Treatment of critical limb ischemia

A SHIFTING PARADIGM

Martin Teraa

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The research described in this thesis was financially supported by grants from Stichting Vrienden UMC Utrecht (the Netherlands, grant CS06.007), the Dutch Heart Foundation (the Netherlands, grant 2008B094), and foundation "De Drie Lichten" (the Netherlands, grant 10/06).

Publication of this thesis was financially supported by W.L. Gore & Associates, Merck Sharp & Dohme, Willpharma, and Chirurgisch Fonds UMC Utrecht. Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

The sponsors, who are kindly acknowledged, were not involved in the design, data collection, analysis, and interpretation of results or decision whether to publish results of studies related to thesis.

ISBN	978-90-88916-37-3
Design & Lay-out	Wendy Schoneveld, www.wenz iD.nl
Cover	Adapted from Seasonscape by Vitaly S Alexius
Printing	Uitgeverij BOXPress, www.boxpress.nl

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A SHIFTING PARADIGM

Behandeling van kritieke ischemie

EEN VERSCHUIVEND PARADIGMA

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus,

prof.dr. G.J. van der Zwaan,

ingevolge het besluit college voor
promoties in het openbaar te verdedigen op
donderdag 27 juni 2013 des middags te 2.30 uur

door

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geboren op 13 oktober 1983
te Boxtel

Promotoren

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Voor Christa
en Stijn

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CHAPTER 1

General introduction and thesis outline

INTRODUCTION

Critical limb ischemia

Peripheral arterial disease (PAD) of the lower extremity is usually caused by atherosclerotic obstruction of the arteries supplying the limb and estimated to affect 27 million individuals in Western Europe and North America alone.[1] Its incidence is expected to increase even further in concert with the growing burden of cardiovascular risk factors,[2-4] such as aging, diabetes mellitus, and sedentary lifestyle.[5,6] The clinical spectrum of PAD varies from asymptomatic disease to critical limb ischemia (CLI). CLI is defined as chronic rest pain or tissue loss due to insufficient perfusion of the extremity, and has an estimated incidence of 500-1,000 individuals per million per year. Presence of PAD in all stages of the disease is associated with increased cardiovascular morbidity and mortality.[7-9] CLI patients are at particularly high risk for limb loss, with reported rates for major amputation of 40%,[10,11] as well as cardiovascular events with mortality rates of 20%[12,13] 6 months after diagnosis. These numbers exceed those for every other form of occlusive cardiovascular disease,[14,15] and reflect the major systemic atherosclerotic burden in CLI. CLI is associated with poor quality of life[16] and high treatment costs.[17]

Treatment of CLI primarily aims at limb salvage by restoring blood flow to the lower extremity.[10,11] In the past decades surgical and endovascular revascularization strategies for CLI have evolved, with the development of novel devices such as drug-eluting balloons, drug-eluting stents, and re-entry devices.[18-21] Despite these innovations approximately 25-40% of the CLI patients is still not eligible for revascularization.[10,11] The poor quality of life[16] and high treatment costs of these so-called no-option CLI patients underline the need for novel strategies to restore tissue perfusion in CLI.

Atherosclerosis, arterial regeneration, and cell-based therapies

Atherosclerosis is the principal cause of CLI. It is a complex systemic multifactorial disease, which is influenced by genetic traits and environmental factors, such as presence of diabetes mellitus, hypertension, dyslipidemia, and aging. The healthy endothelial layer has an active function in regulating vascular tone, maintenance of a selective and active barrier, and protection against inflammation and unwanted coagulation, hereby protecting the vessels against atherosclerosis and thrombosis.[22,23] Endothelial dysfunction, characterized by reduced nitric oxide bioavailability and increased oxidative stress,[22,24] characterizes the early stages of the development of atherosclerotic lesions.[24,25] This subsequently induces the attraction of a range of inflammatory cells into the vascular wall, resulting in accumulation of lipid-laden macrophages, enhancing endothelial dysfunction.[26] Endothelial dysfunction results in adhesion and activation of circulating platelets, which further exaggerates the inflammatory and atherogenic response.[27,28] This altogether causes a vicious cycle resulting in progressive loss of arterial patency and reduced tissue perfusion. The clinical presentation of atherosclerotic disease depends on whether arterial occlusion occurs acutely or more gradually.

