blood-Outgrowth Endothelial Cells; OECs: Outgrowth Endothelial Cells; EPDCs: Endothelial Progenitor Derived Cells. PAC: Proangiogenic Cells

The colony-forming assay introduced by Hill et al. (48, 135) seemed to produce a third, mixed, EPC phenotype, as the colonies were shown to consist of both monocytes and lymphocytes (93, 101, 124).

How the cultured phenotypes relate to cEPCs depends on how the latter are defined. A common definition of cEPCs is based on the coexpression of CD34 and KDR; this definition includes mostly cells from the hematopoietic lineage (17, 69, 93, 120) that comprise a subset of the cells that will form CACs in culture (103). For clarity, we adopt the following nomenclature for the various circulating and cultured progenitor cells (**Table 2**). We refer to circulating EPCs, as detected by flow cytometry, as cEPCs; monocytic early outgrowth cells are denoted as circulating angiogenic cells (CACs); colonies obtained with the Hill assay will be called CFU-Hills; and late-outgrowth endothelial cells will be called endothelial colony-forming cells (ECFCs). The term EPCs will be used to describe relevant progenitor cells in general.

Definition and characterization of progenitor cells is historically based first and foremost on cell-surface antigens associated with either or both endothelial cells and progenitor cells, which can be detected by using flow cytometry and immunohistochemistry (120). The antigens selected to define EPCs are selected on the basis of previously established associations with either stem cell characteristics, as, for instance, CD34 (5) and CD133 (89), or endothelial cells, such as KDR/VEGFR2, eNOS, or von Willebrand factor (vWF) (5). Although this reasoning has proven very effective in advancing the field of regenerative medicine, characterization by flow cytometry is not very specific, as much overlap occurs in markers between cells in the hematopoietic line, especially in early progenitor cells (39, 93). Furthermore, this approach is prone to errors with regard to cultured cells, as markers may be lost (89) or acquired (20, 94, 107, 120) during mononuclear cell isolation and culture (33). The introduction of a classification on the basis of functional capabilities that are relevant for cell therapy, such as the secretion of growth factors (110), the ability to proliferate (57, 142) or to form tubular structures on Matrigel, has been a leap forward in creating a conceptual framework to understand the roles of circulating and cultured EPCs in the restoration of blood flow to ischemic tissues (141, 142).

Starting from a cell population that displays certain phenotypic or functional characteristics, transcriptional profiling may be used rationally to identify unique markers or genes associated with a particular cell-type function. Transcriptional profiling provides a characterization method that is relatively unbiased because it indiscriminately includes the entire set of transcribed genes. In addition, the existence of online repositories for gene-expression profiles allows comparisons to be made between cell types of different origins. To illustrate how gene-expression profiles may aid in the classification of EPCs, we conducted an analysis of publicly available transcriptomes from different cell types and tissues of mesodermal origin (see Supplementary Data). With this approach, at least one

transcriptome of CACs, ECFCs, and CFU-Hill each on the Affymetrix HG-U133 Plus 2.0 array could be identified. In addition, we included a further 45 transcriptomes from 30 studies, representing various cell types from hematopoietic and mesenchymal tissues. Results of the hierarchic clustering of all transcriptomes show a clear distinction between hematopoietic and other mesenchymal lineages (Figure 3). The cord blood-derived ECFCs cluster with the endothelial cell lines included, showing high similarity to human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMECs). Interestingly, the cord-blood-derived CACs cluster with the hematopoietic cells and show greater similarity to adult CD14+ monocytes than to CD34+ cells from cord blood. The correlation between CACs and monocytes was not as high as correlations between samples of similar origin, making the exact position of CACs in this hierarchy less clear cut. The CFU-Hills seem to cluster loosely with CD34+ cells and were previously found more closely to resemble T-cells (26). Possibly the ambiguous clustering reflects the fact that CFU-Hill colonies consist of both myeloid and lymphoid cells (101).

These results demonstrate that it is possible to use transcriptome analysis to distinguish between different cell types and to place cell types of unclear or disputed origin in an established biologic context. An important caveat when comparing transcriptome data from different sources is that culture conditions may influence gene expression, resulting in altered gene-expression profiles. The transcriptome data used in this study are derived from primary blood or tissue samples, as well as cell lines cultured in different media.

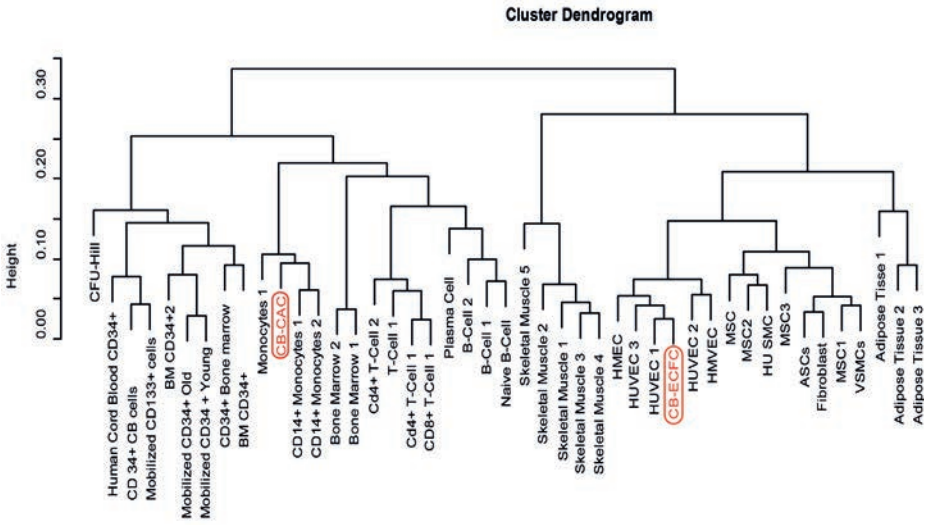

Figure 3. Dendrogram generated by unsupervised hierarchic clustering of transcriptomes of various mesenchymal tissues. The Y-axis represents similarity based on correlation (Pearson's R). ASC, adipocyte stem cell; HM(V)EC, human microvascular endothelial cell; HUSMC, human umbilical cord smooth muscle cell; HUVEC, human umbilical vein endothelial cell; MSC, mesenchymal stromal cell.

Despite the varying conditions, the clustering correctly identified the interrelatedness of the included expression profiles. Furthermore, it is underlined that CACs are myeloid cells, showing similarity to CD14+ monocytes, whereas ECFCs show a high similarity to endothelial cells.

Similar results are described by Medina et al. (82), who directly compared the transcriptomes of CACs and ECFCs from single donors with microvascular endothelial cells and CD14+ monocytes. Clustering analysis showed a pattern similar to our analysis, with CACs more closely resembling monocytes and ECFCs closely resembling microvascular endothelial cells. Genes involved in hematopoietic development, such as RUNX1, WAS, and LYN, and genes involved in the immune response, including TLRs, CD14, and HLAs, were significantly more highly expressed in CACs. In contrast, genes involved in angiogenesis, such as Tie2, eNOS and Ephrins were highly expressed in ECFCs. In addition to transcriptional profiling, Medina et al. also compared the proteomes of the different cell types and were able to identify several unique proteins in CACs and ECFCs. The authors have yet to characterize the spots with mass spectrometry to identify these potential marker proteins for the different progenitor cells.

The described microarray data should be interpreted in conjunction with those from other recent studies. Piaggio et al. (91) and Otten et al. (87) investigated progenitor cells in patients diagnosed with chronic myeloid leukemia (CML). This type of leukemia is characterized by a supernumerary chromosome arising from translocation of the BRC and ABL genes on chromosome 9 and 22, respectively. The aberrant chromosome, called the Philadelphia chromosome, can be detected with fluorescent in situ hybridization (FISH) karyotyping or PCR. Piaggio et al. (91) showed that a significant portion of CACs, but none of the ECFCs, of patients with CML showed the translocation. As CML tumor cells arise from an early progenitor in the myeloid lineage, this study confirms that CACs are myeloid/monocytic cells and that ECFCs do not arise from CACs directly, but from a distinct endothelial lineage. An explanation for the fact that CACs show a large number of endothelial surface markers is provided by Prokopi et al. (94). The authors used proteomics to identify a large number of surface markers on CACs and compared these with genes expressed in these cells. It was shown that many of the endothelial surface markers are not expressed but are acquired from platelet microparticles during isolation and culture. These findings indicate that the different EPC phenotypes that arise in culture not only are functionally different, but also have differing expression profiles and arise from distinct lineages, which was confirmed by the comparison of transcriptome profiles.

Changes in Gene Expression during EPC Culture

Ex vivo culture and expansion of progenitor cells is one of the most important strategies to overcome a low number of EPCs and to develop future regenerative therapies. Moreover, an interim culturing step provides a window for interventions, as culture

conditions can be designed to overcome functional deficits that progenitor cells from patients may have. Transcriptomics provides a method of systematically comparing changes that cells undergo in culture, and of identifying genes that may be important for optimizing cell therapy.

The majority of preclinical studies investigating therapeutic applications of progenitor cells have hitherto been conducted with CACs (61, 122), perhaps because, in contrast to ECFCs, CACs are relatively easily and reproducibly isolated. It is interesting to note that although many studies have shown that CACs are able to restore perfusion *in vivo* (16, 18, 122, 143), CD14+ monocytes, from which CACs predominantly arise (33, 65), do not show this effect (14, 122). This suggests that an active subset of CD14+ monocytes (27) is selectively enriched during attachment in CAC culture (102, 122), or that differentiation activates proangiogenic mechanisms in CACs. Only a handful of studies have investigated transcriptional changes that occur during selection and differentiation from PBMCs or CD14+ monocytes to CACs. Dernbach et al. (25) were the first to describe differences in the transcriptome between CACs, monocytes, and differentiated endothelial cells (HUVECs and HMECs). The authors show that a number of antioxidant genes are selectively upregulated in CACs, in particular, manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPX), and catalase (Cat). These genes confer a high resistance to hydrogen peroxide-induced oxidative stress, as was demonstrated by a fluorescent oxidation-sensitive dye. It was demonstrated that CACs are less likely than endothelial cells or monocytes to go into apoptosis in the presence of high concentrations of reactive oxygen species (ROS) or during serum starvation, a condition associated with increased oxidative stress. Finally, it was shown that the antioxidant capacity of CACs is dependent on MnSOD, GPX, and Cat by using knockdown or specific inhibitors against these antioxidant enzymes. To reach intracellular ROS levels similar to those in HUVECs, inhibition of all three proteins was necessary, showing the redundancy of the antioxidant system in CACs. Interestingly, the authors also showed that CAC migration is inhibited by elevated levels of ROS, and that migration is further reduced by inhibition of antioxidant genes.

A study by Urbich et al. (123) also compared expression profiles of CD14+ monocytes, CACs, and HUVECs. The authors identify a number of genes that are upregulated in CACs, when compared with HUVECs and monocytes, most notably, a cluster of proteases including cathepsins H, L, and O. The involvement of cathepsin L in ECM remodeling was subsequently demonstrated, as was the requirement of cathepsin L for improvement of neovascularization in a hindlimb-ischemia model after CAC infusion. In a second article, the same group described that a large number of growth factors and chemokines, such as VEGF-A, interleukin (IL)1, IL-2, IL-8, and fibroblast growth factor (FGF)-A, are highly expressed in both monocytes and CACs when compared with endothelial cells (121). During the transition from monocytes to CACs, a second cluster of growth factors

was increasingly expressed, including VEGF-B, platelet-derived growth factor (PDGF) A, stromal cell-derived factor (SDF)-1 α , insulin-like growth factor (IGF)-1, and hepatocyte growth factor (HGF). These growth factors were shown to increase greatly the migratory capacity of HUVECs, suggesting that CACs exert paracrine effects on endothelium.

Collectively, these studies effectively used gene-expression profiling as a screening method to identify important target genes or gene clusters for the function of CACs. Other authors attempted to provide a more-comprehensive overview of transcriptional changes during selection and differentiation. Igreja et al. (56) examined the expression profiles of adherent human cord-blood-derived CD 133+, KDR+ or CD34+, KDR+ cells at day 0 and day 13 of plating the cells in culture. The authors then identified highly expressed genes and divided them into three categories: genes present at day 0 and absent at day 13, genes present at day 13 and absent at day 0, and genes showing a high expression level at both time points. Gene ontologic analysis subsequently showed enrichment in membrane proteins, G protein-coupled receptors, nuclear proteins, and genes involved in cholesterol biosynthesis at day 13. Several well-known markers for primitive endothelial progenitors were found to be highly expressed. KDR, angiopoietin 1, hypoxia-inducible factor (HIF)-1 α , heme oxygenase (HO-1), HOXA5, and Delta-like ligand 4 (Dll4) were found to be highly expressed throughout differentiation; expression of TEK could be detected only on day 0, whereas Flt1 could be detected only on day 13. Furthermore, the expression of two genes associated with homing of progenitor cells, SDF1 α and the IGF2 receptor, was increased on day 13, although CXCR4 expression decreased during differentiation. The authors also noted that cell-adhesion molecules, such as L-selectin, integrins α 4, α 5, α V, and β 2 are highly expressed throughout the differentiation process. The authors further report the emergence of endothelial colonies at certain points in the study, but it is unclear how this affects the results of the gene-expression analysis. The study by Medina et al. (82), mentioned earlier in this article, includes samples of both monocytes and CACs. As it was the primary aim of the authors to demonstrate the difference between ECFCs and CACs, however, differences in transcription between CD14+ monocytes and CACs were not investigated in detail. Furthermore, only a single transcriptome of CD14+ cells is included, which does not allow the identification of significantly upregulated genes.

To present a more reliable overview of changes in gene expression that CACs undergo in culture, we included two unpublished microarray expression profiles from our laboratory, obtained with pooled mRNA samples from PBMCs and CACs of 10 healthy human volunteers. Gene-expression profiles of monocytes, PBMCs and CACs were compared by using the rank-product method (12). This nonparametric method ranks genes according to the difference in expression (fold change) between experimental conditions and subsequently identifies genes that are consistently up- or down- regulated (12). The advantage of this method is that it makes minimal assumptions about the

distribution of the data and is therefore very robust, especially in the analysis of small datasets. In addition, it allows identification of differentially expressed genes in a meta-analysis across multiple small datasets (50). In total, 312 genes were found to be differentially expressed in CACs (Supplementary Data). Functional classification by using DAVID (24) revealed activation of distinct functional gene clusters after selection and differentiation (Table 3). The most striking upregulation was observed in genes regulating the uptake and metabolism of lipids. Apolipoproteins and scavenger receptors were found to be highly expressed in CACs but not in monocytes. The exact significance of this finding is unclear, although it is not quite unexpected, given that one of the characteristic features to define CACs is the uptake of acetylated low-density lipoprotein (LDL) as well as the well-known association of genes involved in cholesterol metabolism with progenitor cells (88). In accordance with the findings of the previously mentioned studies (121, 123) a number of ECM remodeling proteases from the cathepsin and matrix metalloproteinase families were also more highly expressed, as were SDF1α, VEGF-B, and a number of antioxidant enzymes, especially genes involved in intracellular thiol redox control. These studies show that CACs consistently upregulate a series of genes when placed in culture. Many of the upregulated genes have been shown to be important for the function of CACs, especially genes involved in homing and ECM remodeling (7, 19, 41, 46, 53, 90, 123, 131, 145). These changes in gene expression may explain the functional differences between CD14+ monocytes and CACs. At this point, however, it is unclear whether these changes occur because of a differentiation mechanism or the selection of an active subset of monocytes in culture. Very little is known about the differentiation of ECFCs in culture, as the circulating progenitor cell from which ECFC colonies develop has not yet been identified. Current evidence indicates that ECFCs arise from a very small fraction of CD34+, KDR+, CD133-, CD45- cells (17, 120). The estimated prevalence of ECFCs is less than one per 1 million nucleated cells (57, 97), however, which poses difficulties for the characterization of ECFCs. A recent study has attempted to use gene-expression profiling to identify genes that are important for the outgrowth of ECFCs (144).

Table 3. Genes upregulated in CAC compared to MNCs/Monocytes

Gene Function	Selection of upregulated Genes in CAC
Lipid Metabolism	ABCA1, ACAT2, AKR1B1, ALDH1A1, ALDH4A1, ALDH7A1, APOC1, APOC2, APOE, DHRS9, FABP3, FABP4, FABP5, FABP5L1, GLA, LIPA, LPL, MGLL, MSR1, NR1H3, OLR1, PLTP, PPAP2B, PPARG3, PTGR1, SCD
ECM Remodeling/ Homing	CTSB, CTSD, CTSK, CTSL1, LGMN, MMP12, MMP7, MMP9, TIMP2, LAMB2, LAMC1, ITGB5
Oxidative Stress	NQO-1, ATOX1, GCLC, GPX3, MT1G, MT2A, PRDX1, TXN,TXNRD1
Chemotaxis	CXCL16, CXCL12 (SDF1?), CCL8 (MCP-2), VEGFB, TNFRSF12A, IGF1

The authors compared different culturing media, with the intention of identifying an animal- serum-free equivalent to the conventional medium, with fetal calf serum. No outgrowths occurred in serum-free media, however, and replated ECFCs showed greatly reduced pro-liferation. Gene-expression analysis showed significant upregulation in genes related to lipid biosynthesis, stress response, and apoptosis, and a marked downregulation in genes associated with proliferation. The authors concluded that lipid supplementation may enhance ECFC outgrowth in serum-free media. This study illustrates how transcriptome analysis can be used to find leads for improving culturing methods in the development of a consistent cell therapy.

EPCs in Health and Disease

Next to providing insight into EPC identity and differentiation, transcriptome analysis provides a powerful tool to elucidate molecular changes in EPCs of patients with (risk factors for) cardiovascular disease. Several studies have shown that numbers of cEPCs (128), cultured CACs (128), and CFU-Hills (48) are reduced in patients with arterial disease or risk factors for cardiovascular disease, such as metabolic syndrome (138), chronic kidney disease (60, 137), and diabetes (119). Furthermore, it has been shown that BM from patients with ischemic cardiomyopathy (45) has an impaired regenerative potential when compared with BM from healthy donors. A role for disease-related oxidative stress in modulating EPC number and function has previously been implied, although the molecular effects of disease on EPC gene expression remain unclear. Sorrentino et al. (111) show increased levels of superoxide in EPC of type 2 diabetes mellitus (DM) patients, and concomitantly reduced availability of nitric oxide (NO) in EPCs. The EPCs from patients show a dramatically reduced re-endothelialization capacity in a carotid injury model.

Patients with severe arterial disease are the primary target population for regenerative cell therapy, which currently most often involves autologous BM-derived cell administration (8, 29, 81, 112). However, because of the impaired regenerative potential of EPCs in these patients, therapeutic effectiveness of such cell therapy is likely to be suboptimal. Importantly, previous studies have demonstrated that EPC dysfunction is at least in part reversible. A number of compounds have been shown to improve the EPC functional capacity or to promote their mobilization, such as antioxidants (52), statins (138), erythropoietin (EPO) (44, 136), and PPAR- γ agonists (111, 134). The mechanisms by which these compounds act on EPCs are incompletely understood, as some act through pleiotropic effects rather than the intended targets for which the drugs have been designed. Transcriptional profiling could elucidate effects of the previously mentioned compounds on the entire EPC transcriptome. Moreover, transcriptome analysis could identify EPC gene clusters with differential expression between patients and healthy controls and facilitate the selection of novel, more- specific therapeutic targets.

The vast majority of studies on EPC transcriptomes in healthy and diseased conditions are conducted in CACs. Fiorito et al. (34) used microarrays to study the effects of tumor necrosis factor (TNF)-α and antioxidants vitamin C and E on CAC gene expression. The authors identified a small set of genes modulated by TNF-α or the antioxidants, although they stated that the biologic significance of these genes in the growth, survival, differentiation, and function of EPCs should be determined by further investigation. Furthermore, two studies investigated the gene-expression profiles of CACs in type 1 DM patients compared with healthy controls (74, 126). Within the modulated gene set, both studies reported increased expression of antioxidant genes in diabetic versus healthy CACs, presumably as a result of diabetes-induced increase in oxidative stress levels. Interestingly, the latter study further demonstrated that folic acid supplementation altered gene-expression profiles of CACs in patients to resemble those of healthy subjects. The effects of EPO on monocyte and CAC gene-expression profiles from patients with combined renal and heart failure are currently being evaluated in the EPOCARES clinical trial (125). To our knowledge, Tan et al. (117) are the first to compare ECFCs from patients with proliferative diabetic retinopathy (PDR) and healthy controls at the transcriptome level. The authors found a number of genes to be upregulated in ECFCs of PDR patients, including two known angiogenesis inhibitors, thrombospondin-1 and tissue inhibitor of matrix metalloproteinases-3 (TIMP-3), possibly explaining (part of) the vascular complications in diabetes patients. Transcriptome analysis of BM samples obtained from patients receiving autologous cell therapy may provide information to predict the response to therapy. Comparing responders and non-responders to autologous EPC therapy could identify signature genes that correlate with functional improvement, while also providing insight into molecular mechanisms of EPC-mediated neovascularization. In an analogous example, Schirmer et al. (106) compared transcriptomes of circulating naïve (monocytes, T-cells, CD34+ cells) and stimulated (LPS-stimulated monocytes and plastic-adherent monocytes/macrophages) cells of arteriogenic responders and non-responders in a patient population with coronary artery disease. Overall, the authors found that in nonresponders, arteriogenesis is inhibited rather than that it is enhanced in responders, as they had hypothesized. Most pronounced differences were found in the stimulated cells, with monocytes from nonresponders expressing more interferon beta after stimulation with LPS. Subsequent experiments showed that interferon β inhibited the arteriogenic response in a murine hindlimb-ischemia model (106). Taken together, these studies illustrate that transcriptome profiling can help to unveil key mechanisms that are modulated by disease in EPCs. Knowledge thus obtained may contribute to ameliorating EPC dysfunction and optimizing EPC therapy.

CONCLUSION AND PERSPECTIVES

At present, transcriptome profiling is a widely used tool for the analysis of gene-expression differences between different cells, treatments, or populations. In this review, we summarized EPC transcriptome analyses to serve as a base for understanding the identity, differentiation, and pathophysiology of different EPC types currently described in the literature. Our meta-analysis of transcriptome studies of cells from the hematopoietic and endothelial lineages demonstrates that transcriptomes can be used for classification and characterization of a particular cell in an unbiased manner. This approach is therefore a powerful addition to characterization based on cell-surface markers only. Once a cell has been characterized, signature genes can be identified from comparative analysis, allowing subsequent easy and high- throughput analyses (77). The currently available transcriptome datasets are few in number, making meta-analyses underpowered, and are hampered by the fact that they are performed by different groups, by using cells from different donors analyzed on different platforms. A direct comparison of transcriptomes from different EPC types to related circulating cells would be a more promising approach to enable the identification of cell-specific properties and marker genes, yielding valuable information about EPC biology. The acquired information may prove to be crucial for future therapeutic use of EPCs. Insight into the ontogeny will provide tools for the optimization of cell selection, culturing, and treatment for therapeutic use. Based on the identification of therapeutically relevant EPC types, endogenous cells may be selectively isolated or targeted. For example, the low efficiency and relatively long expansion period to obtain ECFCs may be improved by selective isolation based on specific cell-surface markers identified in gene-expression profiling studies (64).

Although gene-expression profiles provide insight into the (patho)physiological condition of a cell or tissue at a given moment, many biologic effects do not take place at the transcriptional level. For example, protein abundance does not necessarily correlate with mRNA levels. The secretion of growth factors and other substances is regulated mainly at the protein level, depending on intracellular trafficking and phosphorylation (22, 28). Currently, analyses of biological processes by using proteomics and metabolomics approaches are becoming increasingly widespread (10, 80, 113), and analyses of gene-expression regulation by microRNAs increasingly enter the public domain (84, 147). Fleissner et al. (34) recently used an array-based approach to identify microRNAs expressed in CACs and were able to illustrate the role of miR-21 in asymmetric dimethyl arginine (ADMA)- mediated CAC dysfunction. It was shown that miR-21 downregulates the expression of MnSOD, and that levels of miR-21 negatively correlate with migratory capacity in CACs isolated from patients with CAD (34). Medina et al. (82) included a proteomic approach in their ECFC versus CAC comparison, and the proteomics analysis by Prokopi et al. (94) revealed a previously unknown mechanism for the acquisition of

endothelial antigens by CACs. An article by the Mayr group (95) describes the identification of secreted thymidine phosphorylase as an important paracrine factor in the stimulation of angiogenesis by CACs. These studies illustrate the power of proteomics as an additional tool for cell characterization, and hint about integrating analyses of secreted products by proteomic and metabolomic approaches, based on the transcriptome analyses discussed in this review. Altogether, current and future efforts toward the comprehensive profiling of EPC mRNA, protein, and metabolites will provide detailed insight into the biology, function, and potential therapeutic applications of EPCs.

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Abbreviations Used

ADMA	Asymmetric dimethylarginine
BM	Bone marrow
CAC	Circulating angiogenic cell
Cat	Catalase
CD	Cluster of differentiation
cEPC	circulating endothelial progenitor cell
CFU	Colony forming units
CML	Chronic myeloid leukemia
CXCR4	CXC chemokine receptor 4
Dll4	Delta-like ligand 4
DM	Diabetes mellitus
ECFE	Endothelial colony forming cell
ECM	Extracellular matrix
eNOS	endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
EPO	Erythropoietin
GPX	Glutathione peroxidase
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen (major histocompatibility complex)
HOX	Homeobox
HMEC	Human microvascular endothelial cells
HUVEC	Human umbilical Vein endothelial cells
IGF	Insulin-like growth factor
IL	Interleukin
KDR	Kinase insert domain receptor
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
PDR	Proliferative diabetic retinopathy
ROS	Reactive oxygen species
RUNX1	Runt-related transcription factor 1
SDF	Stromal cell derived factor
TIMP-3	Tissue inhibitor of matrix metalloproteinases-3
TLR	Toll-like Receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelium growth factor
VEGFR	Vascular endothelium growth factor receptor
vWF	von Willebrand Factor
WAS	Wiskott-Aldrich syndrome

SUPPLEMENTARY INFORMATION

Supplementary Methods

An initial search of the GEO(2, 8) and ArrayExpress(1, 23) databases was conducted to identify all published transcriptomes of different EPC types. Different synonyms for CACs, ECFCs and CFU-Hills were used. Series were only included if raw data files (affymetrix *.CEL or unnormalized *.txt files) were available. Initially, transcriptomes on different platforms were cross-annotated on Unigene ID, the different array platforms invariably clustered together, however (data not shown), as has been previously reported(30). The search was therefore restricted to the platform on which the largest number of different EPC transcriptomes was available, the Affymetrix HU 133 plus 2.0 chip. Preliminary attempts at clustering also showed that biological replicates had a high tendency to cluster together, therefore only one sample per series chosen at random. The search was then extended to other mesenchymal tissues using different search terms for monocytes, T-cells, B-Cells, Endothelial Cells, Adipose tissue, skeletal muscle, bone marrow, CD34+ cells, CD133+ cells and MSCs. From studies comparing treated to untreated samples or healthy versus disease conditions the only untreated or healthy samples were included. Series in which the supplied meta-data were insufficient to assure proper identification of tissue or cell type were excluded. For an overview of the included studies, see the table below. Raw data files were subsequently preprocessed and co-normalized using the gcRMA (GC Robust Multichip Average)(34) package in Bioconductor (11). This was done to minimize differences between the laboratories that have supplied the different data series. Density-plots and boxplots of all included samples were subsequently inspected to assure adequate normalization. Unsupervised agglomerative hierarchical clustering was then performed in R(26) on all included data-sets, using 1-R (the Pearson product-moment coefficient) as distance measure, using complete linkage.

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Supplemental Table 1. Studies Included in Meta-Analysis

GEO Series	GEO Sample	Description	Article Citation
GSE3526	GSM80577	Bone Marrow 1	Unpublished
GSM80580	Adipose Tisse 1		
GSE5110	GSM103557	Skeletal Muscle 1	(32)
GSE6054	GSM140248	CD14+ Monocytes 1	(22)
GSE6241	GSM143726	HUVEC 1	Unpublished
GSE7307	GSM175836	Adipose Tissue 2	Unpublished
GSM175883	Skeletal Muscle 2		
GSM175974	Bone Marrow 2		
GSM176003	Monocytes 1		
GSM176005	T-Cell 1		
GSM176007	B-Cell 1		
GSM176283	Cd4+ T-Cell 1		
GSM176285	CD8+ T-Cell 1		
GSE9451	GSM239713	MSC1	(15)
GSE9520	GSM241198	MSC	(16)
GSE9593	GSM242185	MSC2	(33)
GSE9894	GSM250020	MSC3	(6)
GSE9927	GSM251126	Cd4+ T-Cell 2	(28)
GSE11367	GSM287220	VSMCs	(27)
GSE12155	GSM306074	CFU-Hill	(7)
GSE12366	GSM310433	Plasma Cell	(19)
GSM310435	Naive B-Cell		
GSE12891	GSM323168	CB-CAC	(20)
GSM323169	CB-ECFC		
GSE13205	GSM333455	Skeletal Muscle 3	(9)
GSE13899	GSM350085	CD14+ Monocytes 2	(4)
GSE13987	GSM351411	B-Cell 2	(21)
GSM351423	T-Cell 2		
GSE16615	GSM414920	Adipose Tissue 3	(12)
GSE16683	GSM418127	HUVEC 2	(29)
GSE17090	GSM427350	ASCs	(18)
GSE17777	GSM443878	HMEC	(13)
GSE18113	GSM452740	HMVEC	(17)
GSE18583	GSM462218	Skeletal Muscle 4	(31)
GSE19429	GSM483486	BM CD34+	(25)
GSE19735	GSM492833	HUVEC 3	Unpublished
GSM492834	HU SMC		
GSM492837	Human Cord Blood CD34+		
GSE20086	GSM501894	Fibroblast	(3)
GSE20505	GSM515246	BM CD34+2	Unpublished
GSE21164	GSM529756	Skeletal Muscle 5	Unpublished
GSE12277	GSM308241	Mobilized CD34+ Old	Unpublished
GSM308317	Mobilized CD34 + Young		
GSE11092	GSM280220	Mobilized CD34+	(10)
GSE12662	GSM317934	CD34+ Bone marrow	(24)
GSE8023	GSM198051	CD 34+ CB cells	(14)
GSE12646	GSM364548	Mobilized CD133+ cells	(5)

Hendrik Gremmels[1], Joost O. Fledderus[1], Nicole Bleeker [1],Olivier G. de Jong[1] and Marianne C. Verhaar[1]

[1] Departement of Nephrology and Hypertension, University Medical Center Utrecht, The Netherlands

CHAPTER 7

THE TRANSCRIPTION FACTOR NUCLEAR FACTOR E2-RELATED FACTOR 2 (NRF2) PROTECTS ANGIOGENIC CAPACITY OF ENDOTHELIAL PROGENITOR CELLS IN HIGH OXYGEN RADICAL STRESS CONDITIONS

Manuscript in Prepration

Background
Endothelial Colony Forming Cells (ECFCs) are a type of endothelial progenitor cells that has shown great promise in tissue engineering of vascular constructs and as cell therapy for ischemic vascular disease. After implantation ECFCs are subjected to ischemia and a potentially unfavorable patient milieu. Nuclear factor E2-related factor 2 (Nrf2) is a cytoprotective transcription factor that regulates the expression of endogenous anti-oxidant enzymes in response to oxygen radical stress.