The concept of atherosclerosis changed with the discovery of a putative bone marrow (BM)-derived endothelial progenitor cell (EPC) in 1997 by Asahara et al.[29] EPCs were shown to be involved in maintenance of cardiovascular health and postnatal neovascularization. Reduced EPC number and functional impairment thus result in lower vasculoregenerative potential and are associated with increased cardiovascular risk[30,31] and severity of cardiovascular disease.[32] The concept of a single "EPC" as described by Asahara et al. acting as structural building block in neovascularization and vascular regeneration has changed over time.[33-35] Vascular regenerative processes involve a wide range of cells, including resident endothelial cells, circulating monocytes,[36] lymphocytes[37,38] and other BM and non-BM-derived cells,[39] which enhance neovascularization by delicate interaction,[40] wherein paracrine factors are of paramount importance.[34,35]

Soon after the discovery that BM-derived cells contribute to neovascularization, their role as potential therapeutic agent to enhance perfusion of ischemic tissue was explored in experimental *in vitro* and *in vivo* models. Very rapidly this was followed by first-in-human clinical trials to investigate whether BM-derived cells, mainly BM-derived mononuclear cells (BM-MNC), could restore tissue perfusion in ischemic cardiovascular disease, such as myocardial infarction[41] and PAD.[42-44] In 2002 the TACT-trial was the first to show that autologous BM-derived cell therapy was a safe and potentially effective strategy for therapeutic neovascularization in CLI patients.[45] This sparked a great number of subsequent trials, most often small, non-randomized, non-placebo-controlled, and/ or non-blinded trials, to study the therapeutic effect of BM-derived cell therapy in PAD.[44] These trials almost invariably showed improvements of both objective and subjective surrogate endpoints, such as pain scores, pain-free walking distance, ankle-brachial index, and transcutaneous oxygen measurements. Recently, several randomized controlled trials (RCTs) have been published that assess hard clinical endpoints, but still have limitations, such as small sample size and non-blinded or non-placebo-controlled designs.[43] As a result, despite the relatively large amount of studies that report on BM-derived cell therapy for PAD and CLI, still no definitive proof exists on its effectiveness to improve amputation rates, amputation-free survival (AFS) or quality of life.[43] Therefore, larger and well-designed placebo-controlled clinical trials are warranted to investigate whether BM-derived cell therapy exerts clinically relevant effects in CLI patients.

Influences of cardiovascular disease and risk factors on bone marrow and progenitor cell function

The presence of cardiovascular risk factors and overt cardiovascular disease are associated with reduced numbers and impaired function of progenitor cells involved in vascular regeneration.[46-48] Several processes have been implicated in the attenuated progenitor cell numbers observed in cardiovascular disease, such as restriction of the progenitor pool, impaired mobilization of progenitor cells from the BM, impaired differentiation and survival of progenitor cells both in the BM as well as in the circulation, and increased recruitment to sites of vascular damage. Cardiovascular risk factors and cardiovascular

disease also negatively affect progenitor cell function, causing decreased adhesive, proliferative and migratory capacity, impaired angiogenic patency and lower paracrine potential.[32,46,49-52] Since the majority of current cell-based therapies makes use of autologous cells, such BM progenitor cell dysfunction may reduce the therapeutic efficacy. It is therefore mandatory to investigate mechanisms underlying BM progenitor cell dysfunction and search for interventions to reverse dysfunction.[53,54]