Objectives
To investigate the role of the Nrf2/ antioxidant response element (ARE) in ECFC function and explore potential pre-treatment strategies with an Nrf2 activating compound.

Methods
Nrf2 activation was quantified by transducing ECFCs with an ARE-luciferase reporter construct. Gene expression of Nrf2 target genes was measured by quantitative PCR. Stable knockdown of Nrf2 and the physiological inhibitor of Nrf2, Keap1, were achieved by transducing ECFCs with lentiviral shRNA vectors against these genes. Response to sulforaphane (SFN) was assessed. Antioxidant activity of SFN pre-treated or transduced ECFCs was measured by CM-DCFDA fluorescence in the presence of H_2O_2. Endothelial function was assessed by endothelial scratch-wound-closure and by tubule formation on matrigel. All experiments were conducted on primary cells isolated from several donors.

Results
Knockdown of Nrf2 lead to a reduction of antioxidant gene expression and increased intracellular ROS. Functional experiments showed that endothelial tubule formation is disturbed after Nrf2 knockdown even in the absence of extracellular ROS. Conversely, tubule formation is preserved in high extracellular ROS conditions after knockdown of Keap1. ECFCs showed a time- and dose dependent response to Nrf2 activator SFN as measured by luciferase reporter or by expression of Nrf2 target genes. Pre-incubation of ECFCs with SFN reduced intracellular radical oxygen species (ROS) in the presence of H_2O_2.

Conclusion
The results of this study indicate that Nrf2 plays an important role in ECFC angiogenic capacity. Pre-conditioning ECFCs with SFN prior to implantation may be a protective strategy for tissue engineered constructs or angiogenic cell therapies.

INTRODUCTION

The formation of new vasculature is of critical importance for tissue regeneration after ischemic injury. Post-natal neovascularization occurs classically through the extension of pre-existing vascular networks. Increasing evidence shows that *de novo* formation of vascular structures persists in the adult organism (1). It is thought that a rare subset of progenitor cells, also termed Endothelial Colony Forming Cells (ECFCs) resides within the vascular wall (2), which can be mobilized into circulation in response to vascular injury and contributes to the formation of new vessels. ECFCs can be isolated from the blood of both adults (3) as well as cord blood (4). The ability of ECFCs to organize into functional neovessels (5) has found practical applications both in cell therapy for ischemic vascular disease (6) and tissue engineering (7,8)

The tissue microenvironment in ischemic areas is characterized by an excess of Reactive Oxygen Species (ROS), leading to oxidative stress (9). This oxidative stress leads to dysfunction of the endothelium and exacerbates vascular disease (10-12). ECFCs are particularly sensitive to oxidative stress (13,14), which may reduce the effectiveness of ECFCs as cell therapy or in tissue engineered constructs.

The expression of endogenous cellular anti-oxidant defenses is regulated through a common promoter site called the Antioxidant Responsive Element (ARE)(15,16). AREs in turn, constitute a binding site for the transcription factor Nuclear Factor Erythoid Like Related Factor 2 (Nrf2)(17). Nrf2 belongs to the Cap'n'collar subset of basic leucine-zipper (bZip) transcription factors (17). In the resting cell, Nrf2 is sequestered in the cytoplasm by its inhibitor, Kelch ECH associating protein (Keap1), and is continuously targeted for ubiquitination (18). Upon exposure to oxidative stress, Keap1 undergoes a conformational change and releases Nrf2, which can then translocate to the nucleus and initiate an ARE dependent transcriptional response (17,19).

Activation of Nrf2 reduces oxidative stress in endothelial cells (20) and suppresses inflammatory responses that may lead to cardiovascular disease. Age-induced impairments in Nrf2 activation are associated with atherosclerosis and vascular dysfunction (21-23) and treatments activating Nrf2 may attenuate the progression of atherosclerosis (23). Nrf2 knockdown studies have shown that loss of Nrf2 strongly impairs tubule formation of endothelial cells on matrigel (24,25).

In the present study we investigate the role of Nrf2 in ECFCs by examining the effects of Nrf2 pathway modulation on functional progenitor characteristics of ECFCs. Furthermore we explore pre-treatment options with the selective Nrf2 activator sulforaphane (SFN), designed to reduce ECFC susceptibility to oxidative stress, which may provide benefit in clinical therapeutic applications of these cells.

METHODS

Reagents

All reagents were purchased from Sigma Aldrich (Zwijndrecht, NL) unless otherwise specified.

Cell Isolation and Culture

Human cord blood (50-90 ml) was collected from full term pregnancies, in Acid-Citrate-Dextrose anti-coagulated blood collection bags as in (26). Mononuclear cells (MNCs) were isolated by density-gradient centrifugation using Ficollpaque (GE-Healthcare) and resuspended in EGM-2 growth medium (Lonza, Wakersville, MD, USA), containing SingleQuots (hEGF, VEGF, hFGF-2, Long R3-IGF-1, heparin, gentamicin/amphotericin B) and 10% Fetal Calf Serum. MNCs were then plated at a density of $2 \bullet 10^6$ cells/cm^2 on rat tail collagen I (BD Biosciences, Bedford, MA) coated wells and cultured at 37°C, 5% CO_2 in a humidified incubator. After 24 h, non-adherent cells were aspirated and complete EGM-2 medium was added to each well. Medium was replaced every other day thereafter. After 8-10 days, colonies with a typical cobble-stone morphology started to appear, and cells were harvested for further use upon reaching confluency.

Lentiviral Constructs

The ARE reporter construct was kindly provided by Dr. Hanna Leinonen of Kuopio University. It contains three sequential ARE sequences taken from the promoter region of the Glutamate-Cysteine Ligase, regulatory subunit (GCLM) gene, driving a firefly luciferase gene.

The plasmids containing short-hairpin RiboNucleic Acid (shRNA) against Nrf2 and Keap1 were purchased from Open Biosystems. 5 different shRNA sequences against the respective genes were used simultaneously in each experiment. The plasmids contain a Puromycin resistance gene to facilitate selection.

Lentiviruses were produced using the ViraPower expression system (Invitrogen). Plasmid DNA was complexed using polyethyleneiminie (PEI) and added to a 15-cm dish containing 293T cells. 48 hours after transfection the virus containing supernatant was harvested, aliquotted and stored at -80°C.

Lentiviral transduction of ECFCs

Lentiviral particles were charge-neutralized using hexadimethrine bromide (Polybrene) and added to a T-25 flask containing ECFCs at 50% confluency. After 24h medium was replaced and after 72h puromycin selection was initiated, and cells were cultured in the presence of 2μg/ml for 7 days.

Nrf2 pathway modulation

We stably transduced ECFCs of different donors with lentiviral vectors expressing short hairpin Ribonucleic Acids (shRNAs) against Nrf2 and against Keap1 the physiological inhibitor of Nrf2.

To assess the possibility of using Nrf2 activation as a pre-treatment strategy, we used the Nrf2 activator Sulforaphane (SFN) to induce translocation of Nrf2 to the nucleus. ECFCs were incubated with different concentrations of l-sulphoraphane (Sigma) for 4 hours unless otherwise specified, prior to exposure to H_2O_2.

Gene Expression by PCR

Total RNA was isolated using spin columns (RNAspin mini, GE Healthcare, Buckinghamshire, UK), and quantified by spectrophotometry (ND-1000, Nanodrop technologies). First-strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Cat. No. 170-8891, Bio-Rad, Herculus, CA) according to the manufacturer's instructions. Specific primers for Heme-Oxygenase (decycling) 1 (HO-1), NAD(P)H dehydrogenase [quinone] 1 (NQO1), Glutamate-cysteine ligase, catalytic subunit (GCLC), Glutamate-cysteine ligase, regulatory subunit (GCLM) and Nuclear Factor (erythroid-derived 2)-like 2/NF-E2-related factor (NRF-2) were designed, as well as primers for several house-keeping genes (see supplementary table 1 for sequences). Primers were designed to work at an annealing temperature of 60 degrees Celsius, in cases where primers functioned sub-optimally, the optimal annealing temperature was empirically established by setting a temperature gradient on the thermocycler.

The real-time PCR analysis was performed with iQ™ Sybr Green Supermix (Cat. No. 170-8885, Bio-Rad, Hercules, CA), conducted according to the instructions of the manufacturer. The final reaction volume was set at 15 µL. The samples were processed in MyIQ PCR system (Bio-Rad, Hercules, CA) and analyzed using MyiQ System Software, Version 1.0.410 (Bio-Rad Laboratories Inc.). After a hot start of 3 min, each cycle consisted of a denaturation step at 95 °C for 20 s, an annealing step specific for each set of primers for 30 s and an elongation step at 72 °C for 30 s. After 45 cycles a melting curve was obtained by increasing the temperature with 0.5 °C increments from 65 °C to 95 °C. With every run, as internal calibration, a 10-fold dilution series of reference cDNA was included, attained by mixing equal amounts of cDNA from each sample and subsequently diluting the mixture in nuclease free water. The reaction efficiency was calculated by using the formula $10^{-1/\text{slope.}}$ (27)

Data were analyzed using the efficiency corrected Delta-Delta-Ct method(28). The Fold-change values of the genes of interest (GOIs) were normalized using the geometric average of the Fold-change values of multiple housekeeping genes. The best house-keeping genes were selected by implementing the pair-wise variance algorithm introduced in(29), using the geNorm applet (http://medgen.ugent.be/~jvdesomp/genorm/). Expression values

were subsequently analyzed across biological replicates by using an experiment-mean centered approach(30).

Western Blot

Cultured cells were lysed in modified RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X100, 1mM EDTA and cOmplete Protease inhibitors [Roche]) or nuclear extracts were made using the Nuclear Extract Kit by ActiveMotif. Total protein concentration was determined using the Bicinchoninic Acid (BCA) method (Pierce, Rockfort, IL). Equal amounts of protein were loaded in a 4-12% SDS-PAGE gel (life technologies), the gel was set to run at 200V and protein was subsequently transferred to a polyvinylidene fluoride (PVDF) membrane.
After blocking the membrane was stained with anti-Nrf2 (H300, Santa Cruz #sc-13032, Dallas, TX) and developed using a chemiluminescent peroxidase substrate (Sigma). Anti ß-actin was used as loading control.

Oxidative Stress Measurements with CM-H2DCFDA

The effect of Nrf2 pathway modulation on intracellular ROS was determined using 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, (CM-H_2DCFDA, life technologies).
ECFCs were incubated for 1h with 5µg/ml CM-H_2DCFDA, washed once, and were allowed to recover for 30 minutes before being exposed to increasing concentration of H_2O_2. Intracellular fluorescent signal was measured on a fluorescent plate-reader at Ex/Em 485/538nm after two hours. Cell number was corrected for using PrestoBlue® cell viability reagent (life technologies).

Gamma H2AX Staining

ECFCs were grown until confluence on Labtek II chamberslides and exposed to H_2O_2 for 4 hours as indicated. Cells were subsequently fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton. Cells were stained overnight with anti-Ser139 H2AX (gH2AX) (Millipore). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI). Images were taken on an Olympus BX61 fluorescent microscope and analysis of the images was performed using ImageJ. Images were segmented into ROIs on the basis of the DAPI image and for each individual nucleus the total fluorescence of the gH2AX signal was recorded, as well as the number of damage foci.

Scratchwound Assays

To assess horizontal ECFC migration and response to damage signals, a scratchwound-assay was performed (31). ECFCs were grown until confluence in a 24-well plate on which reference lines were drawn using a laboratory marker. A scratch in the monolayer

was made, detached cells were washed off and a range of H_2O_2 dissolved in EGM-2 was added to the wells. Photographs were made on demarcated reference points at baseline and t=6 hours. Average scratch width per high powered field was calculated by dividing the area of the scratch by the length of the scratch and migration was subsequently calculated by subtracting width at t=0 by width at t=6h.

Tubule formation on Matrigel

Tubule formation on matrigel was performed similarly to (26). The inner well of an IBIDI µ-slide angiogenesis (IBIDI, Martinsried, Germany) was filled with 10µl growth factor reduced matrigel (BD). Next 10^4 ECFCs containing various constructs were suspened in 50µl EGM-2 containing different concentrations of H_2O_2 and laid on top of the matrigel. Cells were allowed to form tubular networks for 6 hours, after which photographs were taken. Tubular complex length and number of junctions were quantified using the freeware program AngioQuant (32).

Statistics

Results were analyzed by 1-way Analysis Of Variance (ANOVA) in dose-response experiments and by 2 way ANOVA with interaction the knock-down experiments. Replicates indicate ECFCs from multiple human cord-blood donors. All data are represented as means +/- Standard Error of the Mean (SEM).

RESULTS

The effects of Nrf2 pathway modulation on functional progenitor characteristics of ECFCs

To obtain more insight into the role of Nrf2 in ECFCs, we stably transduced ECFCs of different donors with lentiviral vectors expressing short hairpin Ribonucleic Acids (shRNAs) against Nrf2 and against Keap1 the physiological inhibitor of Nrf2. Nrf2 knockdown efficiency was 60% using the lentiviral shRA construct and largely prevented upregulation of HO-1 in response to SFN stimulation (Figure 1). A similar efficiency of knockdown was observed with shKeap1, which lead to a basal activation Nrf2 signaling as indicated by increased HO-1 expression, regardless of SFN stimulation.

Nrf2 is important in the management of intracellular ROS levels

To evaluate the effects of modulation of the Nrf2/ARE system on oxidative stress, we used the reporter dye CM-DCFDA to assess intracellular ROS levels (Figure 2) Cells were loaded with CM-DCFDA for 30 min and exposed to increasing concentrations of H_2O_2. The cells showed a dose-dependent increase in intracellular ROS upon H_2O_2 exposure,

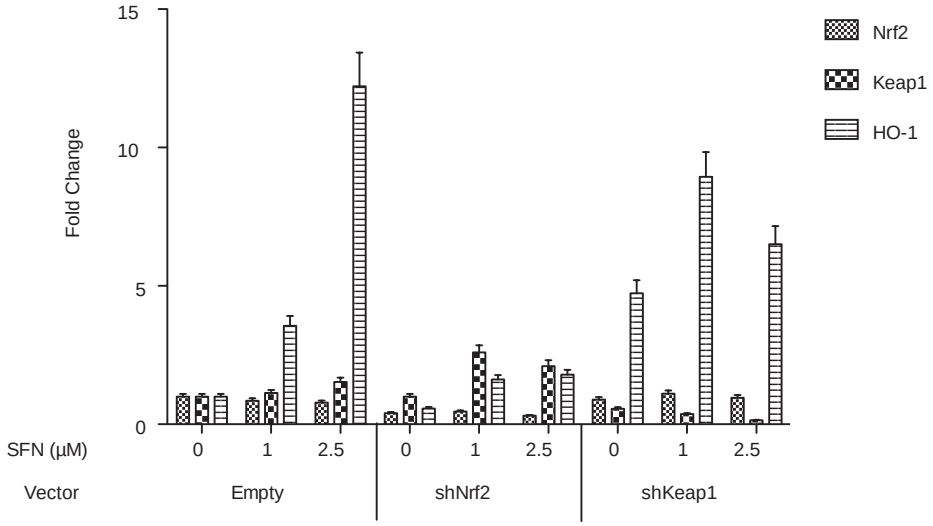

Figure 1. Confimation of Nrf2 and Keap1 knockdown by ShRNA and effects on HO-1 as Nrf2 target gene. Data represent 3 independent biological replicates

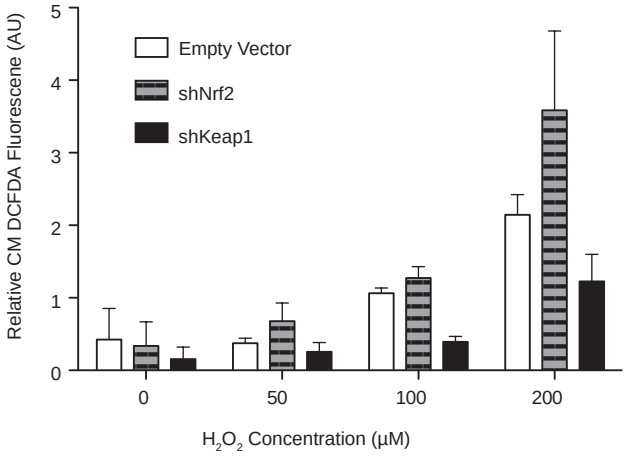

Figure 2. Nrf2 Pathway Modulation and Cell Stress. A. Intracellular ROS levels as measured by CM-H 2 DCFDA Fluorescence in ECFCs transduced with short hairpin vectors to induce Nrf2 and Keap1 knockdown. Keap1 knockdown significantly reduces oxidative stress, whereas Nrf2 knockdown increases susceptibility to higher concentrations of H_2O_2. Graphs represent mean +/- S.E.M., data are from 3 independent biological replicates.

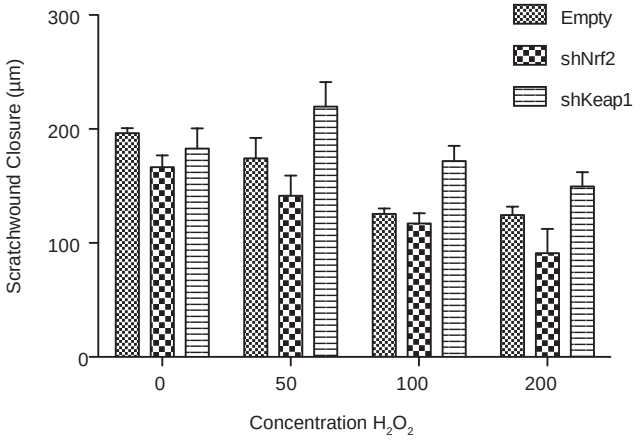

Figure 3. Scratchwound Closure. Endothelial Scratchwound Closure in Nrf2- and Keap1 knockdown ECFCs in the presence of increasing concentrations of H_2O_2. Graphs represent mean +/- S.E.M., data are from 4 independent biological replicates.

knockdown of Nrf2 lead to higher intracellular ROS levels at given concentrations of H_2O_2. Knockdown of Keap1 lead to very low intracellular ROS levels, even in the presence of high concentrations of H_2O_2.

As readout for secondary effects of oxidative stress we used double stranded DNA breaks, as quantified by phosphorylation of pSer139 on histone 2AX (gH2AX). Individual foci were quantified by immunofluorescence on a minimum of 100 nuclei per condition. Untreated ECFCs had very few damage foci at resting conditions (Supplemental Figure 1a), but the number of foci increased significantly after incubation with H_2O_2 (Supplemental Figure 1b). Cells transduced with shNrf2 showed an increased number of damage foci at resting conditions compared to shKeap1 or Empty Vector controls (Supplemental Figure 1c). Upon exposure to H_2O_2 a further increase in the number DSBs was observed (Supplemental Figure 1D).

Involvement of Nrf2 in Angiogenesis

We used a scratch-wound closure assay to assess how oxidative stress impairs ECFC function and the effects of Nrf2 thereupon. A scratch was made on a confluent ECFC monolayer using a pipet tip. Cells were then exposed to increasing concentrations of H_2O_2 and closure of the scratch was monitored over time. We observed a dose-dependent inhibition of scratchwound closure under the influence of H_2O_2. This effect was magnified when Nrf2 was knocked down using shRNAs, knock-down of Keap1 resulted in an increased scratch closure and reduced the effects of H_2O_2 (Figure 3).

We next evaluated the effects of modulating the Nrf2/ARE system in the presence of oxidative stress on ECFC tubule formation on matrigel. ECFCs transduced with different

vectors were placed on matrigel and exposed to increasing concentrations of H_2O_2. Pictures were made after 6 hours and the vascular networks were quantified. In the Empty Vector control we observed a reduction in tubule formation in the presence of high concentrations of H_2O_2, with only minimal reticular organization of ECFCs at 200μM H_2O_2 (Figure 4). Knockdown of Nrf2 greatly reduced network formation even in the absence of H_2O_2, and no networks were formed in the presence of H_2O_2. Knockdown of Keap1 was protective of network formation in the presence of H_2O_2 and tubular networks were still observed at the highest assessed concentration of 200μM H_2O_2 (Figure 4).

PRE-TREATMENT NRF2 ACTIVATOR SULFORAPHANE TO REDUCE ECFC SUSCEPTIBILITY TO OXIDATIVE STRESS

Nrf2 activation using Sulforaphane

We used the selective Nrf2/ARE activator l-Sulforaphane (SFN) to induce nuclear translocation of Nrf2 and subsequent expression of ARE target genes. Nuclear translocation of Nrf2 showed dose-dependent increase in response to SFN (Figure 5 A and B) after 4 hours, with a maximum of ca. 4x increased protein abundance. Nuclear translocation of Nrf2 lead to increased expression of Nrf2 target genes Heme Oxygenase-1 (HO-1), Glutamate-cysteine ligase, catalytic subunit (GCLC) and Glutamate-cysteine ligase, regulatory subunit (GCLM) (Figure 5c). Expression of further genes is shown in Supplemental Figure 2.

To demonstrate binding of Nrf2 to genomic ARE sequences we used an ARE driven luciferase reporter system, containing two ARE sequences from the promoter of the Glutamate-cysteine ligase, regulatory subunit (GCLM) gene. A significant time- and concentration dependent increase in luciferase activity was observed after 4 hours, with a maximum at 24h (Supplemental Figure 3).

Pre-incubation with SFN reduces intracellular ROS and preserves ECFC function

To give a functional correlate of increased anti-oxidant gene expression after pre-incubation with SFN, we quantified intracellular ROS levels. We again show a dose-dependent increase in CM-H2-DCFDA fluorescence in the presence of increasing concentrations of H_2O_2. Pre-incubation of ECFCs with 2.5μM SFN reduces ROS levels, especially at high concentrations of H_2O_2 (Figure 6). Pre-incubation of ECFCs with 2.5 μM SFN did not increase scratch-wound closure at baseline, but preserved reduction of migration in the presence of H_2O_2 (Figure 7).

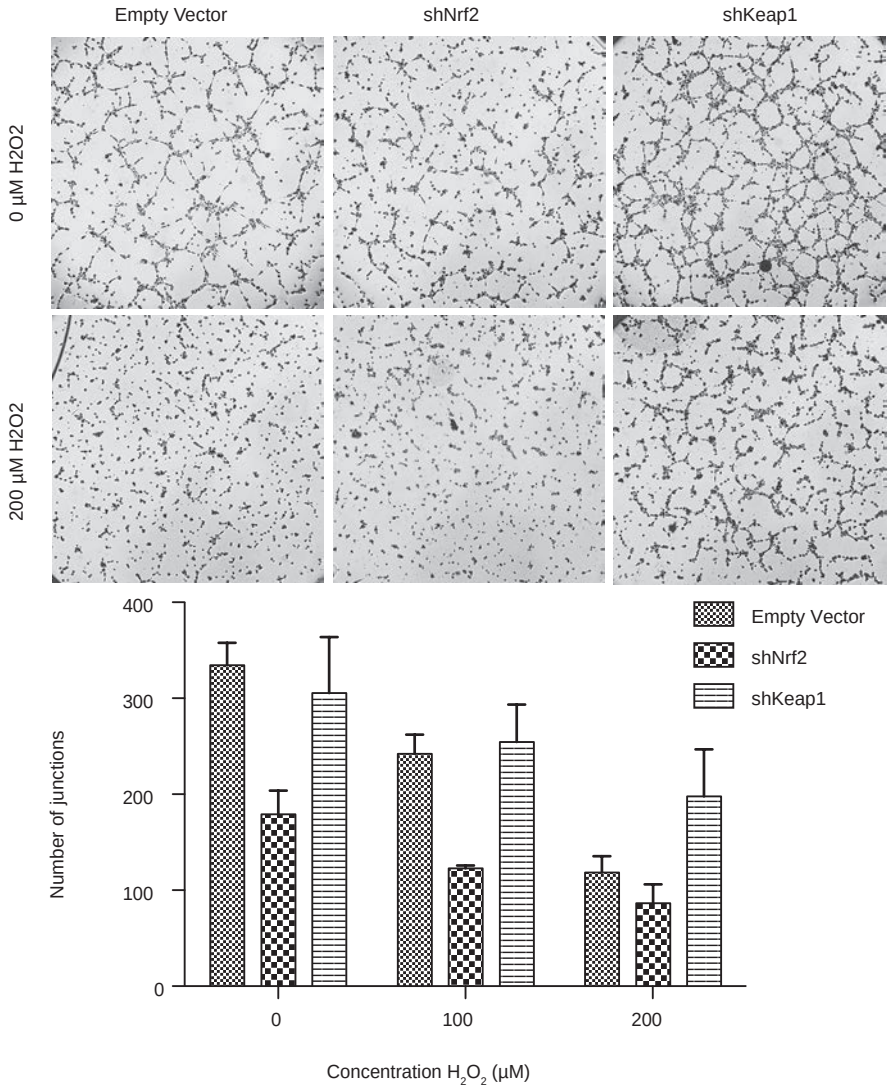

Figure 4. Tubule formation assay. A: Tubule formation assay on matrigel showing that tubule formation is sensitive to ROS. Nrf2 knockdown markedly impairs ECFC ability to form tubules even in the absence of H2O2, whereas Keap1 knockdown acts protective at high concentrations of H_2O_2. B: Quantification of tubule formation by number of junctions. Nrf2 knockdown significantly impairs tubule formation compared to control or Keap1 knockdown ($p=2\times10^{\wedge-7}$). In all conditions there is a dose-dependent inhibition of tubule formation by H_2O_2 ($p=9\times10^{\wedge-11}$). Cells with Keap1-knockdown show a reduced susceptibility to H_2O_2 (p=0.0024). Graphs represent mean +/- S.E.M., data are from 6 independent biological replicates.

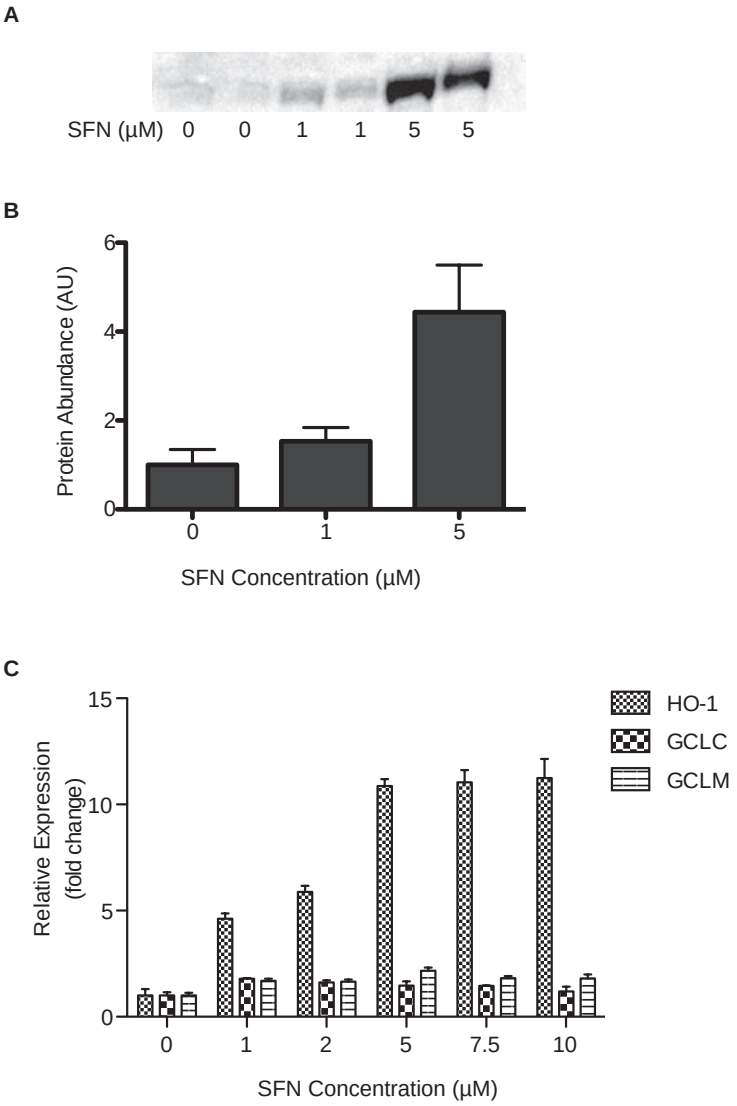

Figure 5. Nrf2 activation by SFN pretreatment in ECFCs. A. Western blot of nuclear extracts of ECFCs exposed to SFN (Nrf2 band at ~ 72kD). **B**. Densiometric quantification of western blot, showing dose-dependent increase of nuclear Nrf2 translocation (p=0.004) **C**. Upregulation of Nrf2 target genes after incubation with sulforaphane (SFN). Glutamate-Cysteine Ligase, Catalytic (GCLC) and regulatory subunits expression is moderately upregulated in ECFCs treated with SFN. Heme-Oxygenase 1 (HO-1) shows a strong dose-dependent increase in expression with a plateau at ca. 5μM. Graphs represent mean +/- S.E.M., data are from 3 independent biological replicates.

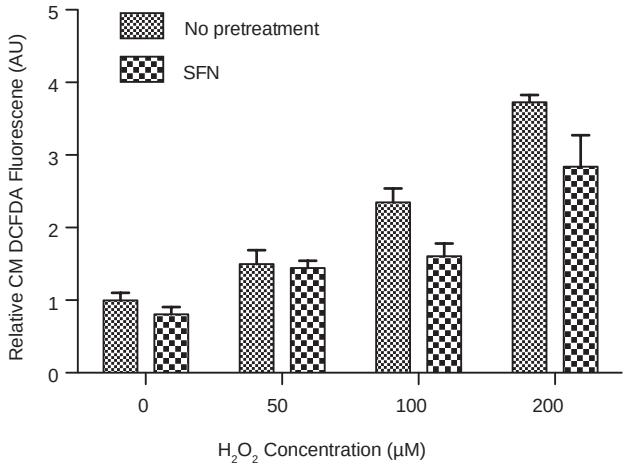

Figure 6. SFN pretreatment reduces ROS. Intracellular ROS as measured by CM DCFDA fluorescence increased dose-dependently with exposure to H_2O_2. Pre-treatment of ECFCs with SFN ameliorated the H_2O_2 induced rise in ROS. Graphs represent mean +/- S.E.M., data are from 3 independent biological replicates.

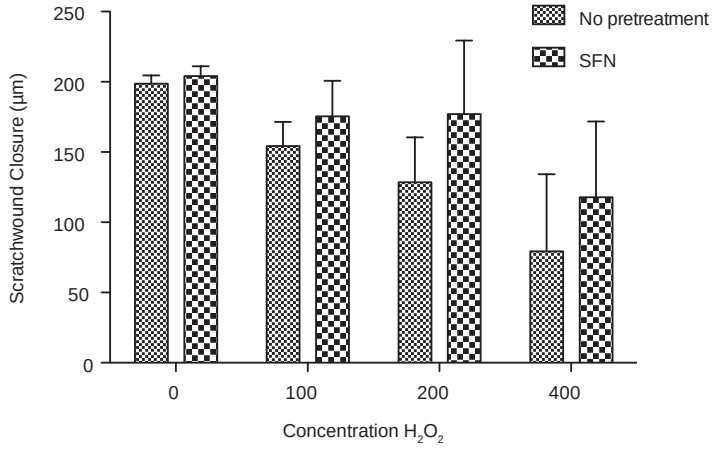

Figure 7. Pre-treatment with SFN protects scratchwound closure. Scratchwound closure was reduced in the presence of increasing concentrations of H_2O_2. Pre-treatment with 2.5µM SFN partially prevented the ROS reduction in migration. Graphs represent mean +/- S.E.M., data are from 3 independent biological replicates.

DISCUSSION

In this study we show that Nrf2 is important in the angiogenic response of Endothelial Colony Forming Cells. Activation of Nrf2 by knockdown of Keap1 preserves cellular functions associated with angiogenesis, such as migration and tubule formation in presence of ROS. Disruption of Nrf2 signaling inhibits these functions, even in the absence of ROS. Pre-treatment with Nrf2/ARE activator SFN preserved ECFC function in conditions with high oxygen radical stress. These findings suggest that Nrf2 is a potential target for modulation of ECFCs in their application as progenitor cell graft.

In the aging western population, there is increasing demand for regenerative medicine to extend the healthy life span. Progenitor cell therapy can be used to restore failing organs or tissues and thus delay the onset of functional decline. Endothelial progenitor cells have applications as separate therapy for vascular disease (33) and are an integral part in vascularized tissue-engineered organ constructs (7,34). ECFCs are the most commonly investigated endothelial cell type, as they are easily obtained from peripheral blood(35) and have the capacity to form vascular networks (36). Both in tissue-engineering(26) and cell therapy (6) survival of ECFC is important for the stability of the newly formed vessels. Studies investigating the cell fate of implanted cells using a luminescent $p67^{phox}$ reporter system have shown that cells are exposed to a great amount of oxidative stress after implantation into ischemic tissue (9). As ECFCs are very susceptible to oxidative stress (13,14), we reasoned that *ex vivo* pre-treatment may be used to protect their *in vivo* function.

We confirm that moderate amounts of ROS can impair survival, migration and angiogenic ability of ECFC, which are important for functioning *in vivo* (6). Our data show that activation of Nrf2 in ECFCs by the knockdown of its inhibitor, Keap-1, limited the effects of oxygen radical stress and preserved endothelial function in an adverse environment. Previous studies (24) have shown an impairment in the migration and tubule-forming ability of coronary arterial endothelial cells upon siRNA knockdown of Nrf2. Kuang et al. (37) observe similar effects in the rat, and show increased susceptibility to hypoxia in Nrf2 knockdown cells. We are the first to show the importance of the Nrf2/ARE system in primary human cells and under adverse conditions these cells may be subjected to in cell therapy or tissue-engineering applications.

Interestingly, our data show that the protective effects of Keap1 knockdown can also be achieved by pre-treating the cells with the Nrf2/ARE activator SFN. SFN is an isothiocyanate compound isolated from broccoli that is a strong inducer of ARE-driven genes (38). It has been administered to patients in several clinical trials and has an excellent safety profile (39-41).

In this study, upon knockdown of Nrf2, tubule formation was profoundly reduced, even in the absence of ROS. These findings have been observed previously (24,25,37) and are

consistent with a role for redox signaling in angiogenesis. ROS have previously been implicated in the signal transduction of angiogenic growth factors such as VEGF and Angiopoietin-1 (42-44). VEGF stimulation has been shown to increase ROS production though NAD(P)H oxidase activation in Endothelial Cells, leading to phosphorylation of VEGFR2 (45). Impairments in growth factor signaling have been observed in Nrf2 knock-out animals, which show impairments in IGF-2 and HIF1α signaling (46,47). This suggests a common role for Nrf2 induced anti-oxidant enzymes in orchestrating redox signaling events, by controlling the activity of the serine/threonine kinase domain of growth factor receptors (24,46). Paradoxically, Nrf2 -/- animals show increased angiogenesis after ischemia, likely due to an upregulation of the inflammatory response (25). This suggests that pre-treatment strategies should be applied *ex vivo* on the cell graft only, rather than a systemic preconditioning of the patient.

The present study has several limitations: firstly the ECFCs used were obtained from cord blood and not from adult peripheral blood as they would for clinical applications. There are indications that functional differences in angiogenic ability exist between neonatal and adult ECFCs, that are related to increased senescence(36). Vascular ageing has been shown to be associated with deficient activation of the Nrf2/ARE system in response to oxidative stress(23), which may limit pre-treatment strategies.

In summary, the present study confirms the role of Nrf2 in angiogenesis and shows its protective role against oxidative stress in ECFCs. These results indicate that Nrf2 modulation, for example with SFN may be a pre-treatment strategy to enhance the survival of ECFCs in cell therapy and tissue engineering applications.

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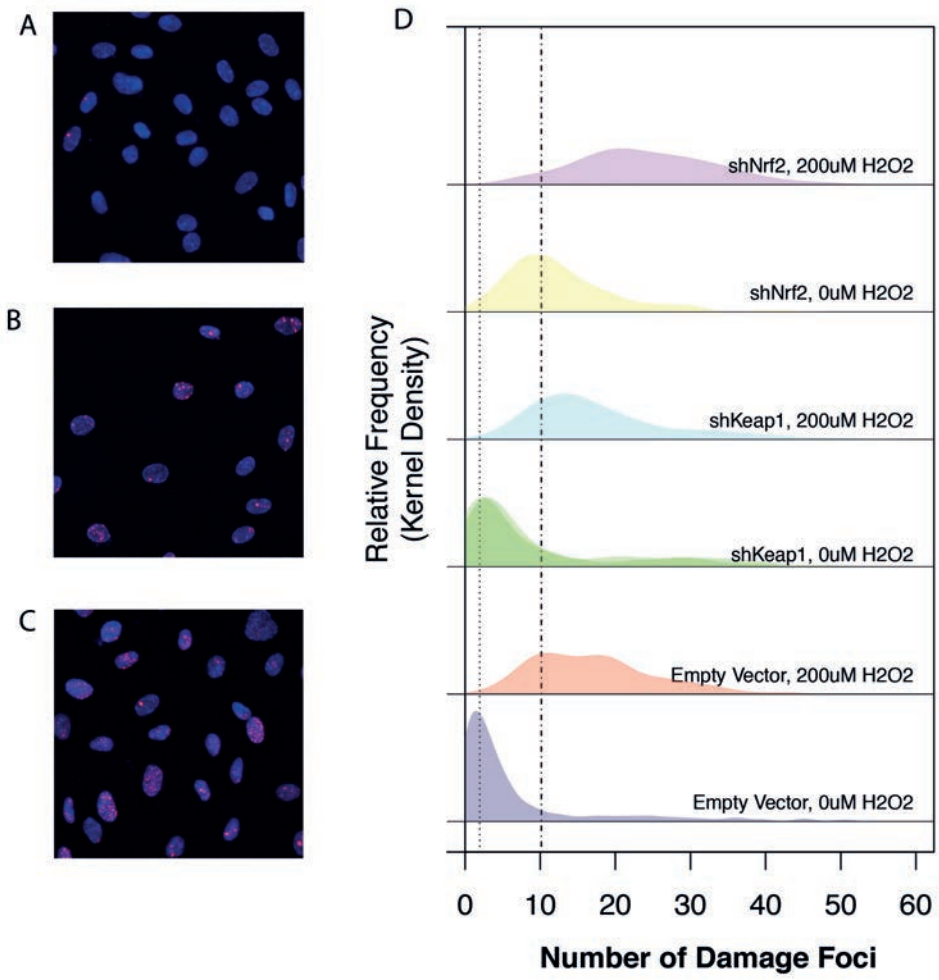
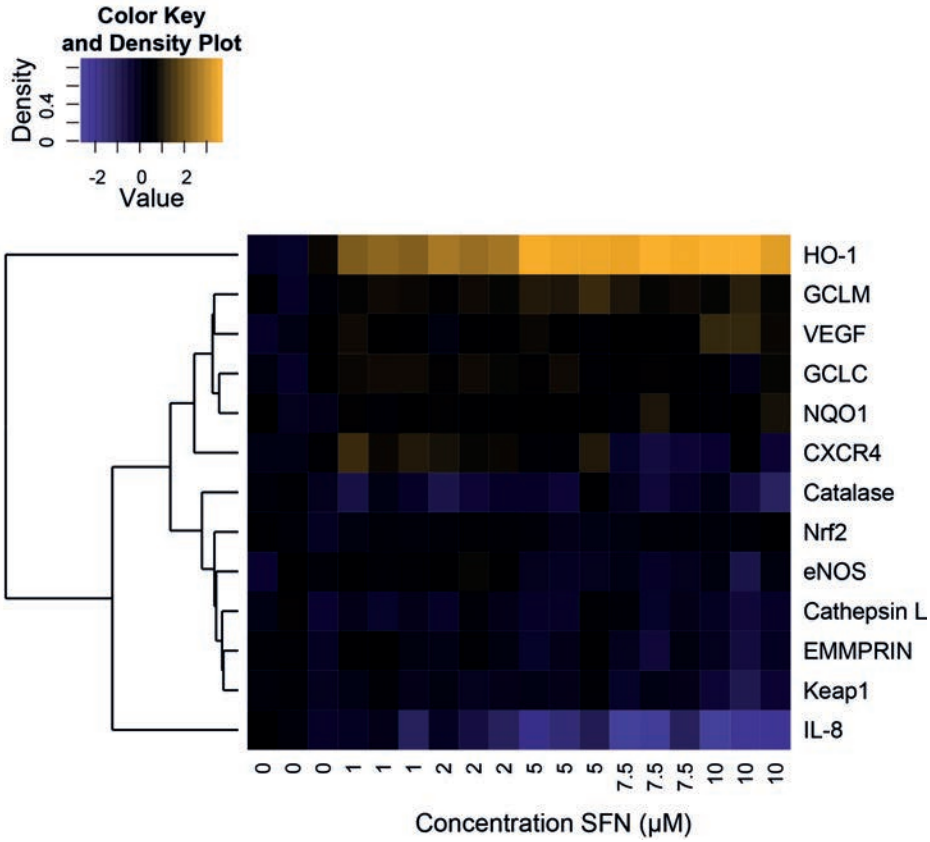
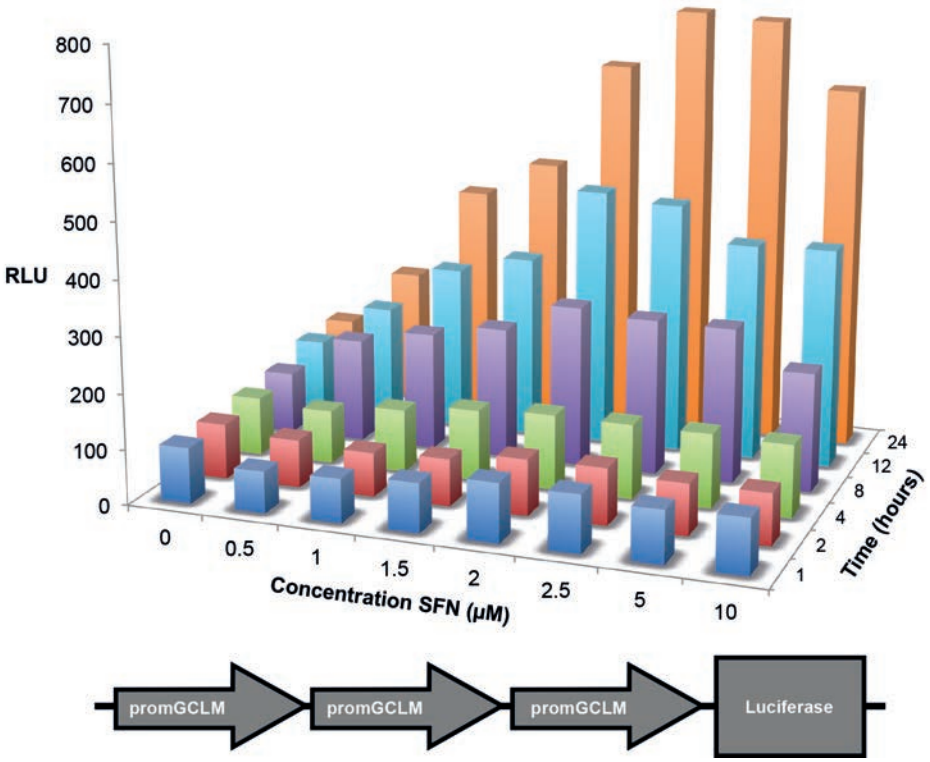
SUPPLEMENTARY INFORMATION



Supplemental Figure 1. DNA damage by γ H2AX staining, typical damage foci reflecting DNA repair after damage. A. Background DNA damage in Empty Vector controls, without induction of oxidative stress by H 2 O 2 . **B**. shNrf2 knockdowns show high levels of spontaneous DNA damage without induction of oxidative stress with H_2O_2. **C.** High levels of DNA damage are seen in all conditions after addition of H_2O_2. **D**. Histograms showing the number of damage foci per individual nucleus in Nrf2- and Keap1 knockdown ECFCs and controls with- or without oxidative stress by 200 μ M H_2O_2. In Nrf2 knockdown cells the number of damage foci is higher at baseline (right dotted line) than in empty vector controls or Keap1 knockdown cells (left dotted line). Each histogram represents ca. 200 nuclei from a minimum of 3 fluorescent photographs, different donors are shown as semi-transparent overlays.



Supplemental Figure 2. Effect of SFN on Gene Expression. ECFCs were exposed to a range of sulforaphane concentrations for 4 hours and differences in expression of several target genes were measured.



Supplemental Figure 3. Time and Dose-dependent ARE activation. ECFCs of three donors were transduced with a luciferase-reporter constuct driven by three GCLM promoter sequences (lower panel). Luciferase signal after incubation with different doses of NRF2 pathway activator sulforaphane (SFN) was measured at different timepoints over the course of 24h.

Supplemental Table 1. Primer Sequences

Gene	Direction	Sequence	Tm (°C)
GCLC	Forward	TCCTGGACTGATCCCAATTC	60
GCLC	Reverse	TGCGATAAACTCCCTCATCC	60
GCLM	Forward	GTTGGGATACTGTGGGCTCT	60
GCLM	Reverse	CGGCCCTGAATTAAGGATTT	60
HO-1	Forward	ACATCTATGTGGCCCTGGAG	60
HO-1	Reverse	CTGGTGTGTAGGGGATGACC	60
Keap1	Forward	GTGTGGAAAGAGCAGGCTTC	60
Keap1	Reverse	TGGGAACCACATTTCCAGAG	60
NQO1	Forward	TACTATGGGATGGGGTCCAG	60
NQO1	Reverse	TCTCCCATTTTTCAGGCAAC	60
Nrf2	Forward	GAGAGCCCAGTCTTCATTGC	60
Nrf2	Reverse	TTGGCTTCTGGACTTGGAAC	60
VEGF	Forward	GCCTTGCTGCTCTACCTCCA	60
VEGF	Reverse	CAAGGCCCACAGGGATTTT	60
CXCR4	Forward	TTTTCTTCACGGAAACAGGG	60
CXCR4	Reverse	GTTACCATGGAGGGGATCAG	60
Catalase	Forward	TTTCCCAGGAAGATCCTGAC	60
Catalase	Reverse	ACCTTGGTGAGATCGAATGG	60
eNOS	Forward	CTCATGGGCACGGTGATG	60
eNOS	Reverse	ACCACGTCATACTCATCCATACAC	60
Cathepsin L	Forward	TGGCCTAATGGATTATGCTTTCC	60
Cathepsin L	Reverse	ATGACCTGCATCAATAGCAACA	60
EMMPRIN	Forward	TTCACTACCGTAGAAGACCTTGG	60
EMMPRIN	Reverse	GTTGATGTGTTCTGACGACTTCA	60
IL-8	Forward	GAATGGGTTTGCTAGAATGTGATA	60
IL-8	Reverse	CAGACTAGGGTTGCCAGATTTAAC	60
Housekeeping Genes			
PPIA	Forward	ATGGTCAACCCCACCGTGT	60
PPIA	Reverse	TCTGCTGTCTTTGGGACCTTGTC	60
P0	Forward	TGCACAATGGCAGCATCTAC	60
P0	Reverse	ATCCGTCTCCACAGACAAGG	60
HPRT1	Forward	TGACACTGGCAAAACAATGCA	60
HPRT1	Reverse	GGTCCTTTTCACCAGCAAGCT	60

Michelle T. Poldervaart M.D.[1], Hendrik Gremmels M.D.[2], Kelly van Deventer B.Sc[1]., Joost O. Fledderus Ph.D.[2], F. Cumhur Öner M.D., Ph.D.[1], Marianne C. Verhaar M.D., Ph.D.[2], Wouter J.A. Dhert M.D., Ph.D[1,3], Jacqueline Alblas, Ph.D.[1]

[1] Department of Orthopaedics, University Medical Center Utrecht, The Netherlands
[2] Department of Nephrology and Hypertension, University Medical Center Utrecht, The Netherlands
[3] Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

CHAPTER 8

PROLONGED PRESENCE OF VEGF PROMOTES VASCULARIZATION IN 3D BIOPRINTED SCAFFOLDS WITH DEFINED ARCHITECTURE

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ABSTRACT

Timely vascularization is essential for optimal performance of bone regenerative constructs. Vascularization is efficiently stimulated by vascular endothelial growth factor (VEGF), a substance with a short half-life time. This study investigates the controlled release of VEGF from gelatin microparticles (GMPs) as a means to prolong VEGF activity at the preferred location within 3D bioprinted scaffolds, and the effects on subsequent vascularization. The release of VEGF from GMPs was continuous for 3 weeks during *in vitro* studies, and bioactivity was confirmed using human endothelial progenitor cells (EPCs) in migration assays. Traditional and real-time migration assays showed immediate and efficient EPC migration in the presence of GMP-released VEGF, indistinguishable from VEGF-solution that was added to the medium. Matrigel scaffolds containing EPCs and VEGF, which was released either in a fast or sustained fashion by application of GMPs, were investigated for their *in vivo* vasculogenic capacity. Implantation in subcutaneous pockets in nude mice for one week demonstrated that vessel formation was significantly higher in the VEGF sustained-release group compared to the fast release group. In addition, regional differences with respect to VEGF release were introduced in 3D bioprinted EPC-laden scaffolds and their influence on vasculogenesis was investigated *in vivo*. The different regions were retained and vessel formation occurred analogous with the results seen in the Matrigel plugs. We conclude that GMPs are suitable to generate sustained release profiles of bioactive VEGF, and that they can be used to create defined differentiation regions in 3D bioprinted heterogeneous constructs, allowing a new generation of smart scaffold design. The prolonged presence of VEGF led to a significant increase in scaffold vascularization when applied *in vivo*.

INTRODUCTION

Vascularization of tissue-engineered constructs is an ongoing challenge in regenerative medicine. Without sufficient blood supply, oxygen and metabolic needs are not met, which can lead to central necrosis of constructs. This limits scaffold size to the diffusion distance nutrients can travel in the used material (1) and (2). A possible solution to this problem could be to introduce a prevascularization step to stimulate the process of efficient vascularization once the construct is implanted. Grafts with new blood vessels that can connect to the host vasculature could enhance the quantity and quality of newly formed tissues. Endothelial progenitor cells (EPCs) that are present in the circulation play an important role in this process as they can differentiate into endothelial cells that line blood vessels (3). Late outgrowth EPCs (sometimes referred to as endothelial colony forming cells or ECFCs in literature (4) and (5)) have a high capacity for proliferation

and vessel formation *in vitro* and *in vivo* (6) and (7). Vessel formation properties of the EPCs have been studied extensively in established 2D tubulogenesis and 3D vasculogenesis assays, and indicate that they are suitable to prevascularize tissue engineered constructs. A potent angiogenic and vasculogenic growth factor that is often applied in regenerative medicine is vascular endothelial growth factor (VEGF), a heparin-binding, homodimeric glycoprotein of 45 kDa of which VEGF165 is the predominant isoform (8). It is a key regulator of physiological vessel formation during embryogenesis, mainly by preventing apoptosis of endothelial cells (9). VEGF is degraded rapidly in the bloodstream with a half-life time of less than 1 h following injection (10) and (11). Controlled release of VEGF to accomplish longer growth factor presence at target locations leads to increased vessel formation in scaffolds (12) and (13). As a suitable system for growth factor release, gelatin or gelatin microparticles (GMPs) are often applied. Gelatin is a natural product that is used in many FDA-approved devices. Growth factor encapsulation is based on electrostatic interactions with the gelatin as well as the gelatin degradation rate (14), (15), (16) and (17). The main advantages of the GMPs are the diffusional loading of growth factors and the non-covalent nature of the interaction between gelatin and growth factor, thus avoiding chemical reactions that could damage the protein. Furthermore, GMPs are non-cytotoxic, biodegradable and they have previously been used to deliver other growth factors such as BMP-2, TGFβ1 and FGF (18) and (19). Intracardial injection of GMPs loaded with VEGF has led to increased neoangiogenesis in a rat myocardial infarct model (20) and (21). GMPs can be incorporated into hydrogel plugs or 3D bioprinted constructs (22). This technique enables production of a new generation of scaffolds with defined architecture and the opportunity to include predefined regions of prevascularization in the scaffold. Furthermore, with 3D bioprinting, scaffold porosity can be introduced, which appears to improve the *in vivo* performance by lowering the diffusion distance for oxygen and nutrients (23).

Based on these considerations the present study aimed to combine controlled VEGF release with 3D bioprinting technology to enable production of novel hydrogel scaffolds with properties that can be tuned both in time and space to optimize vessel formation. Suitability of GMPs for VEGF delivery was first assessed with *in vitro* release studies and real-time EPC migration studies. Subsequently, *in vivo* studies were carried out to investigate whether prolonged VEGF presence in scaffolds would improve the degree of vascularization. The suitability of GMP mediated controlled VEGF release in these novel architectural constructs was studied using the 3D bioprinting technology.

MATERIALS AND METHODS

ECFC Isolation and Culture

Human umbilical cord blood was collected from full term pregnancies, using a protocol approved by the local ethics committee (01/230 K, Medisch Ethische Toetsings Commissie (METC), University Medical Center Utrecht). Mononuclear cells (MNCs) were isolated by density-gradient centrifugation using Ficoll-paque (density 1.077 g/ml). MNCs were subsequently resuspended in Endothelial Growth Medium-2 (EGM-2) containing 10% fetal calf serum (FCS) and EGM-2 SingleQuots (Lonza, Walkersville, MD, USA) and seeded on Collagen I (BD Biosciences, Heidelberg, Germany) coated wells at a density of 2 x 10^6 cells per cm^2 . Medium was exchanged daily for the first week and three times per week thereafter. Cells were harvested when colonies appeared and expanded for further use. All experiments were conducted with cells at passages 3-5.

Flow Cytometric Immunophenotyping of ECFCs

Cells were harvested by trypsinization and labeled with the following antibodies: anti-hVEGFR2-PE (R&D, Minneapolis, MN), anti-hCD34-FITC (BD), anti-hVE-Cadherin-PE, (R&D), anti-CD31-PE (R&D), anti-CD90-PE (R&D), anti-CD105-PE (R&D) anti-CD45-PE (BD), and anti-CD14-PE (Biolegend, San Diego, CA). Antibody labeling was performed for 30 min at 4 ˚C in the presence of FcR-Blocking reagent (Miltenyi), followed by two washing steps using PBS. Directly before each measurement, Sytox® Blue (Life Technologies, Bleiswijk, The Netherlands) was added in order to assess viability. Flow-cytometric analysis was performed using a Becton Dickinson FACSCanto II. After washing and fixing, at least 10^4 cells were acquired and analyzed.

Immunocytochemical Phenotyping of ECFCs

Cells were grown to confluence on Lab-Tek II chamber slides and fixed using 4% paraformaldehyde. Next, cells were permeabilized where appropriate with 0.1% Triton X-100 in PBS and stained with the following antibodies/reagents: anti-CD144 (R&D), anti-CD31 (R&D), and anti vWF (Sigma). Fluorescent second antibody staining was done with Anti-Mouse AlexaFluor 488 and 555 (Molecular Probes) and nuclei were visualized with 4´,6-diamidino-2-phenylindole (DAPI). Images were taken with an Olympus BX60 fluorescence microscope.

Production of Gelatin Microparticles (GMPs)

We adapted the protocol previously described by Tabata et al. (18) and (24). A water in oil emulsion was made using water dissolved gelatin type B (Sigma, St Louis, MO, USA) 10% (w/v) that was added drop wise to refined olive oil (Arcos Organics, NJ, USA) at 60˚C. The solution was stirred (350 rpm) for 15 min and then rapidly cooled with an ice

bath to induce gelation of gelatin droplets. The microparticles were washed with 150 ml of chilled acetone and filtered under pressure (filter paper grade 2, Whatman, Tokyo, Japan). The microparticles were sieved and covalently cross-linked overnight using a 10.6 mM aqueous glutaraldehyde solution (Merck, Darmstadt, Germany) (25) and (26). Three washing steps with 100 mM glycine solution (Sigma, St Louis, MO, USA) were applied to remove residual aldehyde groups. Subsequently, the microparticles were washed in deionized water (MilliQ) three times, freeze-dried overnight, UV sterilized and kept at 4˚C in a vacuum container until use. The GMPs were tested in triplicate for endotoxin content using a chromogenic limulus amebocyte lysate assay (Lonza), according to the manufacturer's protocol. The microparticles were loaded with the dissolved growth factor by diffusional loading.

In Vitro Release of VEGF measured by ELISA

For slow release GMPs (5 mg) were loaded with 5 µl of a 10 µg/ml VEGF in PBS/0.5% BSA solution by diffusional loading overnight at 4˚C, and incorporated into 200 µl Matrigel (growth factor-reduced, BD, New Jersey, USA) plugs (each containing 50 ng VEGF) that were placed in the top compartments of a Transwell system, (0.4 µm pores, Corning Sigma, St Louis, MO, USA). Fast release was also investigated, here the same concentration of VEGF was directly incorporated in the Matrigel plugs. In the lower compartment PBS/0.5% BSA was used as release medium and refreshed at multiple time points. VEGF concentrations in the release medium were determined by ELISA (in duplicate, R&D, Minneapolis, MN, USA). Data are expressed as cumulative release, in mean ± SD.

Transwell Migration Assay

ECFC migration assays were performed using the 24-well Transwell system (10 µm thickness, 8 µm pores, Corning, NY, USA). Microspheres (5 mg) were loaded with 5 µl of PBS, 10 µg/ml or 20 µg/ml VEGF in PBS/0.5% BSA by diffusional loading at 4˚C overnight to allow complete growth factor absorption. The GMPs were suspended in 0.5 ml EGM-2 medium, supplemented with 2% FCS and singlequots (without VEGF), and placed in the lower compartment. For comparison, the same amounts (0, 100 or 200 ng/ml) of VEGF were added directly into the medium (fast release). Medium containing 15% FCS served as a positive control. In the top compartments 10^5 ECFCs (serum deprived, cultured overnight in EGM-2/2% FCS) were seeded. After 24 h non-migrated cells remaining at the upper side of the membranes were carefully removed with cotton swabs, and migrated cells on the lower side of the membrane were fixed with methanol and stained with hematoxylin. High-resolution pictures of four representative fields were taken using a light microscope, and two observers counted the number of migrated cells per region of interest on blinded samples for 3 ECFC donors.

Real-time Migration Assay

Time-dependent cell migration was measured using the xCELLigence system (Roche, Almere, The Netherlands), a microelectronic biosensor method continuously measuring electrical impedance across integrated electrodes over the membrane of a Transwell chamber setup (CIM-Plate 16 containing wells, inserts and membranes). An increase in impedance correlates with a number of cells migrating through the membrane. The CIM-plate membrane was coated with collagen I for one hour at room temperature and rinsed twice with PBS. Serum-free EGM-2 medium was added to all lower compartments and supplemented with VEGF (ranging from 0 to 100 ng/ml, fast release) or the same concentrations of VEGF were laden overnight on 5 mg of GMPs (slow release). 4 x 10^4 serum-deprived ECFCs were resuspended in serum-free EGM-2 and plated into each top compartment. The CIM-plates were assembled and measurements were performed in duplicate following the manufacturer's protocol with ECFCs from three donors. Impedance was measured twice a minute for 9 h at 37˚C and 5% CO2, whereafter the results were translated into a cell index signal and normalized to the migration data of the vehicle group.

Matrigel Tube Formation Assay

Matrigel was mixed with alginate 3% ((w/v), IMCD, Amersfoort, The Netherlands), at different ratios and used for ECFC tube formation assays. 10^4 ECFCs suspended in EGM-2 with 2% FCS were distributed on microwell plates (Ibidi, Martinsried, Germany) coated with alginate, Matrigel or a mixture of these two materials. Cells were allowed to form tubular networks for 24 h. To visualize tubes and cell viability a LIVE/DEAD viability assay (Molecular Probes, Eugene, USA) was performed according to the manufacturer's recommendations. Pictures were taken with a fluorescence microscope (Olympus, BX51, USA), total vascular complex length and branching points were quantified by processing all obtained images with the freeware AngioQuant v1.33 (27).

Dynamic Mechanical Analysis

The hydrogel mixtures that were used for 3D bioprinting were subjected to an unconstrained compression test (2980 DMA, TA Instruments) with a ramp force from 0.1 to 1 N for 10 min. The gels were cast into 200 µl discs and tested with and without 100 mM $CaCl_2$ crosslinking for 15 min. The compressive modulus was calculated from the slope of the stress-strain curve that was obtained.

3D Bioprinting

The Bioscaffolder pneumatic dispensing system (SYS + ENG, Gladbeck, Germany) was used for 3D bioprinting of hydrogel scaffolds (Matrigel/alginate = 3/1 mixture) (28). Scaffold architecture is determined and converted to a computer-aided design (CAD)

file and then combined with specific material settings to a numerical control (NC) code, which directs the robotic arm of the 3D printer (29). One dispensing head contained hydrogel supplemented with 5 x10^6 ECFCs/ml, in the second dispensing head GMPs containing 25 μg/ml VEGF were added to the cell-laden hydrogel. Regionally defined hybrid porous scaffolds measuring 10 x 10 x 3 mm were 3D bioprinted under sterile conditions in a laminar flow-cabinet. After 3D bioprinting the scaffolds were cross-linked by adding 100 mM aqueous $CaCl_2$ 2 for 15 min, then washed with Tris buffered saline (TBS) and cultured in EGM-2 medium. Directly after 3D bioprinting one sample was processed for paraffin embedding and stained with HE, Goldner's trichrome and Safranin-O to assess the distribution of the different components within the construct.