OBJECTIVES AND OUTLINE

The primary objective of this thesis is to study novel strategies for the treatment of CLI focusing on vascular regeneration and neovascularization using BM cell therapy.
PART I of this thesis provides an overview of currently available therapeutic strategies in PAD and CLI, describes how these therapies have changed over the past decades and illustrates why novel treatment strategies are urgently needed.
Chapter 2 reviews the changes in treatment of CLI over the past decades and how these changes have influenced prognosis of CLI. In **Chapter 3** we provide recommendations on the revascularizing treatment of CLI, based on the best available evidence, which was embedded in the "Guidelines for critical limb ischaemia and diabetic foot" of the European Society of Vascular Surgery. In **Chapter 4** we investigated quality of life in patients with CLI as compared to patients with claudication and patients diagnosed with cardiovascular risk factors, without overt cardiovascular disease.
BM-derived progenitor cell therapy is a promising new treatment modality for no-option CLI patients to improve tissue perfusion by enhancing neovascularization. Current evidence and considerations with respect to BM-derived cell therapy in CLI are discussed in **PART II**.
Chapter 5 provides a meta-analysis on all RCTs that have studied BM-derived cell therapy in CLI. After promising results of the first pioneering clinical study on cell-based therapy in CLI, several small clinical trials, often without appropriate controls, have been published. As definite proof on the clinical efficacy of BM-derived cell therapy strategies was still lacking, we initiated a large randomized, double-blind, placebo-controlled trial to study intra-arterial administration of BM-MNC in CLI patients, the Juventas-trial. The results of the Juventas-trial are presented in **Chapter 6**. The Juventas-trial requires BM to be harvested from the patient. As BM cell yield of BM aspiration is a determinant of success of BM transplantation and may be relevant for cell-based therapies in regenerative medicine, we investigated factors that influence cellular yield. In **Chapter 7** the influence on cellular yield of the core diameter of the BM device used for BM aspiration is discussed.
PART III focuses on how cardiovascular risk factors and cardiovascular disease, as present in CLI, influence BM progenitor cell function. Several causative mechanisms have been proposed to be involved in the reduced progenitor cell numbers observed in cardiovascular diseases, such as impaired mobilization from the BM, impaired differentiation, increased cellular death and apoptosis, and increased recruitment due to needs for vascular regeneration.

Chapter 8 provides an overview of the proposed mechanisms leading to reduced progenitor cell numbers in diabetes mellitus and a meta-analysis of the available studies on EPC numbers in diabetes. How EPC mobilization is affected in diabetes, and the role of the BM stroma herein, was studied in *in vitro* and *in vivo* models of diabetes and hyperglycemia in **Chapter 9**. In **Chapter 10** we report how progenitor cell numbers in blood and BM are affected in CLI patients in comparison to healthy controls and discuss disease-specific changes that influence progenitor cell function and number. Animal studies have shown that diabetes has a detrimental effect on BM function and structure, causing microvascular rarefaction, increased endothelial permeability, and BM endothelial dysfunction and apoptosis. Studying **Chapter 11** we investigate whether such changes can also be observed in human subjects with CLI.

Platelets are involved in the development of atherosclerosis and atherosclerotic complications. In **Chapter 12** platelet activity and reactivity in CLI patients is compared to the healthy situation.

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PART ONE

Current treatment of critical limb ischemia and rationale for novel strategies

CHAPTER 2

Temporal trends in critical limb ischemia

In preparation

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INTRODUCTION

Critical limb ischemia (CLI), which is at the end of the peripheral artery disease (PAD) spectrum, is associated with excessively high risk for cardiovascular events, including myocardial infarction, and death.[1-3] Mortality rates as high as 20% within six months from diagnosis and exceeding 50% at 5 years have been reported for CLI,[4,5] whereas one-year mortality rates in non-revascularizable, so-called no-option CLI patients range from 10-40%.[6,7] The high mortality rates exceed those for every other form of occlusive cardiovascular disease, including symptomatic coronary artery disease (CAD),[8,9] and reflect the systemic atherosclerotic burden associated with CLI. Besides poor survival rates, prognosis with respect to limb preservation in CLI patients is poor,[10] particularly in no-option CLI patients, where 6-month major amputation rates have been reported to range from 10-40%.[6,7] Additionally, CLI is associated with poor quality of life[11] and high treatment costs,[12] especially when amputation is inevitable.[12,13] With an estimated yearly incidence of 500-1,000 new cases per million individuals in Western Society,[6] which is ever increasing in concert with the increase in cardiovascular risk factors,[14-16] CLI poses a substantial burden on patients, health care providers, and resources.

In the current review we will describe how management strategies in CLI have evolved over the past decades and discuss issues that could facilitate more rapid and evidence-based improvements in CLI management and care. Focus will be on factors that may have limited and on actions that could promote progress in this field.