Scaffold Preparation for *In Vivo* Implantation

Four groups of constructs were produced (Table 1). Three groups consisted of Matrigel (200 μl) plugs containing 5 x 10^6 EPCs/ml, a control group with empty GMPs (PBS laden), a fast release group with empty GMPs (PBS laden) and 25 μg/ml VEGF in the hydrogel, and a slow release group with 25 μg/ml VEGF loaded on 5 mg GMPs. The fourth group consisted of 3D bioprinted Matrigel/alginate = 3/1 mixture containing 5 x 10^6 EPCs/ml. These hybrid constructs contained two regions, half the construct contained 25 μg/ml VEGF loaded GMPs (slow), and the other half served as control. Constructs were trimmed to a size of 10 x 5 x 3 mm and kept in EGM-2 medium overnight before implantation.

Table 1. Experimental Groups

Group	Scaffold	Growth factor presence	ECFCs
Control	Matrigel plugs	PBS laden GMPs, no VEGF	5 x 10^6 cells/ml
Fast	Matrigel plugs	PBS laden GMPs, 25 μg/ml VEGF in Matrigel	5 x 10^6 cells/ml
Slow	Matrigel plugs	25 μg/ml VEGF laden GMPs	5 x 10^6 cells/ml
Hybrid	3D bioprinted Matrigel/ alginate = 3/1	Region 1: no GMPs, no VEGF Region 2: 25 μg/ml VEGF laden	5 x 10^6 cells/ml

In Vivo Implantation

Surgeries were performed with permission of the local Ethical Committee for Animal Experimentation in compliance with the Institutional Guidelines on the use of laboratory animals. Six female nude mice (Hsd-cbp NMRI-nu, Harlan, Boxmeer, The Netherlands), 10 weeks of age were housed in standard cages at the Central Laboratory Animal Institute. Surgery was performed under inhalation anesthesia of 3% isoflurane. After skin incision, subcutaneous pockets were made by blunt dissection. The scaffolds were placed in one

of the four dorsal pockets in the mice in a randomized fashion. Pockets were closed using sutures (Vicryl 4.0). Postoperatively the animals were weighed and given a subcutaneous injection of buprenorphine (0.05 mg/kg, Temgesic, Schering-Plough/Merck, Whitehouse station, NJ, USA) every 8 h (3 times in total).

Sample Processing and Histology

The mice were terminated by cervical dislocation one week after implantation. The scaffolds were retrieved to analyze vessel formation. Samples were fixed overnight in 4% buffered formalin and further dehydrated for paraffin embedding. Hematoxylin/eosin (HE) staining was performed on all samples to investigate the gross morphology of the samples after *in vivo* implantation. Then samples were deparaffinized and incubated with Weigert's hematoxylin followed by Goldner's trichrome to assess vessel formation. The number of erythrocyte-perfused vessels per 100 μm^2 was counted by two independent observers on blinded samples. Subsequently, samples were stained for CD31 (PECAM-1) with rabbit-anti-mouse CD31 primary antibody (cross-reacts with human CD31, B4737, Lifespan, Seattle, WA, USA and 28364, Abcam, Cambridge, UK) at 2 µg/ml, followed by goat-anti-rabbit biotinylated antibody at 5.5 µg/ml (Dako, E0432) and Streptavidin-HRP at 1.4 µg/ml (Dako, K1016). All antibodies were diluted in PBS containing 5% (w/v) BSA. Staining was developed with diaminobenzidine (DAB) and Mayer's hematoxylin was used for counterstaining. Subsequently anti-goat CD31 (staining human and mouse CD 31, SC1506, Santa Cruz, Dallas, Tx, USA) and anti-rat CD31 (staining only mouse CD31, SZ31) stainings were performed, fluorescent secondary antibody staining was done with anti-goat and anti-rabbit AlexaFluor 568 and 594 (Molecular Probes), respectively. Nuclei were visualized with DAPI.

Statistical analysis

Data are represented as mean ± standard deviation, and analyzed using an ANOVA test with post hoc Bonferroni correction. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Characterization of ECFCs from umbilical cord blood (cb-ECFCs)

EPCs were isolated from human umbilical cord blood, expanded and characterized as shown in Figure 1. Flow cytometric analysis shows that progenitor cell markers CD34, CD105, CD133, and endothelial cell markers CD31, CD144 (VE-cadherin), CD90 and KDR (VEGF-R2) were present and leukocyte marker CD45 and macrophage/monocyte marker CD14 were absent in the cell population. Cells were positive for von Willebrand

Factor (vWF), and showed characteristic membrane-bound staining for CD31 and VE-cadherin on immunofluorescence staining. These findings confirm that the cells are late outgrowth EPCs.

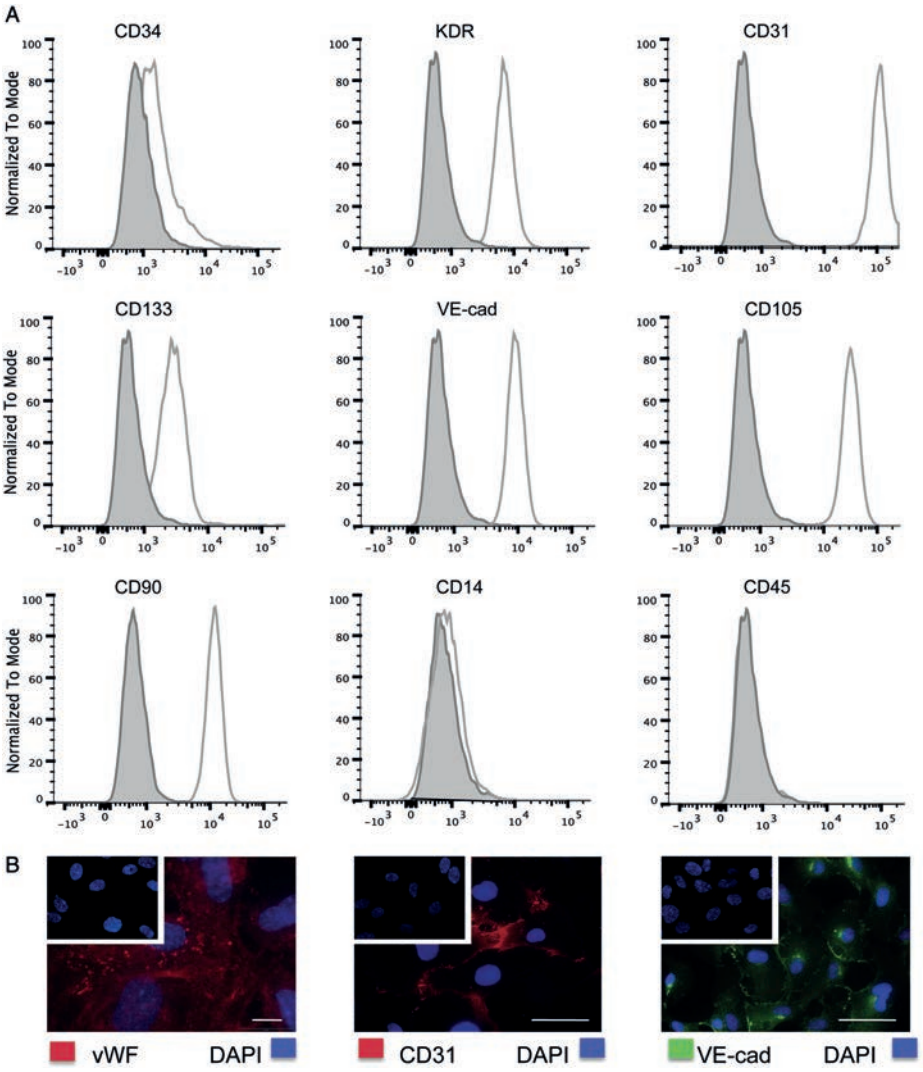

Figure 1. Characterization of Human Cord Blood ECFCs. Cytometric analysis of cultured EPCs for markers CD34, KDR (VEGFR-2), CD31, CD133, VE-cadherin (CD144), CD105, CD90, CD14 and CD45. Open histograms represent cells stained with specific antibodies while solid histograms correspond to isotype-matched control antibodies. One representative donor profile of the 3 donors is shown. B: Expression of vWF (red), CD31 (red) and VE-cadherin (green), in confluent monolayers. Immunofluorescence stainings, nuclei stained by DAPI (blue). Insets of isotype-matched control Ab stainings. Scale bars = 50 μm.

VEGF Release from GMPs

GMPs were produced in a size range of 75-125 µm, based on release profiles from previous experiments (22). Using light microscopy it was shown that the macroparticles are uniform in shape. Endotoxin levels were low (0.03 ± 0.02 EU/mg GMP), remaining well under the FDA accepted range. VEGF was incorporated into the Matrigel (fast release) or into the GMPs by diffusional loading which were then dispersed in Matrigel plugs (slow release) and incubated in PBS/0.5% BSA for release measurements. PBS/0.5% BSA was refreshed at multiple time points and VEGF concentrations (free VEGF, no longer interacting with the GMPs) were determined with ELISA. In Figure 2 the cumulative release profiles of slow and fast VEGF groups are shown. VEGF release was prolonged and more gradual in the slow release group compared to the fast release group. VEGF was continuously released for at least 2 weeks at concentrations relevant for vasculogenesis (The ED_{50} of VEGF is typically 1-6 ng/ml) (30). This indicated that the combined application of GMPs embedded in Matrigel plugs is a suitable system for prolonged VEGF release.

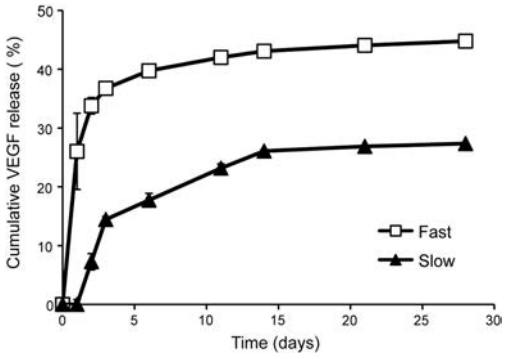

Figure 2. Cumulative release of VEGF. Fast (directly incorporated in Matrigel) and slow release (application of GMPs) of VEGF measured in PBS/0.5% BSA, analyzed with ELISA. Data represent mean ± SD for n = 3.

In Vitro Effectivity of GMP-released VEGF

To establish biological activity of the GMP-released VEGF, migration assays were performed. Migration was assessed using a traditional endpoint measurement chemotaxis set-up and also by real-time migration assays. A statistically significant ($p < 0.01$) dose-dependent migration of EPCs towards VEGF is seen in both assays (Figure 3), with a peak migration observed at 50 to 100 ng/ml VEGF. Increasing growth factor concentration did not lead to higher cell migration. The VEGF-stimulated migration of ECFCs is similar for fast VEGF (added to the medium) and slowly released VEGF (from GMPs). Migration data measured by endpoint cell counting (gold standard) were similar to real-time measurements. Additionally, real-time migration data reveal that EPC migration mainly takes place after 4 to 8 h of incubation.

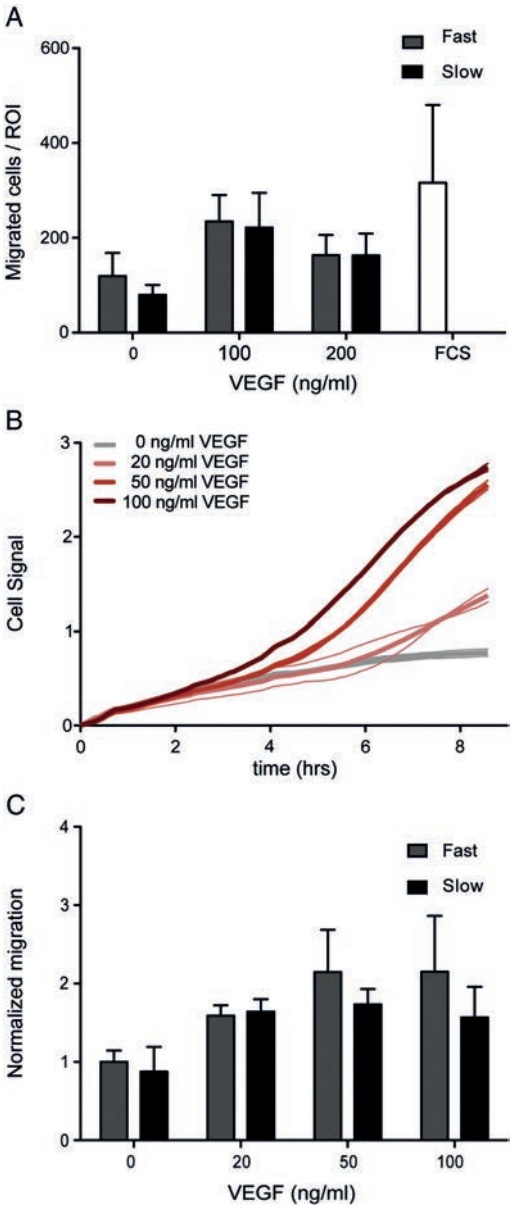

Figure 3. Real-time and conventional migration assays of EPCs towards VEGF. Cells were seeded in the inserts; VEGF was released from GMPs (slow) or added directly to the wells (fast). Performed in triplicate, data represent mean ± SD for 3 donors. **A**. Transwell migration assay, the number of migrated cells was assessed after 24 h of incubation. Migration towards serum (FCS) was included as a positive control. **B**. Real-time cell index signal (calculated from impedance measurement) depicting time-dependent migration of one EPC donor towards fast released VEGF. **C**: Concentration effects were statistically significant for both slow and fast groups; cell migration was normalized to migration towards vehicle.

3D bioprintable hydrogel selection

To optimize Matrigel for 3D bioprinting applications and accelerate cross-linking after 3D bioprinting, it was supplemented with alginate 3% (w/v) at different ratios. Formation of vascular networks was assessed with ECFCs from 4 donors on different hydrogel

mixture ratios. Incubation with Matrigel led to formation of vascular complexes throughout the microwell (Figure 4A). When pure alginate was used, large cell aggregates were visible, no vascular complexes were formed (Figure 4C). The addition of alginate to Matrigel reduced the number of vascular complexes and branching points, depending on the alginate concentration (Figure 4D,E). Some complex formation was seen in the hydrogel mixtures, but this was lower compared to Matrigel only. The addition of alginate enhanced the compressive modulus, which enabled 3D bioprinting (Figure 4F-K). To allow both EPC functioning and bioprinting, the mixture with the lowest rate of alginate that was still printable was selected. As a result, the heterogeneous 3D bioprinted scaffolds for the *in vivo* experiment consisted of a mixture of Matrigel/alginate = 3/1.

Role of Controlled Release of VEGF on Vessel Formation *In Vivo*

After selecting the Matrigel/alginate = 3/1 hydrogel mixture for 3D bioprinting, scaffolds, as described in Methods and Materials were implanted subcutaneously in mice for *in vivo* analysis of vessel formation. After one week of implantation scaffolds were retrieved

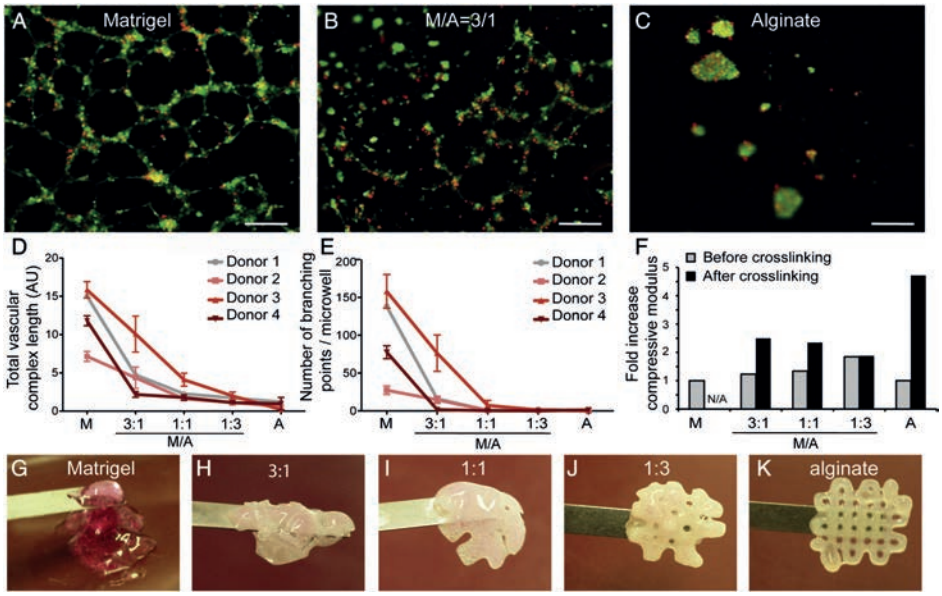

Figure 4. Selection of optimal printable hydrogel mixture by tubulogenesis assay.
A-C. Tubulogenesis of ECFCs after 24 h of incubation on hydrogels, as follows: Matrigel (**A**), Ratio Matrigel/alginate of 3:1 (**B**), alginate 3% (**C**). Scale bar = 200 µm. D: Total vascular complex length for different gel mixture ratios. **E**: Number of branching points for different gel mixture ratios. Performed in triplicate for donors 2-4, donor 1 performed once, data quantification with Angioquant, presented as mean +/- SD. **F**. Compressive moduli of the gel mixtures, before and after $CaCl_2$ -crosslinking. **G-K** Printability of the hydrogel mixtures showing how the printability increases with the addition of alginate to the Matrigel.

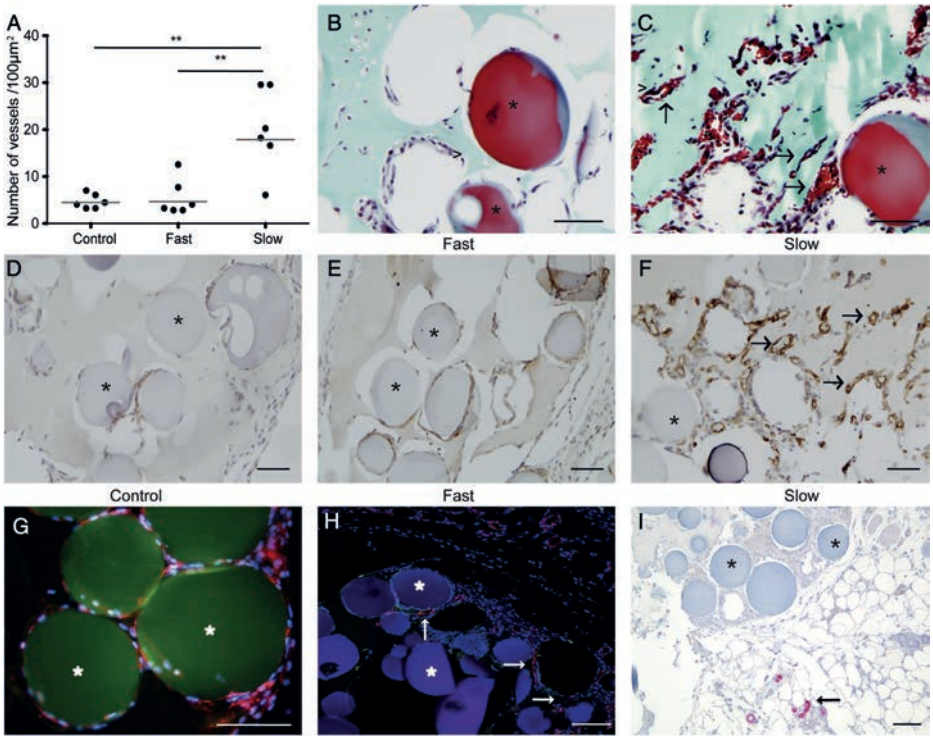

Figure 5. Vessel formation in hEPC seeded scaffolds after a week of subcutaneous implantation in mice. **A**. Quantification of vessel formation, number of vessels per 100 µm² is depicted. **B,C**. Goldner's trichrome staining, representative pictures from fast (B) and slow release groups (C) are shown. **D-F**. CD31 (PECAM-1) staining, human and murine cells stain brown. **G**. Fluorescent anti human CD31 staining, positive signal in red. **H**. Fluorescent anti mouse CD31 staining, positive signal in red. **I**. αSMA staining, only staining mature vessels in subcutaneous tissue (arrow), not the newly formed vessels in the scaffold. Presence of GMPs is indicated with an asterisk in the micrographs. Blood vessels are indicated with arrows. Scale bars = 50 µm.

and paraffin embedded. Presence of GMPs throughout the Matrigel and different amounts of vessel formation were observed in HE stained sections. It was clear that the 3D bioprinted scaffolds were disintegrating faster than the Matrigel plugs, most likely due to the faster dissolving hydrogel mixture combined with scaffold porosity. Cell groups were often seen surrounding the GMPs, which were identified as human ECFCs by immunohistochemical staining against CD31. Mouse ECFCs were identified by specific anti CD31 staining, mainly surrounding the vessels between the GMPs (Figure 5G,H). αSMA staining on the newly formed vessels remained negative, indicating that the formed vessels are not mature yet (Figure 5I). A Goldner's trichrome staining was performed to localize erythrocyte-perfused vessels. Quantification of the perfused vessels in the different groups revealed that slow release of VEGF led to a significant increase in vessel

formation ($p < 0.01$, Figure 5) when compared to fast release of VEGF, or the control group without growth factor. In the 3D bioprinted scaffolds disintegration and deformation have hindered the quantitative analysis, but the two distinct regions could still be discriminated after explantation. It appears that control regions (without GMP or VEGF) contained hardly any vessels, and in regions of slowly released VEGF, more vessel formation was seen (Figure 6). These findings are in accordance with our observations in the Matrigel plugs. The overall vessel formation is lower in the 3D bioprinted constructs than in the Matrigel plugs, most likely due to alteration of the hydrogel properties that result from alginate addition

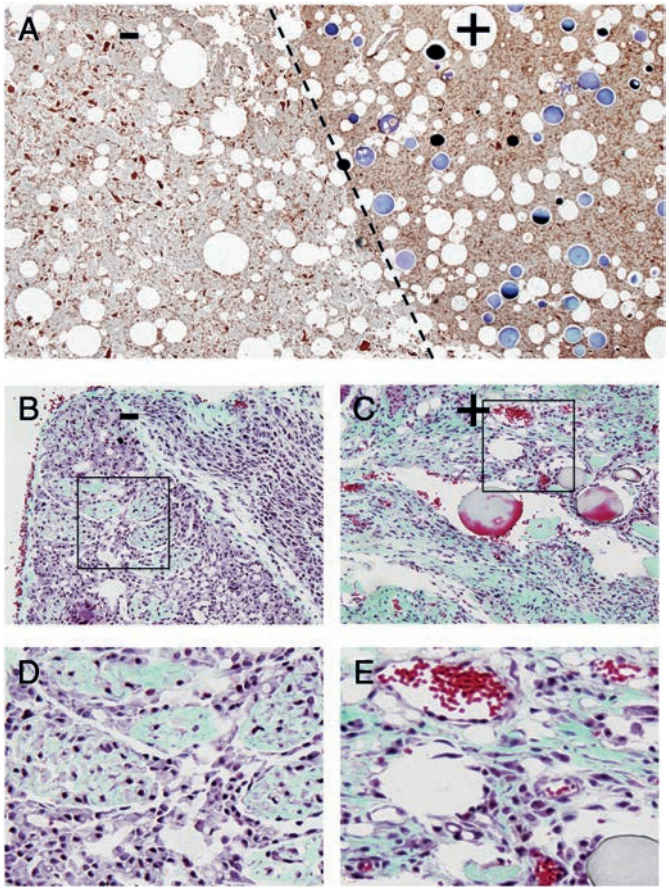

Figure 6. Bioprinted, hECFC-laden, heterogeneous scaffolds. A. Regionally defined scaffold directly after 3D printing. The two regions (- without VEGF, + VEGF-laden GMPs) are indicated by the dotted line. GMPs stained blue. HE staining, scale bar = 500 µm. **B-E.** Vessel formation in regionally defined scaffold after one week of *in vivo* implantation, sections stained with Goldner's trichrome. **B**. No vessel formation in control part of the scaffolds (no VEGF). **C**: Perfused vessels in region with VEGF from GMP (slow release) part of scaffolds. **D, E**. details from B and C respectively (inset region). Scale bars = 100 µm.

DISCUSSION

Slow release of VEGF from GMPs led to a significant increase in the number of perfused vessels in a human EPC containing Matrigel plug compared to fast VEGF release or control group in a vascular ingrowth model. When the VEGF-laden GMPs were regionally applied in 3D bioprinted scaffolds with defined architecture this effect was seen locally, more vessels were present in GMP-containing regions.

In this study, umbilical cord ECFCs were used to assess the effect of prolonged VEGF presence. From previous studies it is known that without these cells hardly any vessel formation would occur in ectopic constructs. In the formation of new capillaries by endothelial cells VEGF stimulation is necessary and sufficient, through its actions on both endothelial cells and myeloid cells. In this study, with high VEGF concentrations, these vessels will remain leaky and sinusoidal due to negative regulation of pericytes, but they are well perfused. In our GMPs the amount of VEGF is finite (in contrast to for instance VEGF plasmids), once it is exhausted we expect a slow recruitment of pericytes and the formation of a more mature vasculature (31). ECFCs are considered an interesting (autologous) cell source in regenerative medicine (32), as they can be harvested from a patient's peripheral blood. However, aging effects when culturing ECFCs are seen, mainly for ECFCs harvested from adults (33). To avoid loss of vessel formation potency of the ECFCs, the current experiment was performed after a minimal number of passages (3-5) (34).

The VEGF release data shown in this article are *in vitro* release data. We know from literature that *in vivo* release rates can be significantly faster than *in vitro* release rates due to higher enzymatic activities (35) and (36). We aimed to compensate for faster release by using the GMPs that showed continuous release for three weeks *in vitro*, in an *in vivo* experiment that lasted only one week. After 3 weeks of *in vitro* release, only 50% of the total VEGF that was loaded into the Matrigel plugs was accounted for. It is known from literature that VEGF does not remain stable in solution (10) and (37). This protein degradation could explain our low retrieval rate. The amount of released VEGF that was measured by ELISA however is more than the ED_{50} , so it is relevant for vasculogenesis. Furthermore, ECFC migration towards the released VEGF took place. Migration assays showed that the biological activity of GMP-released VEGF was intact, which is in accordance with literature on GMPs (15). Real-time migration measurement has benefits when compared to the traditional Transwell chamber, as it provides insight in migration patterns over time (38). ECFC migration towards VEGF already started after a few hours of incubation, therefore, application of GMPs was particularly suitable since diffusion of VEGF out of the GMPs started immediately. Many other release vehicles, such as PLGA, have a shell of material that has to degrade in order for the protein to be released, after which a larger burst release occurs, compared to GMPs (25).

When the ECFCs and GMPs were embedded into hydrogel plugs and subsequently implanted subcutaneously in mice, prolonged presence of VEGF led to a significant increase in scaffold vascularization compared to fast release or control groups. This is in accordance with literature regarding VEGF release from other delivery systems such as nanoparticles or PLGA microspheres (13), (36) and (39). When alginate is added to Matrigel the hydrogel mixture can be bioprinted, and GMPs can be used to generate defined differentiation regions in heterogeneous constructs. Alginate addition led to faster hydrogel degradation, which hindered quantitative analysis of vessel formation in these scaffolds, but regional differences were clearly visible. Also, the overall number of vessels appeared to decrease when alginate was added, compared to Matrigel alone, implicating that the hydrogel used for bioprinting could be improved with respect to gelation time, cell attachment and degradation speed. Combining bioprinting with controlled growth factor release allows scaffold properties to be fine-tuned in a temporal as well as a spatial manner. This is an important step in smart scaffold design, as it enables researchers to reach a higher level of biomimicry.

CONCLUSION

GMPs are suitable to generate sustained release profiles of bioactive VEGF, and are effectively used to generate defined differentiation regions in 3D bioprinted heterogeneous constructs. The prolonged presence of VEGF led to a significant increase in scaffold vascularization when applied *in vivo*.

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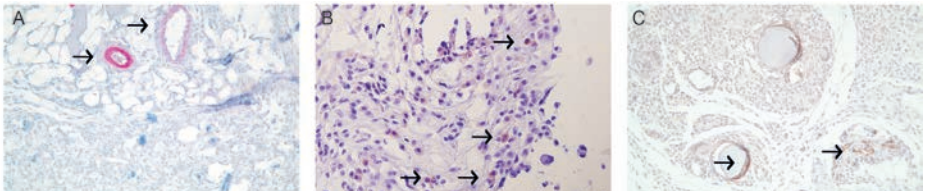
SUPPLEMENTARY INFORMATION

Supplementary Methods

α*SMA*

Samples were stained α-SMA ALP-conjugated primary antibody (1:300, A5691, Sigma). Staining was developed with a basic fuchsin kit (Dako) according to manufacturers protocol, Mayer's hematoxylin was used for counterstaining. MAC-3 Samples were stained for macrophage presence with rat-anti-mouse MAC-3 primary antibody (LAMP-2, 550292, BD Pharmingen) at 0.15 µg/ml, followed by anti-mouse biotinylated antibody at 5.5 µg/ml (Dako, X0931) and Streptavidin-HRP at 1.4 µg/ ml (Dako, K1016). All antibodies were diluted in PBS containing 5% (w/v) BSA. Staining was developed with diaminobenzidine (DAB) and Mayer's hematoxylin was used for counterstaining. CD31 Samples were stained for CD31 (PECAM-1) with rabbit-anti-mouse CD31 primary antibody (cross-reacts with human CD31, B4737, Lifespan, Seattle, WA, USA) at 2 µg/ ml, followed by goat-anti-rabbit biotinylated antibody at 5.5 µg/ml (Dako, E0432) and Streptavidin-HRP at 1.4 µg/ ml (Dako, K1016). All antibodies were diluted in PBS containing 5% (w/v) BSA. Staining was developed with diaminobenzidine (DAB) and Mayer's hematoxylin was used for counterstaining.



Supplementary Figure 1. Additional stainings on the cell influx in a 3D bioprinted sample. A. αSMA staining in the subcutaneous fat tissue, no positive staining around the newly formed blood vessels, or the cell influx. **B**. MAC3 staining, macrophages in pink, showing positive staining among the cell influx. **C**. CD31 (PECAM-1) staining, some positive cells. Positive staining indicated with arrows. Scale bars = 50 µm.

Hendrik Gremmels MD[1], Martin Teraa MD, PhD[1,2], Paul H.A. Quax PhD[3,4],
Krista den Ouden BSc[1], Joost O. Fledderus PhD[1], Marianne C. Verhaar MD, PhD[1]

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
[4] Einthoven Laboratory of Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

CHAPTER 9

NEOVASCULARIZATION CAPACITY OF MESENCHYMAL STROMAL CELLS FROM CRITICAL LIMB ISCHEMIA PATIENTS IS EQUIVALENT TO HEALTHY CONTROLS

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ABSTRACT

Critical Limb Ischemia (CLI) is often poorly treatable by conventional management and alternatives such as autologous cell therapy are increasingly investigated. Whereas previous studies showed a substantial impairment of neovascularization capacity in primary bone-marrow (BM) isolates from patients, little is known about dysfunction in patient-derived BM Mesenchymal Stromal Cells (MSCs). In this study we have compared CLI-MSCs to healthy controls using gene expression profiling and functional assays for differentiation, senescence and in vitro and in vivo pro-angiogenic ability. Whereas no differentially expressed genes were found and adipogenic and osteogenic differentiation did not significantly differ between groups, chondrogenic differentiation was impaired in CLI-MSCs, potentially as a consequence of increased senescence. Migration-experiments showed no differences in growth factor sensitivity and secretion between CLI- and control MSCs. In a murine hind-limb ischemia model, recovery of perfusion was enhanced in MSC-treated mice compared to vehicle controls (71±24% versus 44±11%; $p<1\times10^{-6}$). CLI-MSC and control-MSC treated animals showed nearly identical amounts of reperfusion (ratio CLI:control 0.98, CI95% 0.82-1.14), meeting our criteria for statistical equivalence. The neovascularization capacity of MSCs derived from CLI-patients is not compromised and equivalent to that of control MSCs, suggesting that autologous MSCs are suitable for cell therapy in CLI patients.