CHANGING PROGNOSIS IN CLI

Prognosis with respect to limb salvage and survival in CLI patients, and the PAD population as a whole, has improved over the years.[17-24] In a large population-based study in a heterogeneous PAD population >65 years of age the adjusted odds ratio of lower extremity amputation per year between 2000 and 2008 was 0.95 (95% CI: 0.95-0.95, $p<0.001$).[24] Consistently, Goodney et al. showed a reduction of major amputation rates of 263 to 188 per 100,000 Medicare beneficiaries between 1996 and 2006 (RR 0.71; 95%CI: 0.6-0.8).[20] A similar pattern was suggested for the CLI population in a study by Egorova et al. showing a reduced proportion of the surgical interventions in a US CLI population being major amputations (decrease from 42% to 30% between 1998 through 2007).[22] Since amputations in a PAD population are likely performed in case of CLI, these data suggest a decrease in major amputation rates in the CLI population over the past two decades, while a decrease of CLI incidence might also partially explain these observations. The aforementioned studies also show a trend towards more endovascular as compared to surgical revascularization procedures[18,20-24] and suggest a potential causal relationship between the increased number of endovascular procedures and reduced amputation rates.[22,23] However, Benoit et al. have shown significant improvement of one-year amputation-free survival (AFS) from approximately 28-40% in trials performed during the period 1996-1999 to

48-81% during the period 2006-2010 for patients with non-revascularizable CLI[25] and a tendency for major amputation rates to decline from 20-50% to 10-38% during the same period. Improvements in prognosis over time in this no-option CLI population suggest a role for factors other than increased frequency of endovascular interventions, such as increased public awareness, better medical therapy and secondary prevention.

CHANGES IN REVASCULARIZATION STRATEGIES IN CLI

Endovascular interventions have significantly evolved over the past decades. Since the initial application of plain balloon or percutaneous transluminal angioplasty (PTA), several novel endovascular approaches and devices have been released on the market, for example bare metal stents (BMS), cryoplasty, atherectomy devices, stent-grafts, drug-eluting stents (DES), and drug-eluting balloons (DEB). In general these devices have been studied in relatively small and selected patient populations, often not including CLI patients.[26] The only RCT directly comparing open bypass surgery with endovascular therapy, i.e. plain balloon angioplasty, in CLI patients is the BASIL-trial, which overall showed no differences between the treatment groups with respect to AFS at 1 and 3 years of follow-up based on an intention-to-treat analysis.[4] Patients allocated to the open bypass surgery group surviving for more than 2 years after the initial procedure had improved AFS (adjusted HR 0.37; 95% CI 0.17-0.77; p=0.008) and reduced all-cause mortality (adjusted HR 0.34; 95% CI 0.17-0.71; p=0.004). Patients that had no useable vein graft and hence underwent prosthetic bypass and patients that initially underwent endovascular treatment, but crossed-over to the bypass group fared worse compared to those undergoing initial open bypass surgery using a vein graft.[27,28] Although this study was not specifically designed with this purpose it suggests benefit of bypass surgery in specific subgroups of CLI patients. To date no other randomized studies have compared bypass surgery with endovascular therapy in CLI. However, a tendency to an endovascular-first strategy has evolved over the past two decades, fueled by results of non-randomized comparisons, reports in milder and selected PAD populations, and a perceived short-term favorable balance of an endovascular-first strategy in the high-risk CLI population.[20-22,29]

In line with the BASIL-trial, indirect comparisons of endovascular procedures[30,31] and open bypass surgery using a vein graft[10] show similar results with respect to one-year limb salvage and mortality rates in CLI, both ranging from 85-90%, but a higher need for reinterventions after endovascular therapy.[32] This increased reintervention rate corresponds with an increase in the number of endovascular interventions in both claudication and CLI over the years that outnumbers the decline in open surgical interventions. Goodney et al. reported that over 3 endovascular interventions were performed for every one procedure declined in lower extremity bypass surgery.[20] It is unlikely that this increase in endovascular procedures is merely the result of more reinterventions after endovascular intervention. It may also be due to a lowering threshold for endovascular interventions as reflected by an increase of hospital admissions for endovascular interventions for claudication, i.e. increase

from 10-31% to 26-43% of the PAD-related hospital admissions in 2001 and 2008, respectively.[33] The larger need for reinterventions in endovascular therapy also becomes apparent from cost-effectiveness analyses that focused on the comparison of endovascular strategies with open surgery.[34,35] These studies show an early benefit of endovascular strategies over open procedures but this benefit is lost after approximately one year, due to reinterventions in the endovascular group.