INTRODUCTION

Peripheral arterial disease (PAD) is a manifestation of atherosclerosis that is highly prevalent in aging western populations(1). When arterial occlusion reaches the point where metabolic demands of resting muscle can no longer be met, PAD progresses to critical limb ischemia (CLI), a condition associated with a very poor prognosis with respect to both life and limb(1) and quality of life(2). In a substantial proportion (ca. 40%) of patients conventional and surgical treatment options will be exhausted during the course of disease and amputation of the affected limb is inevitable(1). Consequently, new treatment modalities have been explored in the form of autologous progenitor cell transplantation (3). Initially, bone marrow (BM) mononuclear cells (MNCs) were used in the assumption that this mixture of cells contains pro-angiogenic progenitor cells. Although initial results seemed promising (3,4), restrictions to the clinical utilization of this BM cell therapy for PAD patients arise from the impaired angiogenic activity of patient derived BM progenitor cells (4-6).

A promising alternative are BM-derived mesenchymal stromal cells (MSCs)(7,8). MSCs make up the stromal part of the BM stem cell niche(9) and constitute a multipotent cell

population that can differentiate into several mesenchymal tissue lines(10). Because MSCs are exceedingly rare in BM(11), therapeutic applications generally involve expansion in culture. Culture expanded MSCs possess a pronounced pro-angiogenic capacity, that is likely to be superior to that of BM-MNCs(7,12).

It is thought that MSCs possess strong immunomodulatory properties(13) and low intrinsic antigenicity(14), allowing allogeneic therapeutic strategies. However, differentiation of MSCs after application *in vivo* has been shown to up-regulate expression of MHC complexes, leading to delayed immunization against the injected cells(15,16). This notwithstanding, allogeneic MSCs have been proven safe as single-dose therapy for ischemic cardiomyopathy(17,18).

There is significant uncertainty whether neovascularization capacity of MSCs from patients with cardiovascular disease is impaired, which would limit autologous clinical application in CLI. Previous studies have shown that MSCs from patients with auto-immune diseases, such as systemic lupus erythematosus(19) or systemic sclerosis(20) show alterations in growth factor secretion when compared to healthy donors. In addition, aging has been shown to reduce the efficacy of human MSCs in a murine model of myocardial infarction(21). On the other hand, studies investigating angiogenic cytokine secretion in MSCs obtained from patients with end stage renal disease found no alterations(22,23). Studies involving MSCs from human donors with cardiovascular disease showed that MSCs yields and numbers are not impaired(24,25) compared to age-matched controls.

In this study we investigated whether cultured BM-MSCs obtained from CLI patients are dysfunctional when compared to BM-MSCs from a healthy control population. We have started by global gene expression profiling to identify persistent differences after culture, but found no significantly differentially expressed genes in MSCs obtained from patients versus control MSCs. In a series of functional experiments we quantified MSC response to stimuli inducing differentiation or promoting angiogenesis *in vitro* an *in vivo*. As our starting hypothesis was that there are no differences between CLI- and control MSCs, we used Bayesian inference to give credible intervals of the difference between groups and show statistical equivalence, where present.

MATERIALS AND METHODS

CLI patients and healthy controls

Patient BM was harvested during the JUVENTAS trial, which investigates the efficacy of repeated intra-arterial BM-MNCs injections in patients with CLI (48) (Trial identifier: NCT00371371). Inclusion criteria for the trial consisted of severe infra-popliteal arterial occlusive disease and ineligibility for surgical or endovascular revascularization

procedures. For this study we cultured BM-MSCs from 12 sequentially included patients from March to August 2011. Control BM of 12 donors without PAD was collected during elective orthopedic interventions. Procedures were approved by the local ethics committee, and are in accordance with the Declaration of Helsinki.

MSC isolation and culture

BM from all donors was obtained from the iliac crest by needle aspiration. For each donor 10 x 10^6 MNCs were suspended in MEM alpha, 10% Fetal Calf Serum (FCS) and were left to adhere for 24h in a 10cm^2 tissue culture well. Cells were expanded for three passages until they covered four 75cm^2 tissue culture flasks and were then cryopreserved. All further experiments were conducted with cells in passage 3+1 and were started 48h after thawing and seeding the cryopreserved cells.

Whole-genome gene expression profiling

RNA from each cell sample was amplified and labelled cRNA was hybridized to an Illumina WT-12 v4.0 Expression Beadchip. Samples included RNA from the MSCs of 6 patients and 6 controls.

Differentiation

MSCs were differentiated towards in adipogenic and osteogenic lineages in confluent monolayers, using defined differentiation media (see supplemental information); chondrogenic differentiation was performed in a 3d pellet culture system. Osteogenic differentiation was assessed by Alkaline Phosphatase (ALP) activity using p-Nitrophenyl Phosphate (pNPP) and by Alizarin red retention. Adipogenic differentiation as assessed by lipidtox green staining and chondrogenic differentiation by production of soluble glycosaminoglycans (sGAGs)

Fluorescent staining for senescence with C12 FDG

8 x 10^4 cells were incubated with 100nM bafilomycin A1 for 1 h to induce lysosomal alkalinization. Next 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG) was added to a final concentration of 30μMol/l and cells were incubated for an additional hour. Cells were trypsinized and median cellular fluorescence was quantified using Flow Cytometry.

Migration assays

Real-time measurement of cell migration was performed using the xCELLigence RTCA DP device from Roche Diagnostics. This system is a variant of the two-chamber transwell in which vertical cell migration through a microporous membrane is recorded by measuring impedance on the underside of the membrane. In the lower wells of CIM-

Plates 16 with 8μm pore-size, a short serial dilution of (Platelet-derived growth factor subunit B homodimer) PDGF-BB was prepared in serum-free medium, for each donor. In the upper wells 40000 MSCs were seeded in serum-free medium and allowed to settle for 15 min prior to the beginning of the experiment. All conditions were conducted in duplicate. Data acquisition and analysis was performed with the RTCA software (version 1.2, Roche Diagnostics).
For each well, the end-point migration was calculated by subtracting the baseline value from the point of maximum impedance. A migration index was calculated by subtracting the cumulative migration from the chemo-attractant-free control. MSCs from each donor were allowed to migrate towards a dose-range of 0, 5, 10 and 20 ng/ml PDGF-BB. The half-maximal effective dose was calculated for each donor by fitting a four-parameter log-logistic function.

Collection of Conditioned Medium and Assays for Pro-angiogenic Activity

Conditioned Medium (CM) was collected in serum-free aMEM for 24 h under normoxic (21% O_2) and Hypoxic (2% O_2) culturing conditions. Ability to promote endothelial repair was assessed using a scratch-wound assay. In this assay a scratch is made in a confluent monolayer of immortalized endothelial cells (HMEC-1) using a pipette-tip and CM or control stimuli were added. Scratch-wound closure was quantified by comparing the widths of the scratch on t=0 and t=6h on photomicrographs.
Proliferation of HMEC-1 cells was assessed using real-time Cell Impedance Measurement with an xCelligence® machine. 2000 HMEC-1 cells were seeded per well and cell impedance signal was recorded every 15 min for 72 hours, Population Doubling Time (PDT) was calculated from the linear part of the proliferation curve.
In order to assess the angiogenic effect of MSC-CM in vitro, tubule formation assays using HMEC-1 cells were performed(50). HMEC-1 cells suspended in MSC-CM obtained under hypoxic and normoxic conditions, laid upon gels of Growth Factor-Reduced Matrigel® cast in IBIDI μ-wells, and allowed to form tubules for 24h. Tubule network characteristics were quantified using automated image analysis.

Hind-limb ischemia Model

Hind-limb ischemia was induced in 8-10 week old male nude NMRI FoxN1$^{nu/nu}$ mice (Harlan, Horst, NLD). The femoral artery was occluded with the use of an electrocauterization device (Bovie, Clearwater, FL) directly as it emerged from under the femoral ligament, closing off both superficial and deep branches. The femoral vein and nerve were left intact. The segment of the superficial femoral artery distal to the occlusion was then carefully stripped away from the adjacent vein until the popliteal bifurcation was reached.

Cell injections were performed 24 hours after femoral ligation. For the dose ranging 1, 3.3, and 10 x 10^5 MSCs were injected intramuscularly divided over 5 different sites in the adductor muscle (10µL/injection). In subsequent experiments using patient derived cells 1 x 10^5 MSCs were used. MSCs from 10 donors in each group were injected in 3 mice per donor; in addition a total of 21 vehicle controls was included. Relative perfusion of the ischemic limb compared to the control limb was measured using Laser Doppler Perfusion Imaging, with moorLDI2-HR (Moor Instruments, Devon, UK) imager with an 830nm Helium-Neon laser. Relative perfusion was followed over time by measurements on Day 0, 1, 4, 7, 10 and 14.

Histology

Vessels and muscle fibers in the adductor muscles were stained using anti-Mouse CD31 and Triticum vulgaris lectin. Human nuclei were visualized with anti-human nuclear A/C lamin (see Figure S6c). Staining for arterioles was done using anti-Mouse CD31 and anti-alpha Smooth Muscle Actin (αSMA).

Design and Statistics

Values are presented as relative to the Healthy MSC donor group to allow a comparison between CLI and control MSCs in the various assays performed in this study. Data were analyzed by using generalized linear mixed models, using a restricted maximum likelihood (REML) approach to estimate model parameters. P-values and (highest posterior density, HPD) credibility intervals were estimated by means of Markov Chain Monte Carlo (MCMC) sampling from the posterior distribution of parameter values (10000 iterations). In the analysis of the HLI experiments a hierarchical linear mixed model was used, in which mice were nested within MSC donors. To create a meaningful summary measure for equivalency we calculated areas under curve (AUCs) of relative perfusion over time. Analyses were conducted in 'R' software (version 2.15.3), using the lmer function in the lme4 package.

Groups were considered equivalent when 95% of the posterior distribution was within a pre-defined threshold. As threshold a range of 0.8-1.25, taken from bioequivalency guidelines set forth by drug regulatory agencies such as the FDA or EMA, was used.

In the microarray experiment, a moderated t-test approach as employed in the 'limma' package was used to test for differences between groups. As a lack of a statistically significant difference may for some genes be attributable to a lack of power in the experiment, we took a reverse approach to analysis by excluding genes that with 95% confidence did not differ by more than a factor 2 in expression and are thus considered equivalently expressed (EE).

Table 1. Patient Characteristics

	Controls (n=12)	CLI Patients (n=12)	p-value
Gender (F/M)	8/4	4/8	0.22
Age, years (Median, Range)	50(20-85)	67.5(29-81)	0.43
Diabetes Mellitus	1/12	3/12	0.43
Hypertension	2/12	6/12	0.11
Fontaine Classification (III/IV)	N/A	5/7	-
Rutherford Classification (4/5/6)	N/A	5/6/1	-
Ulceration	N/A	7/14	0.005
Medication			
Antihypertensive drugs	2/12	6/12	0.11
Insulin	1/12	2/12	1
Oral glucose lowering drugs	1/12	1/12	1
Cholesterol lowering drugs	1/12	9/12	0.003**
Anti-platelet drugs	2/12	12/12	<0.0001***

RESULTS

Patient Characteristics

Patient characteristics are shown in Table 1.

Isolation and phenotype

BM-MSCs from 12 patients and 12 healthy controls were successfully expanded to a tissue culture surface of 300cm^2. Cell yields were between 1.8 x 10^6 and 4 x 10^6 (data not shown) and time-to-expansion was similar between groups (19.25 ± 1.39 and 18.44 ± 0.83 days for healthy- and CLI-MSCs respectively). Immunophenotyping showed that the cells were positive for CD90, CD105, CD140b, and CD73, but negative for CD14, CD45, CD19 and CD34 (**Figure S1d**).

Gene expression profiling

Gene expression profiling was performed on MSCs from 6 CLI and 6 control donors. Not a single significantly differentially expressed gene was observed between MSCs derived from CLI patients and healthy donors (Table S1). Only 22 genes showed an on average greater than 2-fold change in expression (Figure 1a); namely *MFAP5, PI16, S100A4, H19, HOXB8, HAPLN1, MMP1, POSTN, SCRG1, TRIB3, HOXB5, FAM162B, DDIT4, SLC7A5, GREM1, HLA-DRB4, ERAP2, IFI44L, BST2, MX1* and *PITX1*, although with substantial variation (Figure S2a). Genes associated with angiogenesis were stably and equally expressed in donors from both groups (Figure 1b).

Analyses on similarity show that 98.7% of detected genes are with 95% confidence less than a factor 2 differentially expressed between CLI-MSCs and control MSCs. The remaining 332 genes (inconclusive, IC) were analyzed for association with known biological processes, in order to find areas of variability or trends towards a difference. This analysis is demonstrated in **Figure 1a**, which depicts the ratio of gene expression (log2) on the y-axis and the average expression level on the x-axis (low to high from left to right respectively). Comparison of the IC gene list with other studies shows that genes associated with chondrogenic differentiation of MSCs(26) ($p<7x10^{-12}$) and aging(27) ($p<2x10^{-17}$) are overrepresented (Figure S2b and c).

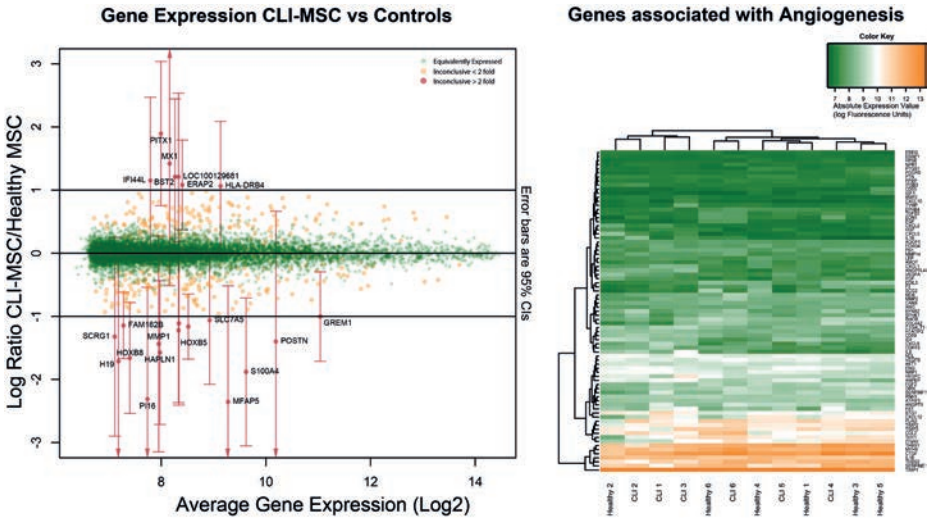

Figure 1. Gene Expression. **A.** Relative expression of genes in CLI-MSCs/Control MSCs. On this M/A plot the x-axis denotes the average expression (in units of fluorescence intensity), the y-axis denotes the ratio of expression in CLI- MSCs/Control MSCs. Expression of genes shown in green is with 95% confidence less than a factor differentially expressed. The remaining genes are inconclusive, genes with an average fold change of >2 are indicated in red and 95% confidence intervals are given. **B**. Heatmap showing genes associated with angiogenesis, clustering on colums shows no clear separation between CLI- and Control MSCs.

Differentiation

Alizarin red S staining showed aggregates of hydroxyapatite-mineralized matrices that stained red after retention of the dye (**Figure S1a**). Quantification of dye retention by acidic extraction showed equivalent amounts of dye retention (Ratio CLI:Control=0.99, 95%CI 0.81-1.17) in MSCs obtained from CLI patients and controls (Figure 2a). Alkaline Phosphatase activity was similar in both groups, but not equivalent (CLI:Control=1.07, 95% CI 0.75-1.39; Figure 2b).

MSCs treated with adipogenic medium displayed lipid droplets that are characteristic for differentiation into pre-adipocytes (Figs. S1b and c). Quantification of the amount of Lipidtox Green uptake by fluorescence measurement showed a trend towards increased adipogenic differentiation in CLI donors (CLI:Control=1.2, 95% CI 0.71-1.49; Figure 2c). Chondrogenic differentiation of MSCs induced the formation of glycosaminoglycans (GAGs). Soluble GAG production was about a 33% lower in CLI-MSC compared to controls (p=0.02, Figure 2d).

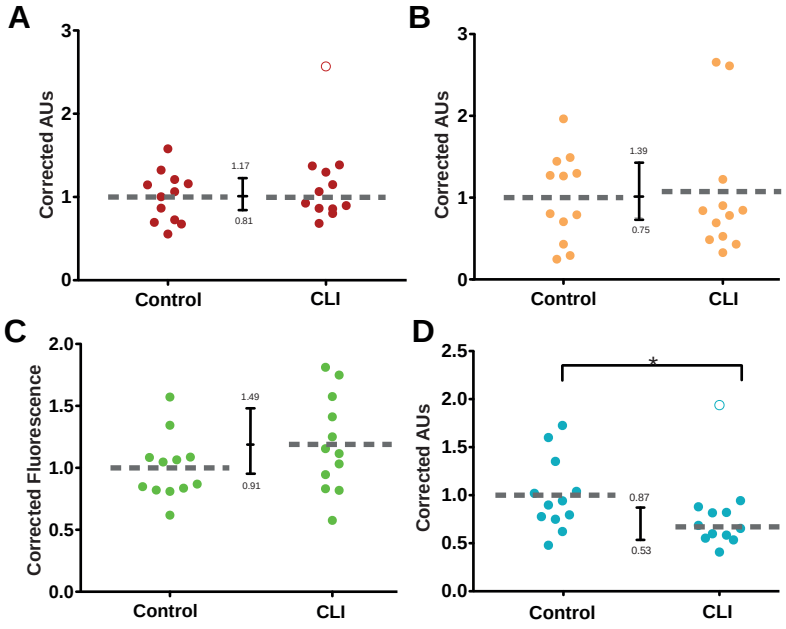

Figure 2. Differentiation. A. Quantification of alizarin red retention in osteogenically differentiated MSCs was found to be equivalent; CLI:Control=0.99 (95% CI 0.81-1.17), the open circle indicates and outlier that has been left out of analysis. **B**. ALP activity in MSCs after osteogenic differentiation; CLI:Control=1.07, 95% CI 0.75-1.39 **C**. Adipogenic Differentiation measured by Lipidtox Green staining, CLI:Control=1.2, 95% CI 0.71-1.49 **D**. Chondrogenic differentiation measured by sGAG production. sGAGs levels were significantly lower in CLI-MSCs.

Senescence

Senescence was assessed by the conversion of C12FDG, a fluorescently labeled ß-galactosidase substrate, and subsequently measured by flow cytometry (Figure 3a). There was a significant increase in senescence associated ß-galactosidase activity in MSCs obtained from CLI donors (Figure 3b). In a model that includes donor age, age is shown to be a significant explanatory variable (Figure 3c, R=0.37). After inclusion of donor age in the model, donor group (i.e. healthy or CLI) ceases to be significantly different, with

a trend towards a larger age associated increase in senescence in CLI-MSCs (p=0.07 for interaction age*group). As an additional marker of senescence the number of spontaneous DNA double strand break damage foci was measured by nuclear staining for gH2AX. CLI-MSCs showed a minor increase in γH2AX signal and number of damage foci (Figs. S3 a-d), but this difference was not significant. γH2AX fluorescence in donors of both groups was associated with both age and CD12FDG signal (Figs S3 e and f). Both donor age and senescence were associated with differentiation potential (Figure S4), leading to a shift from chondrogenic differentiation towards adipogenic differentiation.

Migration and Conditioned Medium

We observed clear time- and dose-dependent migration of BM-MSCs towards PDGF-BB (see Figure 4a for a representative readout, Figure 4b for a concentration response curve).

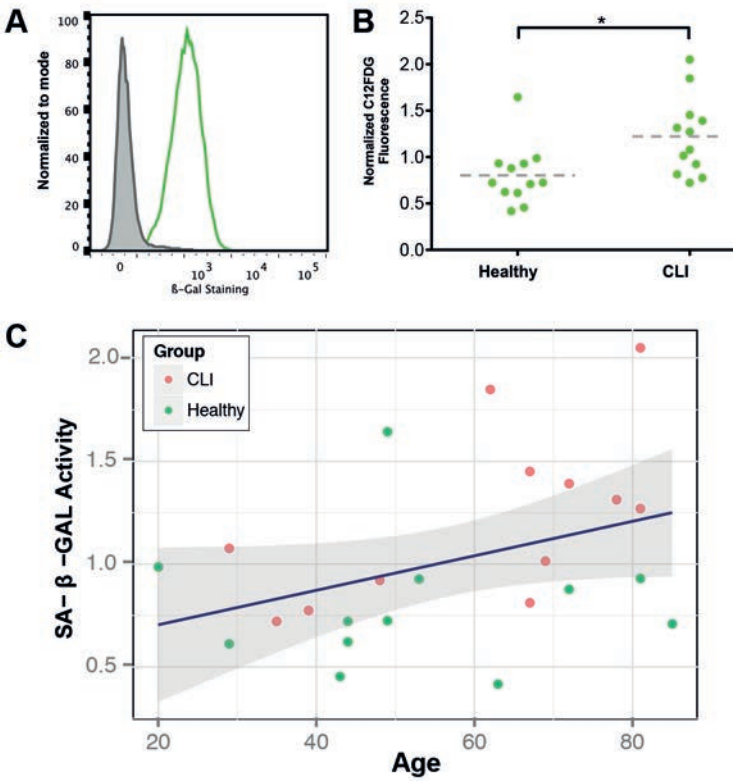

Figure 3. Senescence. A. Representative example of SA-β-galactosidase activity as measured by flow cytometry. **B**. Senescence is increased in CLI MSCs compared to Healthy controls (p=0.01). **C**. MSC senescence is significantly associated with donor age (p=0.02), shaded band indicates 95% confidence interval.

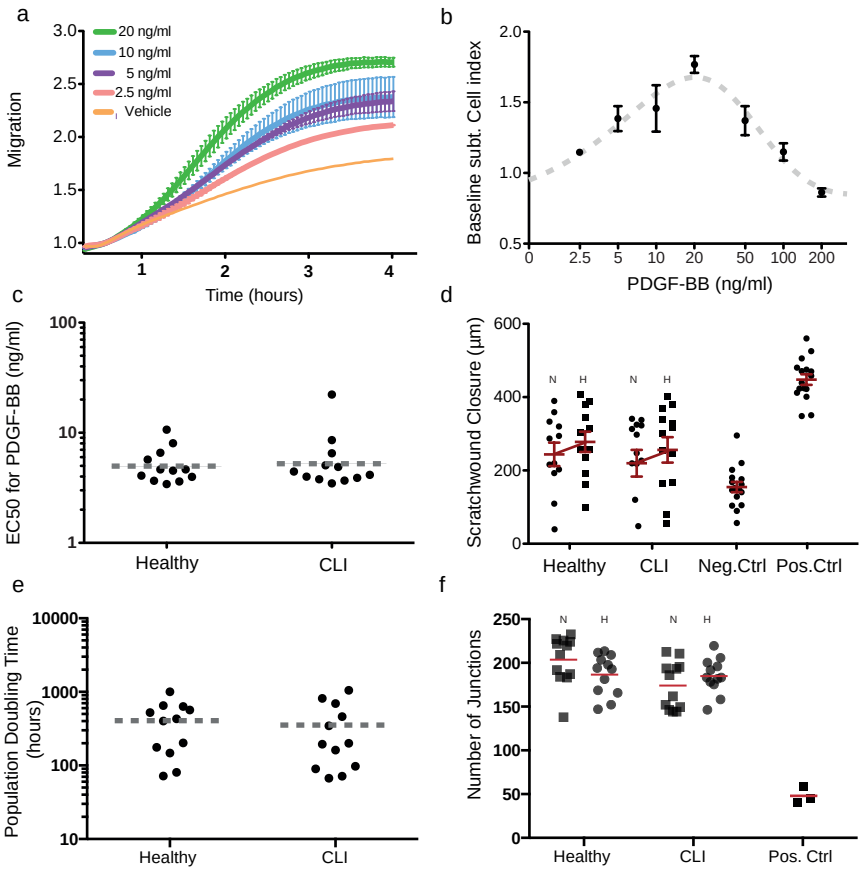

Figure 4. Migration A. Real-time migration of MSCs towards different concentrations of PDGF-BB (lines for higher concentrations have been omitted for clarity). **B**. Dose-response curve of MSCs to PDGF-BB. **C**. EC_{50} values of dose-response curves of CLI- and control MSCs (CLI:Control=1.05, 95% CI 0.66-1.62). **D**. Scratchwound closure of microvascular endothelial cells after exposure to MSC-conditioned medium. N and H indicate CM collected under normoxic and hypoxic culturing conditions, respectively. There is a significant effect of hypoxia (p=0.02), but not of donor group (p=0.33) The Negative control consists of empty medium, the positive control of medium containing 10% FCS. Error bars indicate S.E.M. **E.** Population Doubling Times of HMEC-1 endothelial cells grown in MSC-CM (CLI: Control=0.96, 95% CI 0.47-2.12). **F.** Number of junctions per image in tubule formation assay of HMEC-1 cells on matrigel, in the presence of MSC CM. No differences was observed between CLI and Control MSC CM or between CM collected under hypoxic conditions. N stands for Normoxic, H for Hypoxic CM.

The concentration-dependent response for each donor, as summarized by the half-maximum effective concentration (EC_{50}), was determined as final readout (Figure 4c). MSCs from healthy donors migrated to PDGF-BB with an average EC_{50} of 5.35 ng/ml whereas the average EC_{50} for CLI-MSCs was not different at 5.62 ng/ml (CLI:Control=1.05, 95% CI 0.66-1.62).

The ability of MSC-conditioned medium (CM) to induce endothelial repair was assessed using a scratch-wound closure assay. In addition we examined whether MSC CM was affected equally by hypoxia in either group. A significant effect of oxygen tension was observed, as hypoxic CM significantly increased wound closure by approximately 20% (p=0.02) compared to normoxic CM (Figure 4d). CLI-MSC-conditioned medium did not differ significantly from control CM in wound closure (CLI:Control=1.01, 95% CI 0.77-1.25) or the increase thereof in response to hypoxia.

In examining the effect of MSC CM on population doubling times (PDT) of endothelial cells we did not observe differences between CM of CLI MSCs and Healthy MSCs (CLI: Control=0.96, 95% CI 0.47-2.12), (Figure 4e). Tubule formation assays of endothelial cells in the presence of MSC CM similarly did not show any differences between CM obtained from CLI MSC and controls (CLI:Control = 0.99, 95% CI= 0.88-1.10), nor was there an effect of hypoxia (Figure 4f).

In analogy to the effects of MSC senescence on differentiation ability, we examined whether MSC senescence is associated with pro-angiogenic effects, but no relationship was found (Figure S5).

Hind-limb ischemia

In order to assess the ability of MSCs to promote neovascularization *in vivo,* we performed hind-limb ischemia experiments on $NMRI^{nu/nu}$ mice (Figure 5a). In the initial phase of the experiment, we performed a dose response curve of intra-muscularly injected MSCs obtained from a healthy donor. Animals that were treated with MSCs showed a significant increase in perfusion compared to controls. We observed a maximum effect at the lowest investigated dose of 10^5 cells, with a trend towards reduced effects at higher doses (Figure 5b). In the final experiment we proceeded with the lowest dose of 10^5 cells and compared MSCs from 10 CLI donors and 10 control donors, testing cells from each donor in 3 mice. Animals treated with MSCs showed increased blood flow recovery compared to vehicle controls after two weeks 71 ± 24% (SD) in MSC treated mice and 44 ± 11% in vehicle controls (Figure 5**c**, $p_{MCMC} < 1x10^{-6}$). Time-dependent increase in perfusion did not differ between animals treated with CLI-MSC and healthy MSC. Areas Under Curve (AUCs) of relative perfusion over time for animals treated with CLI-MSCs were equivalent to animals treated with healthy MSCs (Figure 5d, Ratio CLI:Control 0.98, 95% CI 0.82-1.14). Staining for capillaries in the adductor muscles similarly showed an increased number of vessels in MSC treated animals (Figs. 6a and b), but did not differ between CLI-MSC and control-MSC treated animals (Figure 6c). αSMA staining for the formation of arterioles (Figure 6E) showed an insignificant increase in the number of arterioles in MSC treated animals (Figure 6F), as well as a trend towards an increased αSMA+ surface area (Figure S6a) and an enrichment of smaller vessels (Figure S6b).

In accordance with previous studies(28) only very small numbers of MSCs could be traced back 14 days after administration. Although isolated MSCs were found in the muscle stroma, the majority of MSCs were found in localized clusters in between larger muscle fibers, presumably the injection site (Figure 6d). Double-staining for nuclei of proliferating cells with Ki67 and human specific A/C Lamin showed that MSCs did not proliferate after implantation *in vivo,* although clusters of Ki67 positive nuclei were found in the vicinity of injection sites (Figure S6d). While MSCs stained weakly for aSMA, they did not appear to participate in the formation of new aSMA+ vessels (Figure S6e) nor did they appear to integrate into larger existing vessels (Figure S6f).
In order to identify possible predictors for *in vivo* angiogenic ability we performed correlations between the various assays performed in this paper (Figure S7a), paracrine stimulation of endothelial proliferation was most closely associated with neovascularization *in vivo* (Figure S7b).

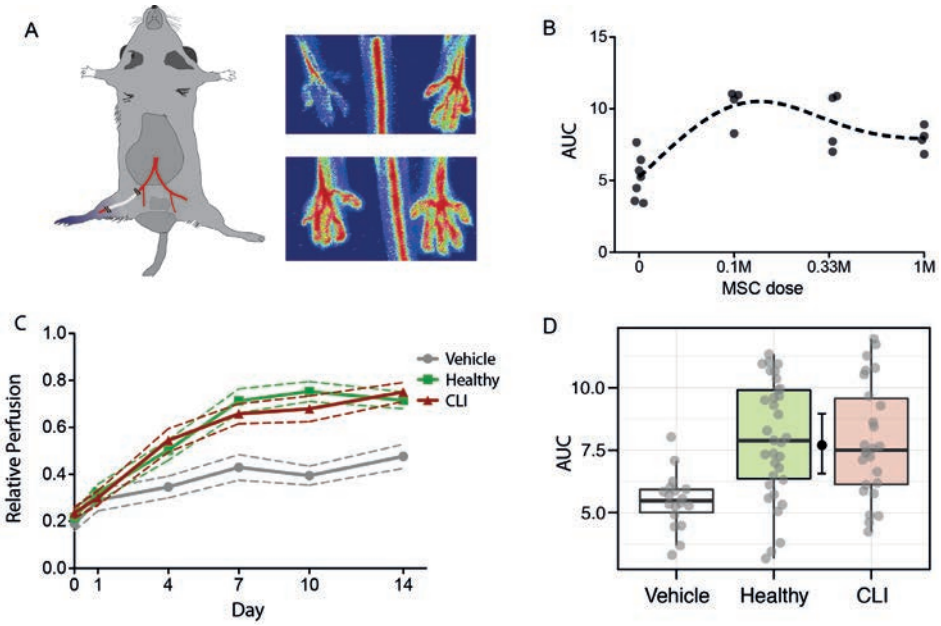

Figure 5. Hind Limb Ischemia Model. A. Hindlimb-ischemia model and representative graphical readout. **B**. Dose-response curve in of intramuscularly injected MSCs in the HLI model. **C**. Relative perfusion over time of ischemic hindlimbs over time in vehicle- and MSC treated animals, MSC treated animals show significantly higher perfusion than vehicle treated animals ($p<1\times10^{-6}$). Error lines indicate S.E.M. **D**. AUCs of relative perfusion over time as a summary estimate for equivalence (CLI:Control 0.98, 95% CI 0.82-1.14).

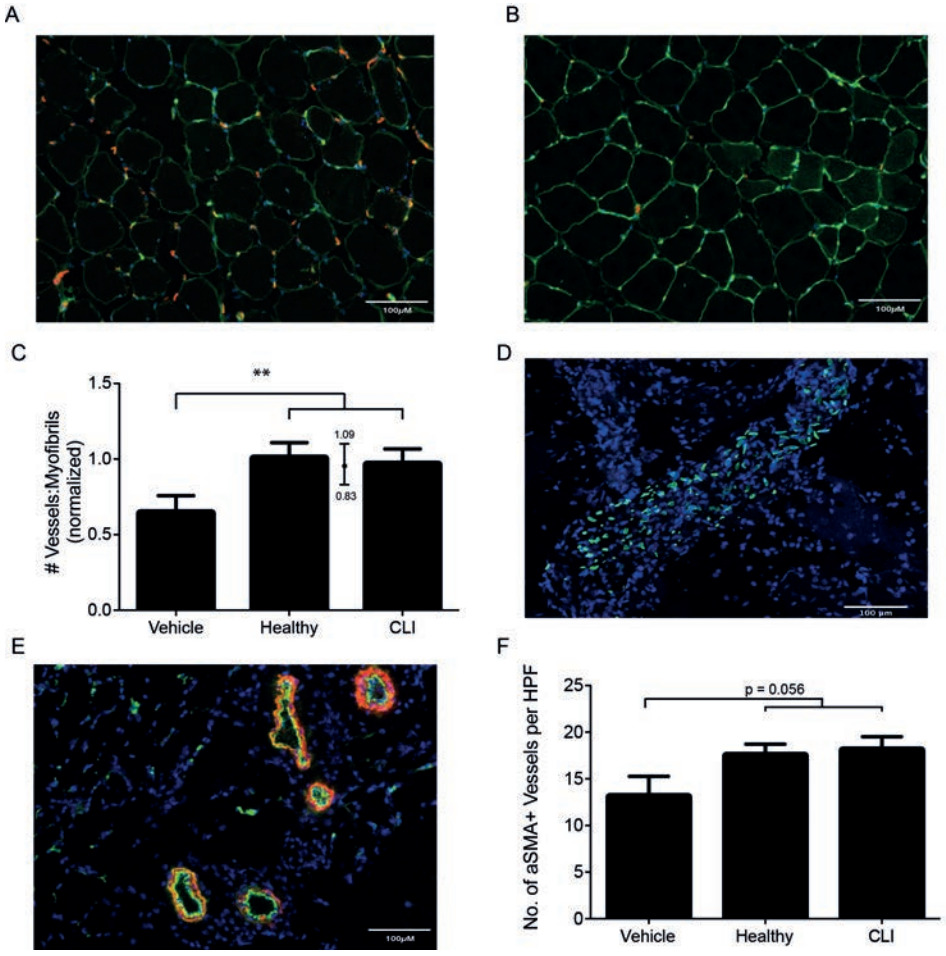

Figure 6. Histology. **A, B**. CD31 staining (red) in CLI-MSC treated (**a**) and vehicle treated (**b**) animals, perimysia are stained in green (scalebar = 100μM). **C.** Number of vessels as ratio vessels:myofibrils; vehicle treated animals show a significant reduction in the number of vessels compared to MSC treated animals ($p<0.001$). The number of vessels in CLI- and Healthy MSC treated animals is equivalent (CLI:Control 0.96, 95% CI 0.83-1.09). **D**. Staining with human specific anti A/C Lamin shows that injected MSCs are predominantly retained in localized clusters, presumably the injection site, after 14 days (scalebar = 100μM). **E.** Representative image of αSMA (red) staining, CD31 staining is shown in green (scalebar = 100μM). **F.** The number of αSMA+ vessels did not differ per group.

DISCUSSION

In this study we show that MSCs obtained from CLI patients are equivalent to MSCs obtained from healthy controls in their ability to restore perfusion in murine ischemic hind limbs. These results are consistent with our initial findings using genome-wide expression profiling, which showed that there were no differentially expressed genes between CLI- and control MSCs. CLI-MSCs show slightly increased senescence compared to controls; while this appears to affect differentiation ability, it does not affect migration, *in vitro* angiogenic effects of conditioned medium or pro-angiogenic ability *in vivo*.

Studies investigating BM-MNCs, which contain a small MSC subfraction (<0.01%)(11), found that BM-MNCs from patients with cardiovascular disease have a reduced neovascularization capacity(5,6). Previous results from our group also show that *in vitro* paracrine angiogenic activity of *ex vivo* cultured early endothelial progenitor cells (EPCs) from the same CLI patient cohort as used in the current study is markedly impaired(29). Similar impairment in *in vivo* models has also been observed in EPCs from diabetic patients(30). Moreover, a study by Yan *et al.* (31) showed that BM-MSCs from diabetic db/db mice are impaired in their ability to promote neovascularization, although it must be noted that in that particular study the effects of diabetes were due to a gene defect, which persists in cell culture.

The results of the present study suggest that selective expansion of human MSCs by *ex vivo* culture yields a cell population that is unaffected by disease with regard to its neovascularization capacity. Gene expression profiling performed at the outset of this study showed strikingly similar gene expression profiles for CLI- and control MSCs, with not a single gene being significantly differentially expressed between both groups. As the number of MSCs is very low in collected BM aspirates, the cells in this study were cultured for 12-15 population doublings before gene expression profiling was performed. *Ex vivo* culture of primary cells rapidly causes changes in gene expression, usually resulting in dedifferentiation of the cells (32,33). Cells can also gain useful properties through culturing, as in the case of early EPCs, which acquire their angiogenic phenotype only after selection and incubation in culture(34). In contrast to the finding in MSCs in this study, disease-mediated dysfunction in early EPCs is not reversed by *ex vivo* culture(35). It may be that unexpanded MSCs show only little susceptibility to disease-mediated dysfunction. Alternatively MSCs may be more amenable to modifying effects of cell culture, perhaps due to their replicative potential.

A report investigating abnormalities in fibroblasts obtained from dogs with congestive heart failure, for instance, showed that phenotypic abnormalities could be reversed after as little as 48 hours in culture(36).

We did observe subtle differences in MSC differentiation capacity, i.e. decreased chondrogenic differentiation and a trend towards increased adipogenic differentiation

in CLI-MSCs. These differences were associated with increased cellular senescence, and may in part be attributed to an overrepresentation of older donors in the CLI group. Donor age has previously been associated with increased MSC senescence and reduced differentiation potential(37-39) with a shift towards adipogenic differentiation(38).

As the primary objective of this paper was to identify differences in the ability to promote neovascularization between CLI- and control MSCs, we performed a series of *in vitro* experiments to study pro-angiogenic activity. In none of these experiments did we observe any differences between MSCs from the CLI- and control group. Interestingly the results also seemed to be unrelated to MSC senescence.

Migration was assessed using PDGF-BB as a chemoattractant, as previous studies identified PDGF as the most potent chemotactic stimulus for MSCs(40). Furthermore, PDGF has been shown to be important in the interplay between endothelium and MSCs during angiogenesis(41). We observed a typical bell-shaped dose-response relationship in migration of MSCs towards PDGF-BB, with an optimum dose of 20 ng/ml. If there would be differences in migration ability between CLI and control MSCs, we would expect a shift in sensitivity towards PDGF-BB. We did not observe such a shift but found that CLI- and control MSCs showed very similar sensitivity towards PDGF-BB.

The currently prevailing theory on the mechanism behind MSC-induced angiogenesis is that it is mediated by paracrine secretion of growth factors(42). We found no differences in migration stimulatory capacity between MSC-CM under basal conditions. We examined whether hypoxia further promotes pro-angiogenic activity(43), as this will be a stress condition that MSCs are likely to be subjected to after implantation *in vivo.* While we did see a significant effect of hypoxic stress on the ability of MSC-CM to promote scratch-wound closure, we did not observe differences between CM derived from CLI- and control MSCs. Similarly we did not find differences in the ability of CLI- and control MSCs to promote endothelial proliferation and tubule formation.

In the *in vivo* hind-limb ischemia experiment we observed a very pronounced effect of MSC-treatment over vehicle controls. We observed a bell-shaped dose-response curve with regard to cell dose, as was previously reported in intra-coronary MSC injections in animals(44) and humans(17), potentially due to increased local competition for nutrients and oxygen at higher cell doses in the injection site. The CLI- and control-MSC treated groups showed identical reperfusion patterns and were statistically equivalent, within the pre-defined thresholds.

Although we observed an association between senescence and differentiation capacity, in accordance with previous studies(37,39), no such associations were found with senescence and assays for pro-angiogenic activity. Others have shown that senescence induces a senescence-associated secretory phenotype (SASP)(45) in fibroblasts. This SASP enhances senescent cells' ability to induce angiogenesis in a mouse xenograft tumor model(46). The differences in senescence observed in this study are comparatively minor

due to the restricted donor age range and strict emphasis on keeping passage numbers equal, which prevents conclusions on the effect of a SASP on MSC-mediated neovascularization. While the primary objective of this study was not to identify a mechanism behind MSC-mediated neovascularization, the differential effects of senescence on differentiation capacity and pro-angiogenic effects as well as the tracing studies in Figure 6 and Figure S6 point towards paracrine affects, unrelated to stemness(47).

Similarly, paracrine stimulation of endothelial proliferation was most closely associated with efficacy of a given MSC donor *in vivo*.

The findings of this study suggest that cell therapy in CLI patients using autologous BM-MSCs will not be impaired by disease-mediated cell dysfunction. As undifferentiated MSCs have very low inherent antigenicity(14) and immunomodulatory properties(13), this cell type potentially offers an opportunity for allogeneic cell therapy without the need for immunosuppression. The question therefore arises whether there is a need for autologous therapy. Allogeneic administration of MSCs allows 'off the shelf' applications for acute ischemic events and does not involve an invasive harvesting procedure in fragile patients, as is often the case in CLI. A recent study by Schwarz et al.(28) showed that MSCs are cleared relatively soon after injection and that only a small number of MSCs remains after more than 3 weeks, which do not contribute to neovascularization. It has been shown, however, that eventual differentiation of remaining MSCs up-regulates expression of MHC complexes, leading to delayed immunization against the injected cells(15,16). In the POSEIDON trial(17) it was shown that 8 out of 27 patients were sensitized to allogeneic HLAs at baseline of the trial, and an additional subject developed alloantibodies after a single MSC administration. In the case of CLI, the wide-spread extent of atherosclerosis, the relative ease of cell administration and the limited effective cell dose per injection and the progressive nature of disease all call for repeated cell administration(48). In addition the findings of the PROVASA study(49) suggest that there may an additional benefit of more than one injection. As repeated exposure will likely aggravate alloimmunization, autologous MSC therapy may be preferable for this indication.

The present study demonstrates equivalent neovascularization potential of MSCs from CLI patients as compared to healthy controls in a murine hind-limb ischemia model. These findings suggest that autologous MSC therapy could be a promising therapeutic strategy, particularly in CLI patients.

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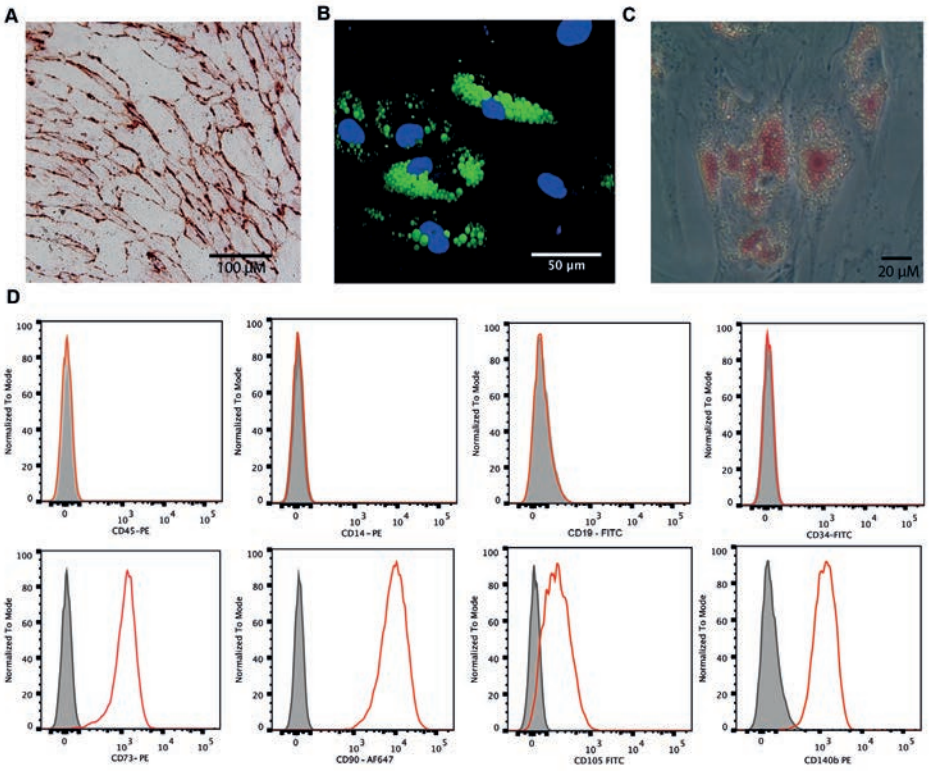
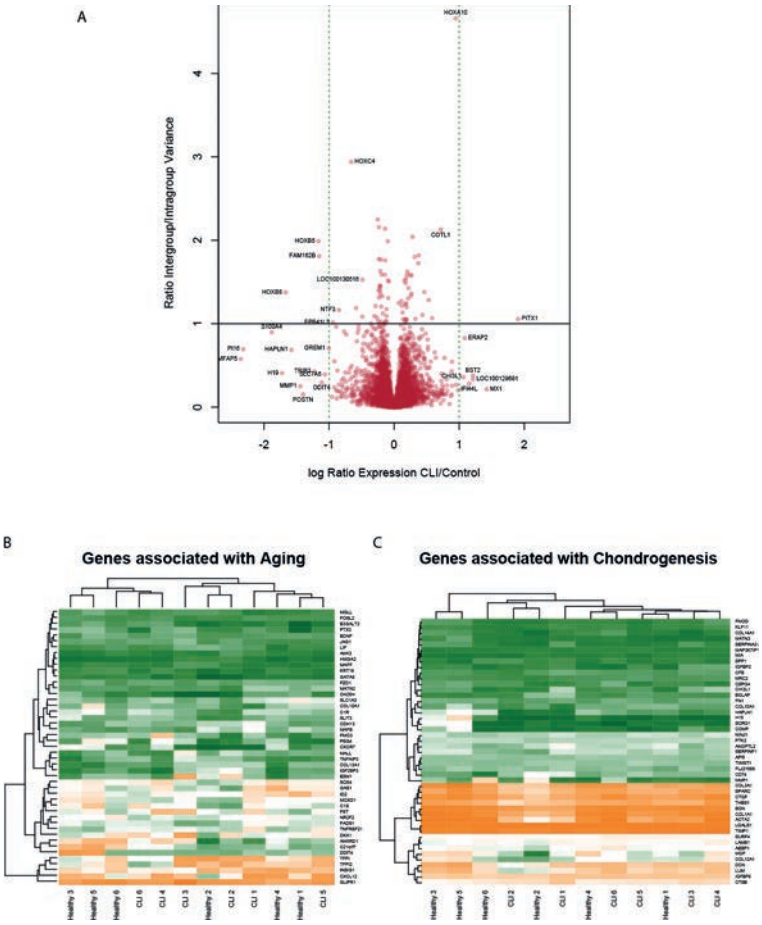
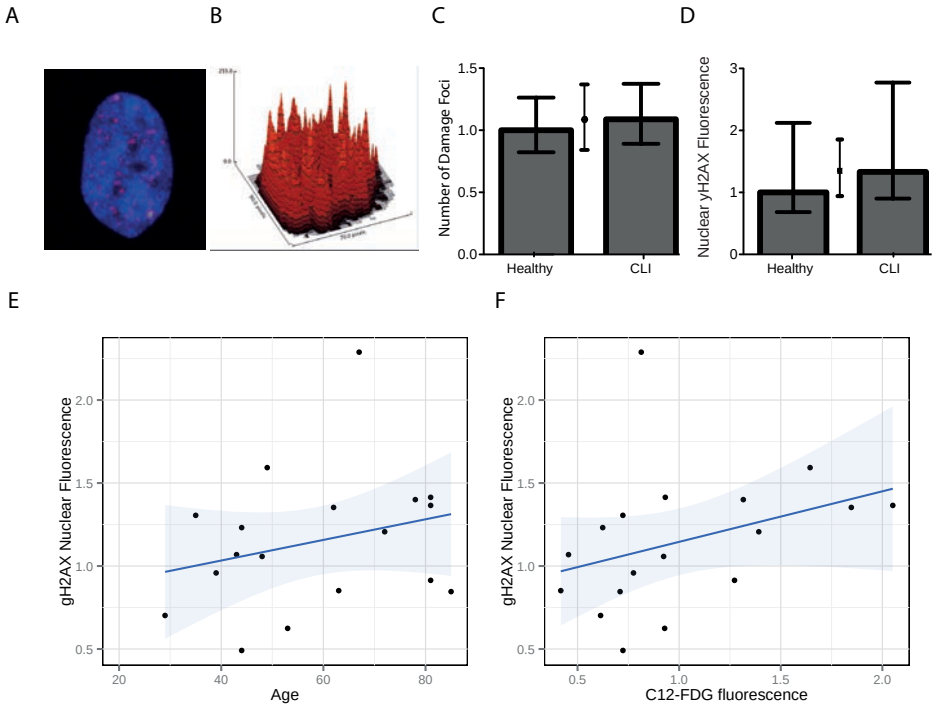
SUPPLEMENTARY INFORMATION



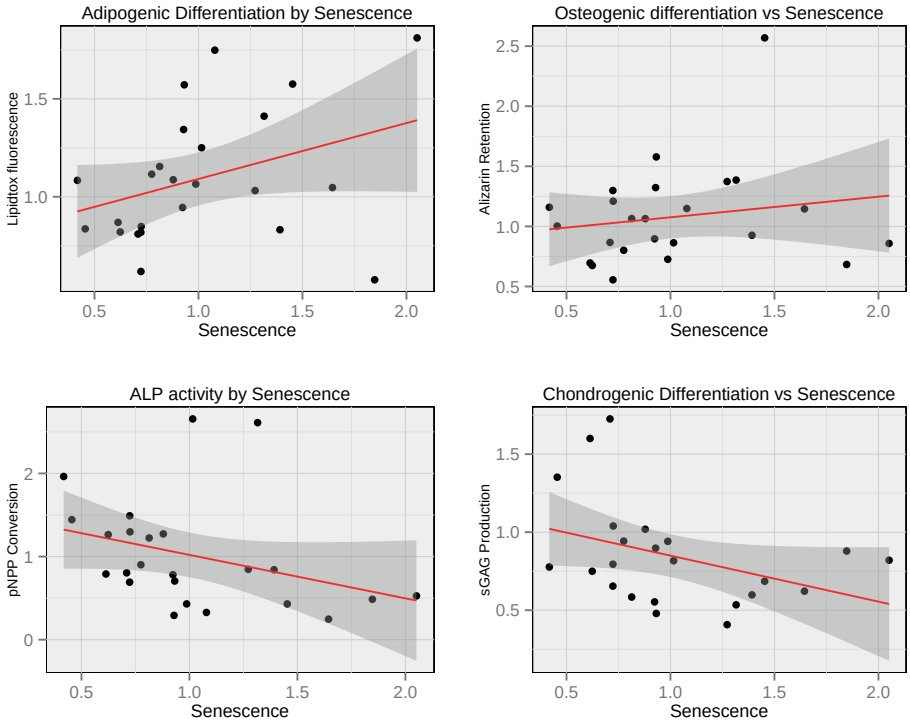
Supplemental Figure 1. A. Alizarin red staining in MSCs after osteogenic differentiation. **B.** Confocal Z-stack of Lipidtox green staining after adipogenic differentiation, nuclei are stained blue with DAPI. **C.** Oil red staining of adipogenically differentiated MSCs **D.** MSC Characterization by Flow Cytometry. The red histograms represent MSCs stained with fluorochorme conjugated antibodies as listed under each graph, the shaded gray histograms are isotype controls for the fluorophore.



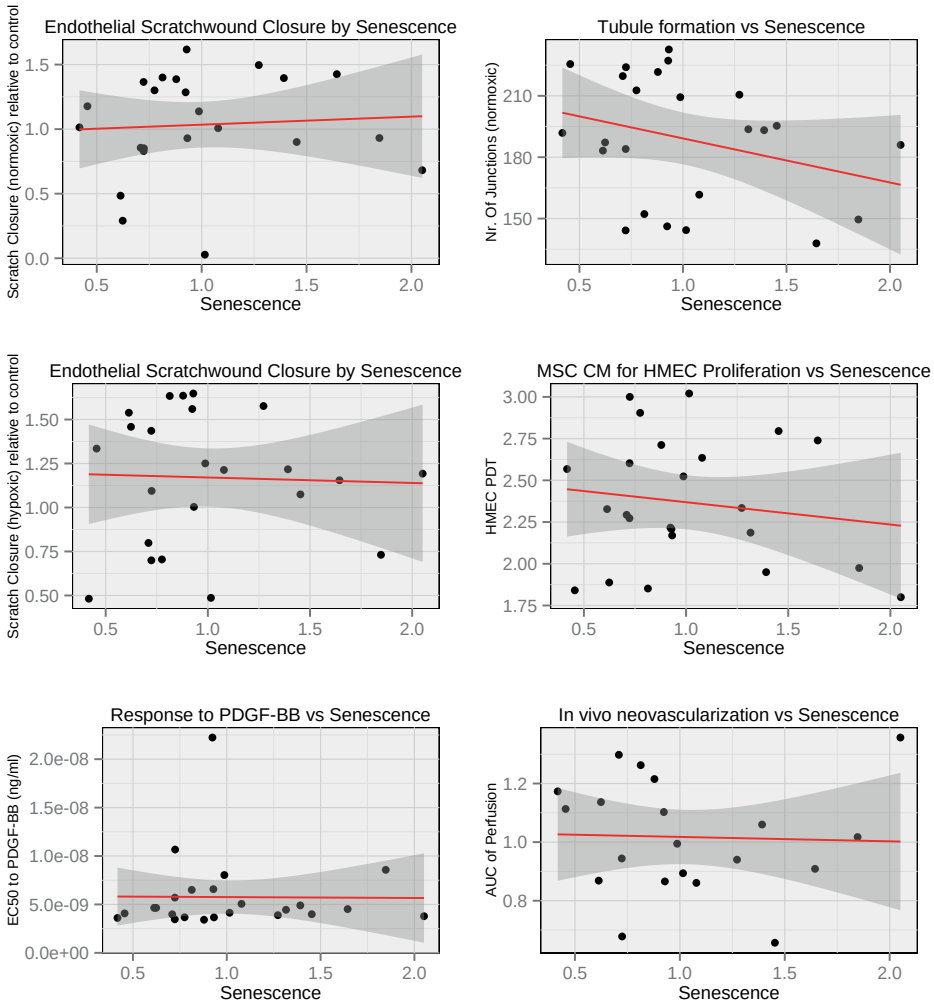
Supplemental Figure 2. A. Modified Volcano plot showing intergroup vs intragroup variance for CLI-MSCs and Control MSCs. **B.** Heatmap showing genes associated with MSC donor aging, note the large variation in expression. **C.** Heatmap showing genes associated with chondrogenic differentation.



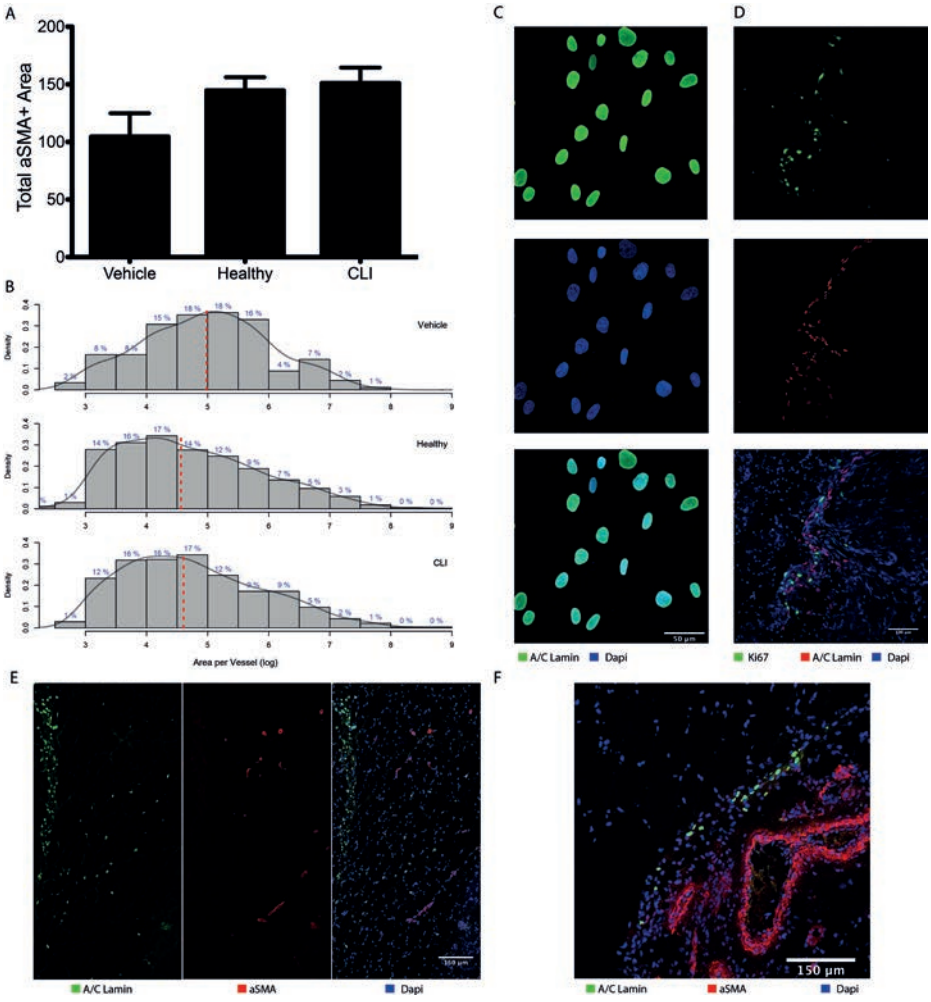
Supplemental Figure 3. A. Representative nucleus showing γ H2AX damage Foci **B.** Maxima as detected by software algorithm **C.** Total nuclear fluorescent signal γ H2AX in nuclei of CLI-MSCs and controls (error bars indicate S.E.M.) **D.** Number of DNA damage foci in nuclei of CLI-MSCs and controls **E.** Association of γ H2AX signal and age (R=0.27) **F.** Association of γ H2AX signal and C12FDG Fluorescence (R=0.34), bands indicate 95% CI.



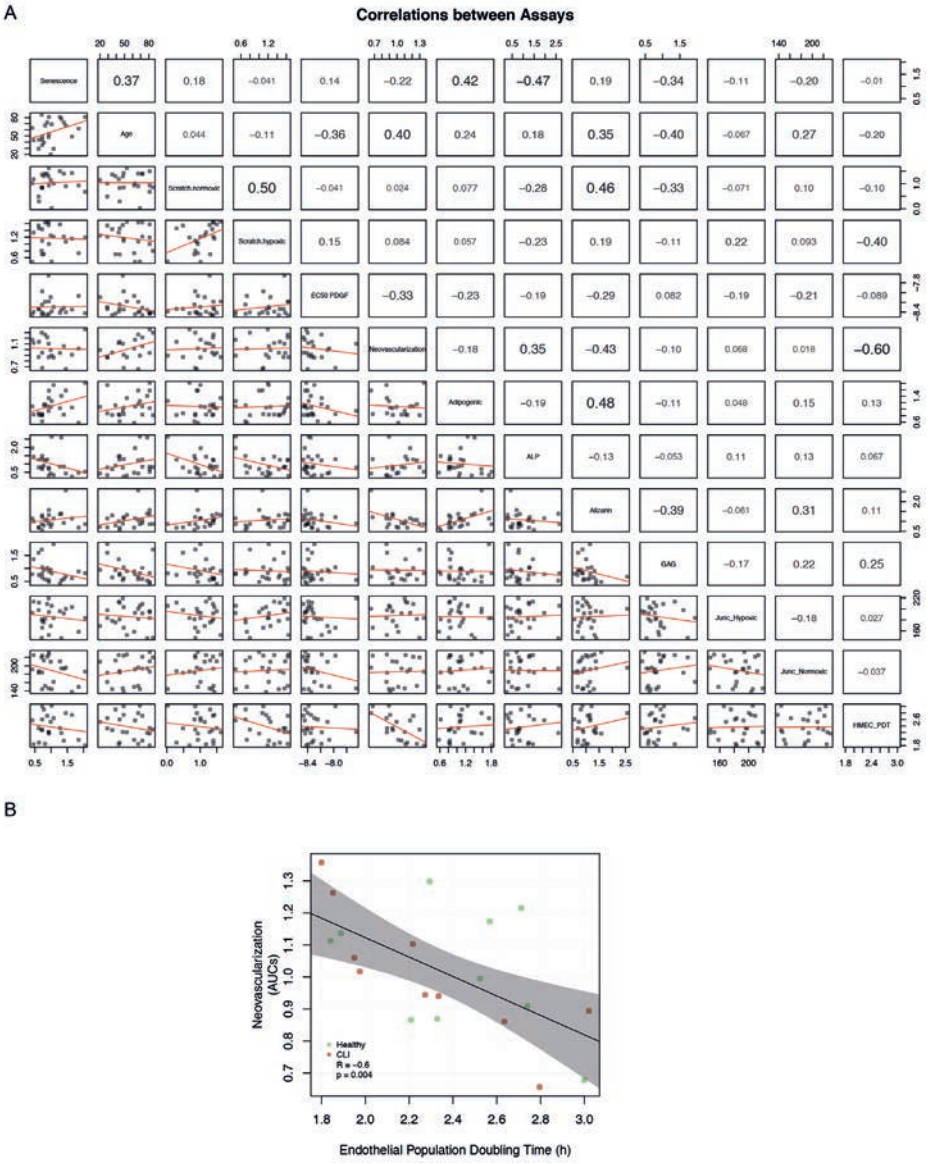
Supplemental Figure 4. Associations of Differentiation Capacity with Donor Senescence. For Adipogenic Differentiation $R_{Senescence}$ =0.41 (p=0.04). For ALP activity (early osteogenic differentiation) $R_{Senescence}$ =-0.34, (p=0.10). For Alizarin retention (late osteogenic differentation $R_{Senescence}$ =0.18 (p=0.4). For chondrogenic differentiation $R_{Senescence}$ =-0.34 (p=0.10). Bands indicate 95% CI.