Based on the available literature, it is not easy to defend either an endovascular-first or a bypass-first strategy in CLI. An argument that is often used to choose for an endovascular-first strategy is that after failure of an endovascular therapy bypass surgery is often still feasible,[26] however this is not based on objective data and there is also evidence that bypass surgery after an initial endovascular intervention has a worse prognosis than initial bypass surgery.[27] Furthermore, the relatively late superiority of open surgery over endovascular intervention is sometimes considered irrelevant in CLI due to the perceived high mortality rates. This argument is challenged by the relatively favorable one-year survival rate of 85% in the PREVENTIII-trial,[36] which studied the effect of edifoligide after bypass surgery in CLI, and 70% 2-year survival rate of the BASIL-trial.[4] The researchers of the PREVENTIII-trial developed an easy to use and highly reliable tool to stratify CLI patients who undergo bypass surgery in low-, medium-, and high-risk categories providing a reliable estimate of the one-year AFS after surgical revascularization.[37,38] The variables that comprise this risk-score include dialysis dependency, tissue loss, advanced age (>75 years), presence of coronary artery disease, and low hematocrit (<30%). Patients in the highest risk group have a one-year AFS after open bypass surgery of approximately 45%, and bypass surgery is therefore not preferred in this high risk population. Other studies also identified risk factors for bypass surgery,[39] and showed that the conduit used is a major procedural factor influencing prognosis after bypass surgery.[26,40]

The initial choice of treatment in CLI patients is not easily made and depends on patient- and procedure-specific factors, such as age and comorbidity, presence of a useable vein graft and the available options for bypass anastomosis. A bypass using an autogenous vein remains the golden standard. Well-designed RCTs investigating novel devices, which are or will become available on the market soon, in CLI, would be highly valuable to determine whether an endovascular-first strategy can be more widely adopted.

CHANGING TRENDS IN MEDICAL THERAPY FOR PAD AND CLI

Current guidelines

In 2000, the first international guideline for the management of PAD was published, the TASC-I.[41] Since then several international guidelines have been published that include secondary prevention in PAD, such as smoking cessation, management of hypertension and diabetes, lipid-lowering, and antiplatelet therapies.[6,41-43] All PAD and CLI guidelines consider the effectiveness of statins, antiplatelet therapy, and ACE inhibitors to reduce cardiovascular events and mortality proven in the PAD population.[6,41-43] Recommendations

for CLI are often, as a result of lacking CLI specific evidence,[39,43] extrapolated from other populations.

Temporal changes in secondary prevention

The ultimate goal of guidelines is to enhance uniform and evidence-based treatment in a specific patient population in order to improve outcome and quality of care. It takes time before guideline-based therapy finds its way to the clinic and treatment conforms to these guidelines. Over the past decade the use of antiplatelet therapy, statins, and antihypertensive drugs in PAD patients has been evaluated in several reports, but no CLI specific data are available. In general an increase in the use of secondary prevention has been observed over time.[44-47] For instance Subherwal et al. reported, in a large population-based study in Denmark, an increase in the use of antiplatelet therapy in patients with the incident diagnosis of PAD,[47] without a history of CAD, from 29% in 2000 to 59% in 2007 ($p<0.0001$). The increase in statin use was even more pronounced from 9% in 2000 to 56% in 2007 ($p<0.0001$), and for use of ACE inhibitors a significant increase was also observed ($p<0.0001$), however its use remained below 20%. Subherwal and co-workers also compared the use of cardioprotective drugs in PAD patients with that of CAD and observed that patients with PAD were approximately half as likely to be treated with cardioprotective drugs for the period from 2000 to 2007. This could be related to the fact that the introduction of guidelines in CAD preceded those in PAD more than a decade.[41,48] The differences between the CAD and PAD population declined over the period from 2000 to 2007 from 28% in 2000 to 19% in 2007 for antiplatelet therapy and 22% in 2000 to 9% in 2007 for statin therapy. The underuse of cardioprotective medication in the PAD population in comparison to patients with CAD has been published previously.[44] The underuse of cardioprotective drugs in the PAD population does not seem limited to PAD patients with relatively mild symptoms. In the PREVENTIII and BASIL-trial, which included patients with CLI between 2001-2003 and 1999-2004, respectively, show that 88% and 46% and 54% and 34% were treated with antiplatelet drugs and statins respectively.[4,36] The relatively poor embracement of PAD guidelines probably results from a relative lack of public awareness about PAD, its unappreciated implications on overall cardiovascular risk, and the fact that the benefits of treatment are not well appreciated.[49-51] Furthermore, overestimation of the treatment given by others, lack of education, training, and organizational facilities to implement guidelines properly play a role,[52] as well as the beliefs of the physician themselves.[53] Cacoub et al. showed that the extent of risk factor management was significantly associated with the type of doctor that treated the PAD patient.[54] Hackam et al. calculated in a systematic review and modeling study that with more widespread implementation of antiplatelet therapy, statins, and ACE inhibition (85% use of each) in the PAD population may prevent more than 200,000 cardiovascular events each year (myocardial infarction, stroke, and cardiovascular death; 212,166 events; 95%CI 95,823-310,392) in North America and Western Europe alone.[55]