Supplemental Figure 5. Associations of Measures of Proangiogenic Capacity with Senescence. Graphs showing the association of Endothelial Scratch wound closure in the presence of MSC-conditioned medium collected in normoxic R=0.07 and hypoxic conditions (R=-0.04), Donor EC_{50} to PDGF-BB (R=0.02), Junctions in Tubule formation of Endothelial Cells with MSC CM (R=-0.24), Endothelial Proliferation with MSC CM (R=-0.15), and neovascularization *in vivo* (R=-0.155) in relationship to senescence by MSC SA- β -activity per donor. Bands indicate 95% CI.



Supplemental Figure 6. Supplemental histology: A. Barplot showing total αSMA+ Area per High Powered Field. **B.** Histograms showing the size distribution of αSMA+ Vessels. In the MSC treated groups an enrichment of of smaller αSMA+ vessels was observed (p=0.02). In conjunction with the trend towards an increased number of vessels in MSC treated animals, this indicates that MSC treatment mainly promotes early arteriogenesis. **C.** Human specific A/C Lamin staining on cultured MSCs **D.** Representative section of staining for proliferating cells (Ki67 - Green) and human nuclei (A/C Lamin - Red), no double staining nuclei were observed. **E.** Confocal photomicrograph showing lack of co-localization of human MSCs (green nuclei) with αSMA positive vessels. **F.** MSC do not appear to integrate into the adventitia of larger vessels when injected in close proximity.



Supplemental Figure 7. Correlations between Assays. A. Correlation matrix between all assays performed in this paper. The upper right half shows the spearman rank correlation coefficient (ρ) between assays. The lower left half shows the bivariate scatterplot with linear regression line. **B.** Graph showing the inverse correlation between the population doubling time of endothelial cells cultured in the presence of conditioned medium from a given MSC donor and the angiogenic effect of the same MSC donor in the hindlimb ischemia model. The confidence bands indicate 95% CI.

Supplementary Table 1.

Top 50 of most differentially expressed genes. Fold Change values are CLI-MSC/Controls

Gene ID	logFC	AveExpr	adj.P.Val	Gene ID	logFC	AveExpr	adj.P.Val
HOXA10	0,943	7,573	0,077	LOC440589	0,245	13,979	0,100
HOXC4	-0,659	7,582	0,447	ISG20L1	0,434	8,205	0,100
COTL1	0,716	8,221	0,778	HOXC8	-0,890	9,653	0,100
HOXB5	-1,161	8,519	0,778	LOC100132439	0,264	6,813	0,100
FAM162B	-1,145	7,279	0,938	GNG12	-0,547	8,825	0,100
MMS19	0,378	8,490	0,100	CCNI2	0,255	6,774	0,100
HOXB8	-1,660	7,402	0,100	LOC729970	-0,286	7,050	0,100
FCHSD2	0,371	7,591	0,100	S100A4	-1,880	9,622	0,100
LOC100130516	-0,486	12,402	0,100	GPR89B	-0,238	7,325	0,100
LOC729466	0,282	12,404	0,100	RWDD1	-0,204	10,363	0,100
ENDOD1	0,315	7,739	0,100	NIT2	-0,213	8,387	0,100
TMEM144	0,280	6,732	0,100	SCG5	-0,768	8,631	0,100
LOC100133517	-0,250	7,349	0,100	CPE	-0,542	6,836	0,100
NLGN4X	0,401	7,013	0,100	NKX3-1	0,325	7,680	0,100
CTNNAL1	0,464	9,465	0,100	STAM2	-0,305	7,703	0,100
LOC646483	0,531	11,292	0,100	LOC404266	-0,354	6,900	0,100
NTF3	-0,844	7,336	0,100	HTRA2	0,204	7,704	0,100
MDFIC	-0,233	7,577	0,100	LOC730996	-0,219	6,958	0,100
MSX1	-0,585	8,265	0,100	C14orf147	-0,217	7,417	0,100
PITX1	1,895	7,996	0,100	RAB11A	0,219	10,350	0,100
C12orf51	0,266	7,367	0,100	NFIB	-0,735	8,672	0,100
MEMO1	-0,235	7,840	0,100	SGCE	-0,560	9,869	0,100
HLA-H	0,583	10,779	0,100	TMEM183A	-0,232	9,038	0,100
EPB41L3	-0,932	6,926	0,100	LOC100133565	0,429	7,794	0,100
STT3B	-0,488	8,055	0,100	LOC100128115	-0,210	7,048	0,100

Hendrik Gremmels M.D., Joost O. Fledderus, Ph.D, Martin Teraa, M.D., Ph.D,
Marianne C. Verhaar, M.D., Ph.D.

CHAPTER 10

MESENCHYMAL STROMAL CELLS FOR THE TREATMENT OF CRITICAL LIMB ISCHEMIA: CONTEXT AND PERSPECTIVE

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ABSTRACT

Cell therapy using mesenchymal stromal cells (MSCs) is a promising new avenue of treatment for critical limb ischemia (CLI). Preclinical studies have suggested that MSCs enhance neovascularization in ischemic limbs. In this commentary, we discuss a recent study by Gupta and colleagues, one of the first human trials using allogeneic MSCs for CLI, in relation to the current state of knowledge regarding cell therapy for CLI.

Recently, Gupta and colleagues (1) reported the results of a randomized double-blind placebo-controlled phase I/II study on the efficacy and safety of allogeneic mesenchymal stromal cells (MSCs), administered by intramuscular injection in patients with critical limb ischemia (CLI). Because the number of randomized controlled trials investigating stem cell therapy in peripheral arterial disease (PAD) is limited, this study is a welcome addition. Gupta and colleagues are also one of the first groups to apply MSCs in PAD.

Roughly a dozen clinical trials investigating stem or progenitor cell therapy in patients with CLI have been reported, but many were of limited size and not placebo- controlled (2). In most studies, the cell therapy product under investigation consisted of autologous bone marrow mononuclear cells (BMCs), which is a heterogeneous mixture of cells obtained by density gradient centrifuga- tion of bone marrow aspirate. Infusion of BMCs has been reported to lead to improvements in ankle-brachial index (ABI) and pain-free walking distance. However, the quality of evidence for efficacy is limited, as most studies lacked a proper placebo or sham group for the invasive bone marrow-harvesting procedure that is required for a study investigating autologous material.

Advances in good manufacturing practice-compliant production of more sophisticated cell products are now opening up the way to a second generation of cell therapy trials. MSCs, which are obtained by expansion of adherent bone marrow cells in *ex vivo* culture, have the capacity to enhance neovascularization and are a promising candidate for cell therapy in CLI. Owing to their inherent low antigenicity, MSCs may be administered in allogeneic recipients (3). Although allogeneic administration has been shown to be safe (4), eventual immunization may occur upon differentiation of residual injected cells (5). Because the pro-angiogenic effects of MSCs occur relatively quickly after administration (6), delayed immunization does not appear to be an obstacle when MSCs are given as a single-time administration. In advanced arterial occlusive disease, as in CLI, it may preferable to give multiple doses, especially considering the relative ease of administration and the lack of an acute indication. It remains to be elucidated whether allogeneic MSCs are sufficiently immunoprivileged to prevent alloimmunization if they are administered repeatedly in a "vaccination scheme".

Animal (7) and human (8) studies comparing efficacy of BMCs and MSCs in ischemic limbs suggest that MSCs are superior to BMCs in promoting neovascularization. The study by Gupta and colleagues, though designed primarily to assess safety and feasibility, shows a substantial improvement in ABI. To illustrate the treatment effect in relation to other studies, we have added a meta-analytic scatterplot plot showing ABI increases after cell therapy observed by Gupta and colleagues and three prior (8-10) placebo-controlled studies that report the same outcome measure (Figure 1).

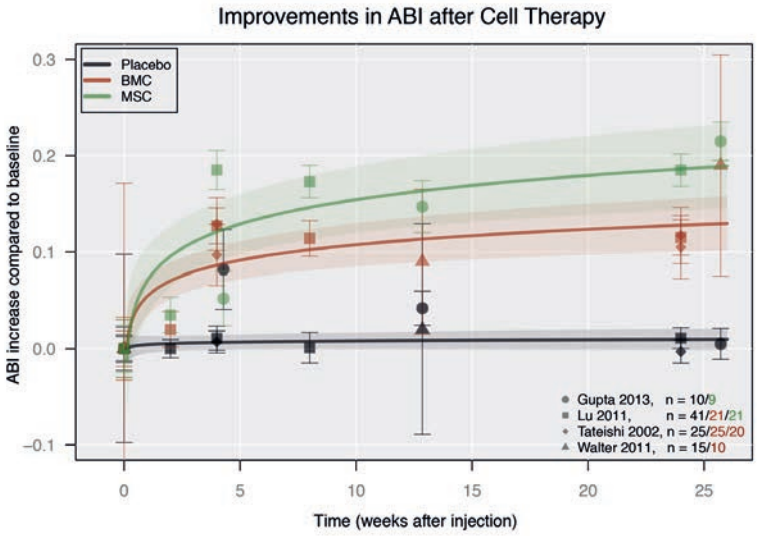

Figure 1. Meta-analytic scatterplot with meta-regression lines shows improvements in ABI compared with baseline after injection of a cell-therapy product or placebo. The two bone marrow mononuclear cell (BMC) cohorts in the study by Tateishi-Yuyama and colleagues (9) were included separately. Regression lines were fit with a mixed model using REML (restricted maximum likelihood) estimation; bands show 95% confidence intervals. This figure was prepared with the "metaphor" (11) package in "R".

Gupta and colleagues are to be commended for includ- ing a placebo arm in their study, as the invasiveness of cell injections often prevents researchers from including controls in early-phase clinical studies. Unfortunately, it is not entirely clear how blinding in this study was performed for either patients or treating physicians or whether blinding was successfully maintained throughout the study. For instance, the placebo administered by the authors is a balanced crystalloid solution that is likely to be easily distinguished from the serum-containing (and potentially dimethylsulfoxide-containing) cryopreservation medium in which the cell product was suspended. A rigorously blind study design is essential to exclude confounding factors, which may lead to an overestimation of treatment effects (12).

Another consideration in the interpretation of the find- ings reported by Gupta and colleagues is that the study population consists of both patients with an arterioscler- otic disease etiology and patients with thromboangitis obliterans, but the authors do not report in which propor- tions. In previous studies, it has been shown that disease etiology is an important determinant of treatment success (13), which makes this omission somewhat unfortunate.

The mechanism of action behind MSC-mediated improvements in perfusion is, at present, still poorly understood. MSC tracing studies in animal models show that MSCs are retained for only a short period of time in injected limbs (6,14) and that incorporation into the vascular bed does not contribute to the observed pro- angiogenic effects (6). Rather, MSCs are thought to act through paracrine effects, either directly on the local endothelium (15) or indirectly through the recruitment of angiogenic monocytes (14). These functions are likely to be unrelated to the multipotent capability of MSCs, and it is unclear whether using whole cells has an added functional benefit (for example, through homing) above their secreted growth factors (16). There is even consider- able discussion whether cell therapy with MSCs deserves the epithet of stem cell therapy at all (17). Regardless of these considerations, the studies by Gupta and colleagues and others show that further development of MSC therapy is, at the very least, a promising avenue in the treatment of patients with very few other options.

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CHAPTER 11

SYNOPSIS

In this work we have investigated the relationship between bone marrow (BM)- derived progenitor cells and Critical or Severe Limb Ischemia (CLI or SLI), with the primary objective of developing a new treatment option for SLI. SLI is the most advanced stage of peripheral artery disease (PAD), in which blood flow is impeded to such an extent that oxygen supply of the limb does not meet metabolic demands, even in rest(1). SLI presents with rest pain and/ or tissue loss, which ultimately leads to amputation in 20-40% of the patients(2). SLI is associated with a poor quality of life(3) and high socio-economic costs(4). A substantial proportion of patients is not eligible for conventional therapeutic options, hence novel treatment modalities are urgently needed. The use of stem or progenitor cells to augment neovascularization in the affected limb is currently one of the most promising options. However, it is at present unclear which progenitor cell type is the most suitable for this approach. This is one of the primary subjects of this thesis. A major confounding factor is the fact that in most cases progenitor cell therapy necessarily occurs with autologous – i.e. the patient's own – cells. A large part of this thesis is therefore dedicated to the biology and functioning of progenitor cells in SLI patients, as this is an important variable in the determination of treatment success.

In **Chapter 2** we have examined the relationship between BM and circulating progenitor cell numbers and the risk of amputation or death. This interconnection is central to the understanding of the contribution of circulating progenitor cells to vascular disease. Many studies have shown that circulating progenitor cell numbers, particularly CD34+ cells, are decreased in different forms of cardiovascular disease(5-8) and that progenitor cell numbers are predictive of cardiovascular events(9,10). Together with the observation that injection of CD34+ progenitor cells promotes angiogenesis in ischemic areas(11), this suggests the existence of an endogenous repair system, that is dysfunctional in cardiovascular disease(12). We show that patients with SLI display an exhaustion of the BM progenitor cell reserve and that in SLI progenitor cell numbers in BM seem to be more important for event-free survival than actual mobilization.

The findings in this chapter have ramifications on progenitor cell treatment. Pre-clinical studies have demonstrated that reduction in progenitor cell numbers in BM isolates from patients with cardiovascular disease are associated with poor pro-angiogenic effects in *in vivo* models(13,14). We show here that reduction of progenitor cells in BM is common in SLI patients, likely causing poor therapeutic efficacy of BM-derived mononuclear cells (BM-MNCs), one of the most studied cell types in (pre-)clinical settings. Furthermore, we show that actual mobilization of progenitor cells is not associated with improved cardiovascular risk. This would also explain why increasing the number of circulating progenitor cells by chemical mobilization with for instance Granulocyte Colony-Stimulating Factor does not show treatment benefits in cardiovascular disease(15,16).

The next two chapters focus on identifying high-risk subgroups within the SLI population. In **Chapter 3** we have identified serum biomarkers that are related to the risk of major

events (amputation or death) in the JUVENTAS cohort. Two inflammatory biomarkers, Interleukin 6 (IL-6) and Interferon gamma-induced protein 10 (IP-10), predicted major events within one year with reasonable accuracy. In **Chapter 4** we examined Diabetes Mellitus as a particular risk factor for amputation in two SLI cohorts. The histopathological form of arteriosclerosis is subtly different in diabetic patients, as they are more prone to media calcification in addition to intimal atherosclerosis. Future trials investigating cell therapy could benefit from selective inclusion of high-risk patients. Prognosis with regard to life and limb has dramatically improved in SLI over the last two decades(17) and this should be taken into account in order to adequately power future trials. Identification of high-risk patients will be an important step in optimizing invasive and expensive treatments such as cell therapy and reducing the number-needed-to-treat.
Chapter 5 aims at the identifications of factors associated with treatment response to BM-MNCs. We used data from the REPAIR AMI trial, a large study investigating BM-MNC treatment for acute myocardial infarction (MI). We have used this study and not JUVENTAS, as cardiac function is comparatively easy and reliable to quantify for any individual patient. We could therefore compare the course of recovery after MI in cell- and placebo-treated patients. We found that younger patient with a large initial infarction and a high risk factor burden benefitted most from treatment. The finding that risk factors such as obesity are associated with a larger treatment benefit was surprising, as cardiovascular risk factors are usually associated with an impairment of progenitor cell function(18,19).

The second part of the thesis focuses on the development of an improved cell therapy product to be applied in a future clinical trial. In **Chapter 6**, we provide an overview of the different progenitor cell types found in BM and circulation and how these are likely interrelated(20). Particular focus is on putative Endothelial Progenitor Cells (EPCs), as at the time of writing, it was considered the most suitable type of stem cells to treat ischemic vascular disease. We quantified relationships between different progenitor and non-progenitor cells in the BM (the most common source for progenitor cells) in an unbiased fashion, by examining the whole transcriptome. We show that there is a great difference between cell-types previously designated as EPCs, which is in agreement with key functional differences between the cells.
In **Chapter 7** we investigate a pre-treatment strategy for Endothelial Colony Forming Cells (ECFCs), an endothelial cell-type obtained from blood. ECFCs enhance angiogenesis in ischemic areas by direct neovessel formation. In contrast to most other cell types functioning via paracrine effects, ECFC survival in the long term is important for maintenance of the newly formed vessels(21). ECFCs have been shown to be very sensitive to oxidative stress(22). We aimed to enhance ECFC function by pre-treating the cells with compounds that activate Nuclear factor (erythroid-derived 2)-like 2 (Nrf2),

a key transcription factor that regulates expression of anti-oxidant enzymes, hence preparing the cells for a potentially hostile environment. In this chapter we show that Nrf2 is very important in angiogenic function of ECFC, by creating constitutional Nrf2 knock-down and activation cell lines. Our findings show that knock-down of Nrf2 has dramatic effects on ECFC function, in line with previous studies on endothelial cell lines(23,24).Furthermore, we show that Nrf2 activation via pre-treatment with sfn can rescue ECFC function in the presence of oxidative stress. Given the potential of ECFCs in cell therapy and tissue engineering applications, our findings may have clinical relevance for enhancement of cell survival after implantation.

In **Chapter 8,** we have investigated a co-implantation strategy of ECFCs with a slow-release system for growth factors. ECFCs have been shown to achieve better neovascularization if they are co-implanted with another cell type with paracrine activity(21). In this study we have used gelatin microparticles to deliver growth factors, with the intent of creating spatially defined vascular structures in tissue-engineered constructs. We show that using this slow-release system we can enhance the local formation of vascular structures in three-dimensionally printed constructs.
Chapter 9 investigates autologous Mesenchymal Stromal Cell (MSC) therapy in SLI patients. As we have shown earlier in the thesis, the number of CD34+ - hematopoietic - progenitor cells in BM is reduced in SLI patients, which likely impairs the efficacy of BM-MNC treatment. It is not known if a similar reduction in number and function of progenitor cells also occurs for progenitor cells of different lineages. MSCs are a progenitor cell population that arise from pericytes in the BM niche(25) and which can differentiate into several mesenchymal tissues, such as bone, cartilage and fat(26). Culture expanded MSCs secrete a plethora of growth factors and microvesicles that contribute to tissue repair and neovascularization(27) and are considered as cell therapy for SLI(28). We show that BM-MSCs from SLI patients are not impaired in their neovascularization capacity compared to MSCs obtained from healthy controls. This suggests that autologous MSC therapy does not suffer from the same limitations as autologous BM-MNC therapy and may indeed be suitable for clinical application. In **Chapter 10** we discuss autologous versus allogeneic MSC therapy in light of a recent trial using allogeneic MSCs in SLI(29).

After this synopsis we present our current outlook on cell therapy for SLI, and our considerations for a successor trial to JUVENTAS in **Chapter 12.**

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CHAPTER 12

CELL THERAPY FOR SEVERE LIMB ISCHEMIA: THE NEXT STEP

Severe Limb Ischemia (SLI) poses a great unmet need for novel treatments, as 20-40% of the SLI patients are not eligible for conventional revascularization, ultimately leading to amputation(1,2). No-option patients frequently have diffuse atherosclerotic lesions, with profound infra-popliteal disease, which is difficult to treat either surgically or endovascularly(3,4) and requires novel approaches that target neovascularization. Such therapies could consist of the local delivery of growth factors or progenitor cells, of which the latter has been proven to be more efficacious in preclinical models(5). Most progenitor cell trials have been conducted using bone marrow-mononuclear cells (BM-MNCs), on the supposition that the BM, as the reservoir of hematopoietic stem cells, also contains endothelial progenitor cells (EPCs)(6,7). Early, uncontrolled clinical studies using BM-MNCs were promising(8), but placebo-controlled trials gave conflicting results(9). A large, double-blind, placebo-controlled randomized trial by our group, the JUVENTAS trial, showed no treatment effects of BM-MNC administration over placebo(10). Furthermore, an aggregated study with over thousands of patients that were treated with BM-MNCs for myocardial infarction also failed to find a consistent positive effect(11).

The Physiology of Endothelial Progenitor Cells as Rationale for Cell Therapy

The rationale behind using (progenitor) cell therapy as a treatment modality for cardiovascular diseases was historically motivated by observations that made an attractively coherent picture(12), which can be summarized as follows: Endothelial Progenitor Cells (EPCs), that can differentiate both into endothelial cells and the hematopoietic line - similar to hemangioblasts in the developing embryo - are found in circulation in humans(13). These cells mobilize from the BM to home to sites of ischemic damage and can differentiate into endothelial cells to promote neovascularization(13),(14). Patients with cardiovascular risk factors have fewer progenitor cells(15) in their circulation, as these risk factors inhibit mobilization from BM(16). Isolation of progenitor cells and direct injection into ischemic areas promotes neovascularization(17). However, advancements in scientific understanding and technology have demonstrated problems with this paradigm.

Firstly, it has proven difficult to identify and define a progenitor cell that fulfills the criteria to be called EPC. There are multiple different phenotypes of cells isolated from blood(18,19), which display both endothelial and progenitor cell markers and have consequently been designated as EPC(20). Closer examination, however, shows that the most commonly isolated phenotype, the early EPC or Circulating Angiogenic Cell (CAC)(18), is of myeloid origin(21), displays functional monocytic characteristics(22,23) and likely acquires progenitor cell surface markers due to isolation artifacts(24,25) (see also **Chapter 6**). CACs do participate in angiogenesis, but only as an adjuvant cell, a role well-established for monocytes/macrophages(26,27). While it is possible to isolate angiogenic cells of endothelial origin from blood, called late EPCs or Endothelial Colony

Forming Cells (ECFCs)(28,29), it is unclear if these also reside in BM(30). ECFCs, while colony-forming and highly proliferative(28), lack progenitor cell characteristics such as asymmetric cell division or lineage hierarchy. Moreover, they retain the characteristics of the endothelial bed from which they are isolated, indicating a high level of differentiation(31).

Aside from characterization of cell types isolated *ex vivo*, it is unclear whether circulating progenitor cells contribute to endothelial repair and neoangiogenesis *in vivo.*

Two observations have historically lead to this hypothesis: Firstly, it is consistently observed that the number of progenitor cells in circulation is reduced in various forms of cardiovascular disease(32). Furthermore, there is an acute increase in circulating progenitor cells – mobilization – after ischemic events such as myocardial infarction(33),(34), which is blunted in patients with a high burden of cardiovascular risk factors(35,36). Secondly, injections of progenitor cells, both of culture expanded cells and raw isolates, have been shown to augment angiogenesis in animal models of tissue ischemia(17,22,37-39).

More recent insights show that this relationship is more complex and possibly only circumstantial. There is strong evidence that circulating cells do not contribute to re-endothelialization(40,41) or newly formed vessels *in vivo*(42). Rather, all new vessels sprout from the resident endothelial cells(40). Studies investigating BM-derived cell treatments do show homing of injected cells into ischemic areas, but these cells serve an auxiliary role and do not incorporate into the vasculature(43,44). Closer examination shows that the injected cells temporarily reside in the periphery of blood vessels, presumably promoting angiogenesis via a paracrine mechanism, *viz.* the secretion of growth factors or microvesicles(43,45). Whether similar homing of progenitor cells occurs physiologically, without isolation and (re-)injection, is unclear. After acute ischemia, CD34+ progenitor cells are mobilized into the circulation, due to release of certain cytokines, such as VEGF or SDF-1α(46). The subsequent homing of mobilized progenitor cells to sites of vascular or ischemic damage has not been conclusively shown, and may occur only at low levels(47,48). The BM-derived cells that do migrate into hypoxic tissue mostly consist of neutrophils and monocytes(49).

An independent indication that progenitor cell mobilization does not play a major role in cardiovascular outcomes in the chronic phase of atherosclerotic ischemic disease is given in **Chapter 2.** We show that there is a quantitative exhaustion of the BM progenitor cell reserve in SLI patients, which is independent of the circulating cell numbers and mobilization. This reduction of BM progenitor cells is statistically more closely associated with patient event-free survival than progenitor cell mobilization, supporting that reduced mobilization of progenitor cells is a secondary phenomenon. Together these observations suggest a less prominent role for circulating progenitor cells in vascular homeostasis than has previously been proposed.

A new approach to cell-therapy

The tenuous relationship between progenitor cells, vascular repair, and the development of vascular disease does not disqualify cell therapy as therapeutic modality. In various pre-clinical models of vascular disease, cell therapy including studies using BM-MNCs, has shown consistent enhancement of neovascularisation(38,39). Perhaps, alternative explanations for this effect can be identified that do not presuppose a central role for progenitor cells. For example *ex vivo* manipulation of BM-MNCs, even by simple isolation and re-injection, may lead to an activation of progenitor cells that has been a confounder in interpreting the physiological role of circulating progenitor cells(50). This might also explain why enhancing mobilization of progenitor cells with Granulocyte Colony Stimulating Factor (G-CSF), does not appear to show any benefit(9,51,52) in recovery after myocardial infarction. In any case, the pro-angiogenic effects of cell therapy appear to be due to a complex mixture of secreted factors and modification of the immune response, rather than regeneration by progenitor cells(53).

For this reason we advocate a more pragmatic approach to cell therapy. Here, the focus is not as much on identification of the exact progenitor cell population but rather on enhancing different aspects of angiogenesis using specific cell-types, regardless of progenitor status. From a mechanistic standpoint there are several possibilities to use cell injections to enhance specific aspects of angiogenesis, each exemplified by a specific cell-type.

- Induction of an inflammatory response by direct introduction of immune cells, such as macrophages(27,54) or CACs(22), that induce remodeling of the local vasculature and guide endothelial cells in angiogenesis (figure 1, "A").
- Direct introduction of endothelial (progenitor) cells, such as ECFCs, that will repair endothelial defects and participate in the formation of new vessels (figure 1, "B")
- Indirect induction of inflammation or endothelial recruitment via chemical signals such as growth factors or microvesicles (figure 1, "C"). While this method does not necessarily involve cell therapy, the use of cells as 'delivery vehicle' for growth factors and signaling molecules has many advantages, including prolonged secretion of signals, homing to ischemic sites and a wide repertoire of secreted factors that can be modified to the local milieu.

A particularly interesting candidate for this latter application are Mesenchymal Stromal Cells (MSCs), a culture-expanded cell-type which is thought to arise from pericytes (55,56). MSCs have been implicated in a variety of biological processes including wound repair and show great potential in aiding the recovery of various tissues, including skin, joints, the liver and kidneys(57). MSCs are thought to act via a variety of paracrine mechanisms(57), that modulate inflammation(58), aid angiogenesis(59) and limit fibrosis(60). While MSCs are grown from pericytes, they rarely take up this role after injection in ischemic areas (61). While MSCs have progenitor cell characteristics, such as the ability to differentiate into different mesenchymal tissue lines(62), it is not likely

that this aspect is of importance for their regenerative effects(53). Arguably, progenitor cells are not well suited for the indirect methods that rely on paracrine effects. Progenitor cells generally display a quiescent and glycolytic(63,64) metabolism, with limited secretion of growth factors. Differentiated or senescent cells on the other hand expand their Golgi apparatus and show a greatly increased secretion of paracrine factors(65-67). In addition, the high replicative potential of stem cells poses potential risk of malignant transformation(68), whereas differentiated cells that undergo senescence are unlikely to form tumors(69).

It is difficult to identify the optimal cell type to enhance neovascularization on theoretical or biological grounds such as progenitor cell characteristics or markers. It is conceivable that, as the mechanisms by which different cell therapy products promote angiogenesis differ, combinations of different cell-types may exert a synergistic effect.

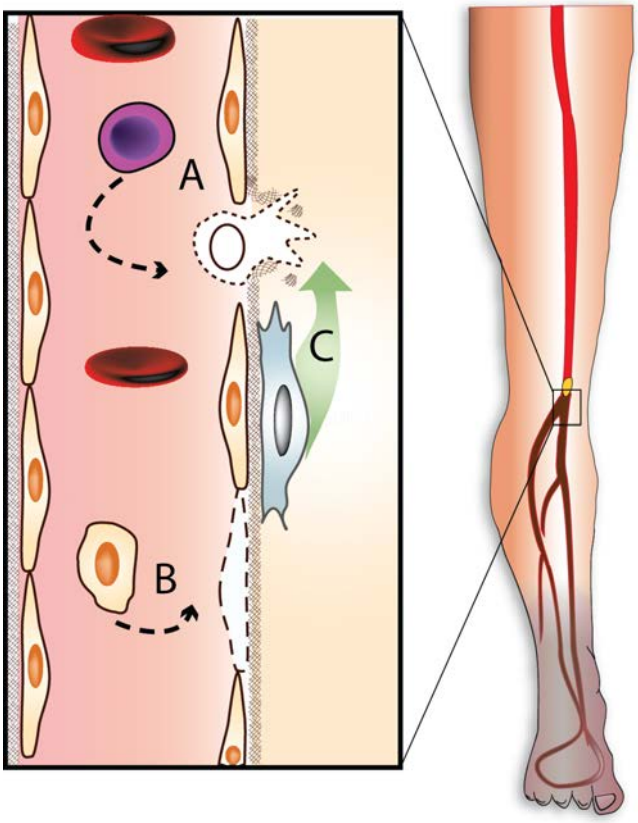

Figure 1. Modes of action for cell therapy. A: Remodeling of the vasculature by immune cells. **B:** Repair of endothelial defects and angiogenesis by ECFCs: **C**: Recruitment of leukocytes and endothelial cells by paracrine effects.

The pragmatic approach proposed here requires direct, head-to-head comparisons of different (progenitor) cell applications, or as the old adage goes: the proof of the pudding is in the eating(70).

Comparative Studies of Efficacy using Different Cell Types

Several studies have compared the angiogenic potential of different cell types head-to-head in animal models of vascular disease. Some caution should be applied in extending these findings to SLI as the contribution of neovascularization to functional recovery in different animal models differs. In hind-limb-ischemia models distal perfusion is almost solely dependent on angiogenesis and arteriogenesis. In models of myocardial infarction, functional recovery quantified by left ventricular ejection fraction also depends on preservation of tissue-at-risk. In the heart, progenitor cell therapy ideally also regenerates lost myocytes; in SLI myocyte loss contributes less to disease.

Myocardial infarction models have been used extensively to investigate cell therapy, including many studies in large animals(71). This is of importance as small animal studies generally greatly overestimate effect sizes and are less representative of human physiology(72,73). Direct comparisons between cell types generally favor cardiac specific progenitor cells, results which are also corroborated by a meta-analysis(73,74). Results using cell types that mainly act through promotion of angiogenesis remain variable. A study by Li et al. has directly compared cardiac progenitor cells, MSCs and BM-MNCs in a porcine model of myocardial infarction(74). While cardiac stem cells were the only cell type to increase myocardial function after infarction, MSCs did prevent loss of cardiomyocytes and preserved cardiac function compared to controls or BM-MNC treatment. Similar results were obtained by other groups(75),(76) but two studies reported the opposite result with a greater improvement in BM-MNC treated animals(77,78). Lastly co-transplantation of MSCs and BM-MNCs may be better than either cell type alone(79). Fewer studies investigated ECFCs, but results indicate that they preserve myocardial function to a similar extent as MSCs(80,81).

Preclinical studies for SLI have only been conducted in rodents, using the hind-limb ischemia model(82). A direct comparison between BM-MNCs and BM-derived MSCs showed that MSCs induced a more rapid and more complete recovery after ischemia(83). Similar results were obtained with adipose tissue derived MSCs(84). ECFCs alone induce substantial neovascularization, though not superior to CACs(85) but together these two cell-types possess a synergistic effect(86). Similarly ECFCs and MSCs show a synergistic effect, wherein ECFCs permanently form new capillaries and MSCs secrete paracrine factors that are only necessary in the initial phase of angiogenesis. (87).

In direct comparisons, there appears to be trend towards superior angiogenic effects for culture-selected cells such as CACs, MSCs and ECFCs compared to the most often used BM-MNCs; especially in hind-limb ischemia models. Cells undergo a great

number of transcriptional changes in cell culture(88-90), which perhaps adds to their pro-angiogenic effects.
It must be noted that almost all preclinical studies have been performed under optimal laboratory circumstances, using healthy or neonatal cell donors. Not all of these approaches can be directly translated to clinical applications, as there may be difficulty in obtaining autologous cells or there may be disease-mediated dysfunction of progenitor cells(91)

Clinical Application and Translation to the Bedside

An important requirement for a clinically applicable cell therapy is consistent isolation and availability of clinical-grade cell culture additives. MSCs have been safely applied in over 200 clinical trials(92) and clinical grade production is available in many centers. There have been two phase I/II trials for SLI, that show that sufficient numbers of cells can be expanded and that administration in patients can be performed safely(93-95).
ECFCs on the other hand prove very difficult to isolate reproducibly from adult subjects, with success-rates for obtaining a single colony varying from 10-60% (28,85,96) Moreover, ECFCs colonies will only appear when blood mononuclear cells are cultured in one specific cell culture medium, EGM-2, which contains many recombinant growth factors, making it difficult and expensive to produce in clinical grade. Attempts to obtain ECFCs with clinical grade reagents have been unsuccessful(97), as have been attempts to isolate ECFCs from other sources such as BM-MNCs(30). Furthermore, most ECFC studies are proof-of-concept studies using cord-blood derived cells. Adult ECFCs have been shown to senesce quickly, potentially limiting their clinical application(98).
CACs are comparatively simple to isolate from peripheral blood, although isolation also requires the relatively complex medium EGM-2. Furthermore the absolute cell number obtainable is quite low, as CACs do not divide in culture(88). For this cell-type there is another hurdle to clinical translation, namely that cell numbers and angiogenic function are reduced in patients with cardiovascular disease(15,99). Studies have shown that patient-derived CACs are less responsive to growth factors, show increased signs of senescence(100) and induce less neovascularization *in vivo*(101,102). Progenitor cells from a diabetic mouse have even been shown to inhibit neovascularization(103). Interventions with pharmacological therapy, including statins(104-106) and PPARγ-agonists(107-110) have been able to increase cell number *in vitro* but restoration of angiogenic potential *in vivo* showed only limited effects.
Whether disease-mediated dysfunction is a common feature of all patient-derived progenitor cells remains unclear. It has been demonstrated in BM-MNCs(111-113), which is partially reversible by pre-treatment(114,115). In MSCs, which are most often cultured from BM-MNCs we have shown that there does not seem to be an impairment in pro-angiogenic potential, perhaps due to the significant expansion in culture(61)

(see Chapter 9). Studies on ECFCs show conflicting data, and both a decreased(116-118), unaltered(119) or increased(120,121) number of colonies have been reported in patients with cardiovascular disease.

In a balance of therapeutical efficacy and clinical feasibility, MSCs currently appear to be the best candidate cell type for further development as a therapy for SLI. In preclinical studies, MSCs consistently induce a high degree of neovascularization in ischemic limbs(83), and preliminary data support efficacy in clinical trials(95) (**Chapter 10).** Furthermore, MSCs are comparatively simple to produce in a clinical setting and appear not to be affected by disease processes. In the long term, the field may switch to combinations of cell types, as is currently proposed in the treatment of myocardial infarction(122). The combination of an endothelial substrate such as ECFCs and an adjuvant cell-type such as MSCs seems the most promising, but is currently limited by ECFC isolation. Current limitations may be overcome in the future by the use of other endothelial sources such as the vessel wall(123) or reprogrammed cells(124).

Design considerations for future studies using MSCs

The potential efficacy of cell treatment does not only depend on selection of the best cell type, but also on the method of application. MSCs, rather uniquely among transplanted cell grafts, are only minimally immunogenic(125) and display strong immunomodulatory properties(58), which makes allogeneic application possible(126). Several clinical studies using allogeneic MSCs have been performed in patients with myocardial infarction and allogeneic cells did not cause an acute rejection or detectable allo-antibodies(127,128). On the other hand, pre-clinical studies have shown that immunization against allogeneic MSCs does occur(129,130), likely after differentiation *in vivo*(131-133), leading to clearance of injected cells. It is likely that in a clinical setting, immunization against MSCs will also occur, especially after repeated administration(95).

Allogeneic administration of MSCs has several advantages. Firstly, the burden on the patient is significantly less(134), as patients will not have to undergo a BM harvesting procedure. This is also of importance in clinical trial design, where patients in the placebo-arm of an autologous cell trial will also have to undergo a BM harvesting procedure, making the risk/benefit ratio for trial participation unfavorable(135). A second advantage of allogeneic MSC therapy is that the pro-angiogenic capacity of the cell isolate can be tested in advance. In **Chapter 9,** we observed a considerable heterogeneity between donor MSC isolates, with about 25% of isolates not inducing increased neovascularization compared to placebo. In autologous application of MSCs, this heterogeneity will likely affect trial results. In allogeneic application, a selection of the best donor isolates can be made to reduce variability in treatment response. Lastly, there is considerable difference in treatment cost. In allogeneic MSC application, MSCs from altruistic donors can be expanded on a large scale prior to the study and cryopreserved as off-the-shelf available

therapy. Many aspects of quality control such as karyotyping need only to be performed once per batch, making the logistics less laborious.
From these considerations, allogeneic MSC therapy would be clinically preferential and should be prioritized in clinical trials. Concerns about rejection remain valid, and would limit the interpretation of negative trial results. However, sufficient indication for a positive treatment effect of allogeneic MSC application from phase I/II studies exists, although these preliminary results should be confirmed in larger, double-blind randomized placebo-controlled trials(93-95).

Patient Selection

A hitherto little-explored subject is which patients are most likely to benefit from cell-therapy. One of the complicating factors is that in autologous application of cell therapy, patient response can both be affected by the intactness of the (progenitor) cell reservoir, as well as by the extent of disease in the target organ. We have shown in **Chapter 5** that treatment response in BM-MNC therapy for myocardial infarction (MI) can be predicted to some extent by baseline factors. The most important factor proved to be the severity of the infarction, as defined by reduction in left ventricular ejection fraction. Interestingly, factors that are likely to negatively affect the angiogenic capacity of the stem cell product, such as increased patient weight(111,112) may even be associated with a greater treatment response. Together, these findings suggest that treatment success may depend at least to some extent on the patient, and not only on the cell product. Treatment responders will likely be easier to identify in studies using allogeneic MSC therapy, as the cell product is more consistent in such studies. This is exemplified by a recent study by Golpanian et al, who identified patient age as a significant determinant to treatment response in a small study investigating MSCs for the treatment of chronic ischemic cardiomyopathy(136).
In the case of SLI less is known about factors associated with treatment response. Meta-analyses suggest that patients with tromboangiitis obliterans generally showed greater treatment benefits than patients with western type atherosclerosis obliterans (ASO), such as in JUVENTAS(9). Even within ASO patients, there is substantial heterogeneity in disease etiology, due to factors such as inflammation and diabetes, as we show in **Chapters 3 and 4.** It is conceivable that response will also vary between such subgroups. Unfortunately, considerations of statistical power do not allow meaningful sub-group analyses in the JUVENTAS dataset. However, future endeavors in this direction may be attempted using individual data meta-analysis, as has also been initiated in BM-MNC treatment for myocardial infarction(11).

Conclusion and Future Directions

Scientific understanding of the role of progenitor cells in cardiovascular disease has changed over the past two decades. The idea that cell therapy using progenitor cells

recapitulates the developmental process of vasculogenesis has been replaced by a more complex picture involving complex interactions between injected cells, the immune system and resident endothelial cells. In the meantime the clinical landscape for SLI patients has changed. Clinical management has improved and secondary prevention by medical therapy, such as the widespread use of platelet aggregation inhibitors and statins has become widely adopted(137). As a consequence amputation-free survival for SLI patients at 1 year has nearly doubled since the first trials with BM-MNCs(138). The improved prognosis for SLI patients makes the design of trials that are powered to detect differences in major events increasingly difficult. Patient inclusion for the JUVENTAS trial, which was designed to include 160 patients, required 6 years, from 2006 to 2012(10). In order to prevent future trials from being underpowered they should include even more patients. Better risk-stratification of patients by using a prediction model as in **Chapter 3 and 4** or high-risk subgroups such diabetics can be used to decrease the number-needed-to treat. Ideally, responders can be identified *a priori* as we have shown in patients with a myocardial infarction (**Chapter 5**).

Ultimately, however, collaborative efforts, both on the national and international level are required to push the field forward and gain more definitive answers regarding the efficacy of cell therapy and development of novel treatment modalities.

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APPENDIX

NEDERLANDSE SAMENVATTING

DANKWOORD

LIST OF PUBLICATIONS

CURRICULUM VITAE

NEDERLANDSE SAMENVATTING

Perifeer arterieel vaatlijden ontstaat als gevolg van ernstige verkalking (ook wel atherosclerose) van de slagaders (arteriën), met name in de benen. De ernstigste vorm wordt kritieke ischemie genoemd, in het Engels *Severe* (of soms *Critical) Limb Ischemia (SLI).* Bij kritieke ischemie is de bloeddoorstroming door slagaderverkalking dermate beperkt dat het aangedane been onvoldoende zuurstof en voedingsstoffen krijgt. Patiënten met kritieke ischemie hebben dientengevolge pijn in het aangedane been en in veel gevallen begint het weefsel uiteindelijk langzaam af te sterven en ontstaan er ulcera of necrose. In sommige gevallen is het mogelijk de slagadervernauwing te openen met een stent of een bypass aan te brengen, maar 40% van de patiënten raakt op een gegeven moment uitbehandeld en zal amputatie van het been moeten ondergaan.
Er is een dringende behoefte aan nieuwe, effectieve behandelingsmogelijkheden voor kritieke ischemie. Voorlopercelltherapie, het toedienen van uit eigen beenmerg afkomstige stam- en voorlopercellen, lijkt een veelbelovende nieuwe behandelingsoptie. Om dit te onderzoeken heeft onze groep de Juventas studie verricht, een gerandomiseerde, placebo-gecontroleerde studie naar de klinische effecten van toediening van eigen beenmerg cellen in patiënten met kritieke ischemie. Deze studie toonde echter aan dat er geen duidelijk effect was van beenmergcel therapie in vergelijking met een nep-behandeling (placebo). Hiervoor zijn verschillende mogelijke oorzaken aan te wijzen. Een van de mogelijke oorzaken is dat in patiënten met risicofactoren voor hart- en vaatziekten de functie van de beenmergcellen minder goed is. Daarnaast werd hier een ongeselecteerde fractie van beenmerg gegeven, waarin behalve gunstige stamcellen ook andere cellen aanwezig waren die mogelijk kunnen bijdragen aan het ontwikkelen van slagaderverkalking en ontsteking.
Het doel van dit proefschrift was het ontwikkelen van een optimale vorm van stamceltherapie voor patiënten met kritieke ischemie. We hebben vanuit dit oogpunt ons onderzoek verdeeld in twee hoofdlijnen: Onderzoek naar patiënten met kritieke ischemie en hun ziektebeloop, onder andere om te zien welke patiënten de grootste kans hebben op amputatie en hoe de lichaamseigen voorlopercellen hierbij betrokken zijn. Ten tweede onderzochten wij of het mogelijk was gericht voorlopercellen voor therapie uit het beenmerg te isoleren en expanderen voor een klinische toepassing.

In **Hoofdstuk 2** hebben we de relatie tussen voorlopercellen in bloed en beenmerg onderzocht en hoe deze zich verhoudt tot de kans op het ondergaan van een amputatie of dood in patiënten met kritieke ischemie. Het is bekend dat het aantal voorloper cellen in het bloed lager is in patiënten met verschillende vaatziekten, en dat het aantal voorspellend is voor de ziekte uitkomst op lange termijn. Een achterliggende theorie is dat er in patiënten minder vaatherstel kan optreden omdat er minder voorlopercellen in

de bloedvaten beschikbaar zijn. Tevens is bekend dat de voorlopercellen in het bloed afkomstig zijn uit het beenmerg. De toestand van het beenmerg in patiënten met vaatlijden is nog relatief onbekend, mede omdat het moeilijk te onderzoeken is door de invasieve aard van een beenmerg punctie. Er werd tot op heden altijd aangenomen dat de lagere aantallen voorlopercellen in bloed vooral het gevolg zijn van een verstoorde communicatie tussen de vaten in het lichaam en het beenmerg. De signalen van het lichaam dat de vaatvoorziening verstoord is, zouden niet meer aan komen in het beenmerg en er worden dus minder voorlopercellen gemobiliseerd om vaatherstel te bevorderen. In recent onderzoek wordt de oorzakelijke rol van voorlopercellen in het vaatherstel steeds meer in twijfel getrokken, het verband tussen aantallen in bloed en ziektetoestand blijft echter bestaan. In dit hoofdstuk laten wij zien dat afname van voorlopercellen in het beenmerg juist bepalend is voor het ziekteverloop en dat lagere aantallen voorlopercellen in het bloed grotendeels daarop terug te voeren zijn. De verhouding van cellen tussen bloed en beenmerg – een maat voor mobilisatie – lijkt niet geassocieerd met de kans op amputatie of dood. Dit is een onverwachte bevinding die lijkt te bevestigen dat er misschien slechts een indirect verband is tussen aantallen voorlopercellen en cardiovasculair risico.

De volgende twee hoofdstukken zijn erop gericht hoog-risico patiënten binnen de groep van patiënten met kritieke ischemie aan te wijzen. In **hoofdstuk 3** hebben we geprobeerd stoffen in het bloed (biomarkers) van patiënten aan te wijzen die voorspellen of iemand amputatie of dood (een 'event') zal ondergaan. Wij hebben twee moleculen, IL-6 en IP-10, geïdentificeerd, waarvan de bloedwaardes voorspellend zijn. Aan de hand daarvan hebben we een wiskundig model gemaakt dat de kans op een 'event' redelijk nauwkeurig kan voorspellen. In **hoofdstuk 4** hebben we apart gekeken naar de rol van suikerziekte (Diabetes Mellitus) in het ziektebeloop van kritieke ischemie. Hiervoor hebben we de resultaten van twee studies, de eerder genoemde Juventas studie en de PADI studie gepoeld. Wij laten zien dat patienten met suikerziekte een groter risico lopen op amputatie, maar dat dit niet los staat van andere risicofactoren, met name de bloeddruk in het been. Een mogelijke verklaring zou zijn dat patiënten met suikerziekte en iets andere vorm van aderverkalking hebben.

Tezamen kunnen hoofdstuk 3 en 4 dienen om klinische interventies te prioriteren, zowel in de kliniek als in studieverband. Door vooruitgangen in de medische kennis is de prognose van patiënten met kritieke ischemie in de laatste 20 jaar drastisch verbeterd. Met de identificatie van de patiënten met een hoog risico op een ongunstig ziektebeloop kunnen invasieve en dure therapieën zoals voorlopercelltherapie effectiever worden ingezet. In **Hoofdstuk 5** hebben wij getracht om aan te wijzen welke patienten zullen reageren op beenmergceltherapie. We hebben hiervoor gebruik gemaakt van de REPAIR-AMI studie, waarin beenmergceltherapie werd gebruikt in patiënten die een hartinfarct hebben ondergaan. We hebben juist deze studie gebruikt omdat hartfunctie goed over de tijd te

volgen is, en het mogelijk is om binnen één patiënt verbetering of verslechtering aan te tonen. Voor een zwart-wit uitkomstmaat zoals amputatie of dood als in de juventas studie is dit veel lastiger. Onze resultaten laten zien dat vooral jonge patiënten met een groot infarct en een slecht risicofactor-profiel het meeste baat hadden bij de beenmergcelbehandeling. Dit is een onverwachte bevinding omdat uit dierstudies juist is gebleken dat beenmergcellen van patiënten met een slecht risicofactor-profiel ook slechter werken. Het moet echter vermeld worden dat de resultaten van onze studie niet conclusief zijn en in andere studies gevalideerd moeten worden.

Het tweede deel van dit proefschrift is gericht op het ontwikkelen van een celtherapieproduct dat we zouden kunnen toepassen in een volgende klinische studie. In **Hoofdstuk 6** geven we een overzicht van de verschillende voorloperceltypen in bloed en beenmerg en hoe deze aan elkaar gerelateerd zijn. De nadruk ligt op endotheel-voorlopercellen gezien deze lange tijd als het meest geschikte celtype gezien werden om vaatherstel te bevorderen. Endotheelcellen vormen de binnenwand van bloedvaten en kleine haarvaten bestaan vrijwel alleen maar uit endotheel. Er zijn meerdere types endotheel voorlopercellen beschreven, maar het is onduidelijk in hoeverre ze van elkaar verschillen. Ook is het onduidelijk of deze cellen daadwerkelijk endotheel vormen of dat ze alleen helpen in de vorming van nieuwe vaatjes door het bestaande endotheel te stimuleren. Wij hebben hier getracht een kwantitatieve maat voor de verschillen te zoeken door naar de expressie van alle van genen gelijktijdig te kijken (het transcriptoom). Wij laten zien dat er een groot verschil is tussen verschillende celtypes waarvan van elk gedacht werd dat het endotheel voorlopercellen zijn, en dat deze verschillen overeenkomen met de functionele verschillen.
In **hoofdstuk 7** onderzoeken we een voorbehandelingsstrategie voor één type endotheel voorlopercel, de *Endothelial Colony Forming Cell* (ECFC). Onderzoek heeft inmiddels aangetoond dat de meeste voorlopercelbehandelingen een indirect effect hebben: de voorlopercellen stimuleren vooral de bestaande vaatjes in bijvoorbeeld het been om zich uit te breiden. Voor ECFCs geldt dit echter niet, dit celtype vormt actief zelf nieuwe vaatjes. Het is echter gebleken dat ECFCs erg gevoelig zijn voor schade, met name voor oxidatieve stress, wat juist veel voorkomt in ischemisch weefsel. Wij laten hier ook zien dat relatief kleine hoeveelheden oxidatieve stress de vaatvorming van ECFCs al verstoren. Het lichaam heeft echter een ingebouwd verdedigingssysteem tegen oxidatieve stress dat bestaat uit een reeks anti-oxidatieve enzymen. De genexpressie van al deze enzymen wordt gereguleerd door een gezamenlijke transcriptiefactor, een soort schakelaar, genaamd Nrf2. In dit hoofdstuk laten we zien dat Nrf2 zeer belangrijk is voor de vaatvorming van ECFCs. Ook onderzoeken we of het mogelijk is om Nrf2 te activeren vóórdat de cellen in contact komen met oxidatieve stress, zodat de cellen zich alvast hebben kunnen wapenen tegen de stress.

In **hoofdstuk 8** hebben we een co-implantatie strategie onderzocht van ECFCs met een vertraagde-afgifte systeem van groeifactoren. Zoals boven reeds beschreven werken de meeste voorlopercellen behalve de ECFC via een indirect effect op reeds bestaande vaatjes. Men ziet dan ook een additief effect van ECFCs, die zelf vaatjes vormen, met andere voorloperceltypes in het verbeteren van doorbloeding. Wij hebben hier echter onderzocht of we de indirecte effecten ook kunnen bewerkstelligen met groeifactoren. Gezien groeifactoren snel worden afgebroken, hebben we hier een systeem voor vertraagde afgifte ontwikkeld, bestaande uit kleine gelatinebolletjes (*Gelatin Microparticles, GMPs).* We laten zien dat de GMPs de ECFCs helpen in het vormen van nieuwe vaatjes. Ook laten we zien dat we d.m.v. de GMPs heel gericht de vorming van vaatjes kunnen controleren in 3D geprinte weefsels.

In **hoofdstuk 9** onderzoeken we de functie van Mesenchymale Stromaal Cellen (MSCs) van patiënten met kritieke ischemie. MSCs zijn een type voorlopercel dat, in vergelijking met bijvoorbeeld endotheel voorlopercellen, vrij gemakkelijk op grote schaal voor klinische toepassingen geproduceerd kan worden. In de nabije toekomst is de MSC dan ook de meest realistische kandidaat voor een celtherapieproduct in een klinische studie. MSCs worden gekweekt uit het beenmerg en wij hebben eerder (o.a. in hoofdstuk 2) laten zien dat de aantallen van andere voorloperceltypes in het beenmerg van patiënten met kritieke ischemie verminderd zijn. Ook is er aangetoond dat beenmerg voorlopercellen van patiënten met hart- en vaatziekten minder goed vaatnieuwvorming kunnen induceren dan cellen van gezonde donoren. Onze vraag in dit hoofdstuk was of dit ook voor MSCs geldt. We hebben derhalve een hele reeks eigenschappen van MSCs van patiënten met kritieke ischemie onderzocht: gen expressie; differentiatie in verschillende weefsels; *senescence* (biologische leeftijd); migratie en het stimuleren van vaatnieuwvorming *in vitro* en *in vivo.* We hebben gevonden dat MSCs van patiënten met kritieke ischemie meer senescence vertonen en wellicht daardoor een ander differentiatie-patroon vertonen waarbij ze meer neigen naar vet dan naar kraakbeen te differentiëren. Belangrijker echter is gebleken dat de toegenomen senescence niet belangrijk is voor hun vermogen om vaatnieuwvorming te bewerkstelligen. In dit, voor de behandeling van kritieke ischemie cruciale opzicht, zijn ze equivalent aan controle MSCs. De resultaten impliceren dan ook dat er geen theoretische bezwaren zijn tegen een autologe (d.w.z. patiënt-eigen) therapietoepassing.

Hoofdstuk 10 is een kort hoofdstuk waarin we de toepassing van allogene (d.w.z. van een andere donor) versus autologe (patiënt-eigen) MSCs discussiëren aan de hand van een recente klinische studie met MSCs. **Hoofdstuk 11** is een Engelstalige samenvatting van dit proefschrift. In **Hoofdstuk 12** tenslotte, geven we onze visie over wat met de huidige stand van kennis de meest geschikte aanpak zou zijn voor een klinische studie met voorlopercellen voor de behandeling van kritieke ischemie.

DANKWOORD

Welkom beste lezer, bij het dankwoord van mijn proefschrift! Na het doorwaden van dit bondige stukje proza – wat u ongetwijfeld van-kop-tot-staart gedaan heeft – nu eindelijk een iets luchtiger stukje. Zoals elk proefschrift, was dit boekje niet tot stand gekomen zonder hulp van vele anderen. Sterker nog, voor mij waren altijd de gezellige en uitdagende samenwerkingen mijn voornaamste motivatie zijn om dit werk te doen. Op dit punt wil ik dan ook de mensen bedanken die dit boekje mede mogelijk hebben gemaakt.

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Gisela, Rockstar! Superheld! Meesterdief! Ik kom superlatieven te kort om jouw tijd in ons lab te beschrijven. Ondanks de drie Cell of Nature papers die je altijd tegelijk aan het schrijven was en het kleine leger studenten dat je commandeerde wist je altijd nog tijd te vinden voor je collega's. Het was leuk dat we samen nog een klein projectje hebben kunnen doen en veel succes in de Heimat! **Nynke,** de rouwdouwer van ons groepje, eigenlijk vond ik het stiekem altijd grappig als je weer eens je geduld met de computer verloor. Al denk ik wel dat de agressie significant minder is geworden nadat je bent gaan rugbyen. Ik ben erg onder de indruk van je kennis van de nefro-pathologie, toch mooi dat je daarin inmiddels al internationaal aanzien geniet. Veel succes met het afronden van je boekje, ik ben benieuwd wat er allemaal voor stukken in gaan komen.
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Gedurende de tijd van de PhD waren er ook veel samenwerkingen, al hebben deze niet allemaal dit boekje gehaald. **Michelle,** het idee om samen een paper op te zetten was één van de beste überhaupt! De meetings bij Tricolore of in de micaffè waren altijd supergezellig en ik verbaas me nog steeds over het resultaat. Veel succes bij de Ortho en ik hoop dat we binnenkort nog eens een keer een koffie/quiz kunnen doen. **Annelien,** alhoewel je officieel de substudie als student gedaan hebt, was je eigenlijk meteen al even zelfstandig als elke AIO. Ik was erg onder de indruk over hoe snel je alles oppikte en ik

hoop dat het resultaat nu eindelijk ergens blijft plakken. Helaas zijn we er vorige winter niet aan toe gekomen om een Glühweinborrel te doen, maar zodra het weer kouder wordt ga ik hem zeker inplannen. **Marlon,** eigenlijk wouden we maar één klein verhaaltje schrijven, maar het blijven er steeds meer worden! Veel succes met het afronden van je specialisatie en straks de fellow! **Peter-Paul,** de (nieuwe) koning van de meta-analyses! Volgens mij ben je me qua R skills allang voorbij gestreefd en dat in een fractie van de tijd. Het is heel leuk en een beetje een voorrecht geweest om met je samen te mogen werken; ik ben altijd zeer onder de indruk van je energie en van wat je allemaal voor elkaar krijgt. Ik hoop dat je nog een beetje bij het onderzoek betrokken blijft als je straks de kliniek in gaat, maar eigenlijk twijfel ik daar nauwelijks aan.

Deze PhD zou ook niet mogelijk geweest zijn zonder de collega's van de experimentele cardio, bij wie ik altijd om advies kon vragen (en zo-nu-en-dan eens een pipetpuntje of twee kon lenen). Veel van wat in dit proefschrift staat heb ik van jullie geleerd, m.n. van **Chaylendra** en **Willy** destijds. Ook wil ik mijn oud-kamergenoten: **Dries, Alain, Krijn, Zhiyong, Janine en Judith** bedanken voor de koffie, gezelligheid en vele discussies. Natuurlijk mag in dit rijtje **Frederieke** niet ontbreken, ondanks het feit dat je altijd mijn kryptonite – Tequila – met me wilt drinken en ik daar elke keer weer spijt van krijg. Leuk dat we op het allerlaatste moment nog een projectje samen zijn begonnen en dat je mij voor de neutro gewonnen hebt. Veel geluk nu met de afronding van je boekje, als het af is drinken we samen een swizzle (de enige vorm van Tequila die ik nog kan hebben). **Sailay,** my weirdest friend (I mean that in a good way), I miss our (well, your) smoke-breaks and your stories. De rest van het lab – eigenlijk zijn het er bijna teveel om op te noemen - : **Lena, René, Geert, Marten, Jelte, Sander van der Laan, Sander van de Weg, Sanne, Patricia, Erik, Danny, Ellen, Hamid, Roberto, Mirjam, Dennie, Noortje, Loes, Arjan, Corina, Esther, Hanna, Jesper, Vera, Pleunie, Vince en iedereen die ik nog ben vergeten:** bedankt voor de gezelligheid bij de borrels en dat ik altijd op cardiofeestjes mocht komen partycrashen.

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De mensen van de quiz: **Linda, Heidi, Ronald, Merel, Anne, Wijnand** en natuurlijk ook **Larry en Simon,** het was altijd gezellig en het verliezen van de quiz is een trotse traditie geworden. **Olaf en Sjoerd,** menige kater op een zaterdagochtend is door jullie vakkundig genezen. Bij jullie kon ik altijd overtollige energie (ook letterlijk in de zin van calorieën), samen met de **Kaputters** kwijt. **Brooke,** my fellow adventurer in all things related to food and drinks. You're the only person who thinks that it's perfectly normal that I order an entire side of beef and then still offer me part of your food. I always loved hanging out, and there are plenty of places that we still need to check out!

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MANUSCRIPTS IN PREPARATION

Gremmels H, Teraa M, van Rhijn-Brouwer FCC, Papazova DA, Fledderus JO, Verhaar MC on behalf of the JUVENTAS study group: Higher Circulating and Bone Marrow Progenitor Cell Numbers are Associated with Increased Amputation-free Survival in Severe Limb Ischemia. *Submitted*

Spreen MI, Gremmels H, Teraa M, Sprengers RW, Verhaar MC, Statius van Eps RG, de Vries JPPM, Mali MPThM, van Overhagen H: Decreased limb survival in diabetic patients with critical limb ischemia compared with non-diabetic patients. *Submitted*

Zwetsloot PP, Gremmels H, Assmuss B, Koudstaal S, Zeiher AM, Chamuleau SAJ: Responder definition in clinical stem cell trials in Cardiology; will the real responder please stand up? *Submitted*

De Kat AC, Gremmels H, Verhaar MC, Broekmans FJM, Yarde F: Early vascular damage and its relation to anti-Müllerian hormone: a cross-sectional study. *Submitted*

Van Kesteren CFMG, Gremmels H, de Witte LD, Hol EM, van Gool AM, Falkai PG, Kahn RS, Sommer IEC: Immune involvement in the pathogenesis of schizophrenia: a meta-analysis on post-mortem brain studies. *Submitted*

Oosterhuis NR, Papazova DA, Gremmels H, Joles JA, Verhaar MC: T-cells contribute to hypertension and renal injury in mice with subtotal nephrectomy. *Submitted*

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CURRICULUM VITAE

Hendrik Gremmels was born on the 15th of November 1983 in Isernhagen, Germany. The first conscious years of his life he spent in Kulmbach, a small town in Franconia. He then went on to live in Bilthoven, the Netherlands, where he completed primary education and later attended the Nieuwe Lyceum where he finished the Gymnasium track.

In 2001, he enrolled in the University College Utrecht, where he opted for the Pre-Med track, which he finished with honors in 2004. In the same year, he was admitted to the SUMMA medical master's program, which he finished in 2010 obtaining the title of Medical Doctor.

Next, he commenced his PhD under the supervision of prof. dr. Marianne C. Verhaar, at the Department of Nephrology and Hypertension at the UMC Utrecht. During his PhD period, Hendrik mainly investigated the interaction between cardiovascular disease and bone marrow-derived progenitor cells. The results of this research are printed in this thesis.