While evidence for secondary prevention on cardiovascular events and mortality in general is not in doubt, there is less evidence whether it can reduce limb-specific events. Statin

therapy is associated with reduced restenosis rates after endovascular intervention,[56,57] and reduced rates of symptom recurrence after revascularization for intermittent claudication.[57] Aiello et al. showed in a retrospective study in 646 CLI patients that statins can improve limb salvage rates after endovascular interventions for CLI (limb salvage 83% vs. 62% at 24 months).[58] The temporal trend of reducing AFS and amputation rates reported by Benoit et al. in no-option CLI patients may also partly reflect a relation between improved secondary prevention and limb-related outcomes.[25] For viable conclusions on the effect of specific secondary prevention measures on limb-related outcomes, amputation rates in particular, larger and well-designed studies should be conducted, based on large patient registries.

FACTORS LIMITING AVAILABILITY OF AND MEASURES TO FACILITATE HIGH-LEVEL EVIDENCE IN CLI

High-level evidence, typically level I, to guide evidence-based clinical decision making in CLI is limited in contrast to coronary artery, carotid artery, and aortic aneurysm disease.[6,26,39,43,59] Few well-designed prospective studies and RCTs in CLI patients are available, which may be related to the fact that studies in this specific population are not easy to conduct, due to the lower incidence compared to milder forms of PAD, issues with respect to follow-up, and related to this less interest from industry to initiate trials in this population. High quality epidemiological data on incidence, prevalence, and prognosis of CLI, particularly more recent data, are also sparse.[6,7]

Essential for the interpretation of study results is a widely accepted definition of the disease under study. Variable definitions are used to classify CLI, which complicate the evaluation of available evidence with regard to CLI.[59] The first formal definition of CLI was proposed by Fontaine et al. in 1954,[60] as being the existence of rest pain or tissue loss due to severe PAD, without including any hemodynamic criteria. In 1986, the first Society of Vascular Surgery/ International Society of Cardiovascular Surgery (SVS/ ISCVS) standards for reporting on lower limb ischemia were published, which included a classification currently known as the Rutherford classification.[61] This classification – in its original form – added objective hemodynamic parameters, i.e. pulse volume recordings and ankle and toe pressure measurements, to the clinical presentation in order to enhance homogeneity and objectivity of the definition. While hemodynamic parameters have remained part of subsequent international consensus guidelines on PAD and CLI,[6,7,41,62] these strict hemodynamic criteria are often not part of routine use in clinical as well as research practice. As the definitions used to define CLI in clinical reports vary from merely clinical criteria,[4] clinical criteria combined with a variety of objective parameters,[36] and definitions based on hospital discharge information[22] research reporting on CLI is inherently variable. Recent consensus statements have proposed stricter definitions that include ankle and toe pressures,[6,41] aiming to improve standardized reporting on CLI.

Given the temporal changes discussed above, one should be careful when considering historical controls and non-randomized cohorts in clinical research in CLI. Recently, the SVS-CLI Working Group published Objective Performance Goals (OPG) which provide benchmark values for various endpoints in CLI, AFS and limb salvage, amongst others.[10] If these benchmark values are to be used as comparator in future CLI trials it should be realized that these values can gradually change over time due to factors not related to the intervention per se, such as secondary prevention measures. It is advisable to regularly update the OPGs to provide contemporary benchmark values. Additionally, the decline in event rates in clinical trials makes it more difficult to demonstrate superiority of a novel intervention,[63] which requires exponentially larger study sizes or are at risk to be underpowered.

There is room for improvement in secondary prevention strategies in CLI.[55] To further improve treatment and outcomes of PAD patients worldwide and in all socioeconomic segments of the population requires collaborative international, national, and local efforts. As funding strategies for PAD are uncommon, public or private initiatives are essential,[64] which could be enhanced by increased public awareness of PAD and its implications for public health, such as cardiovascular risk, influence on quality of life, and expenditure of health care resources.

CONCLUSIONS

Practically, we identified several recommendations with respect to research and clinical management in CLI.

- Initiatives should be taken to enhance widespread use of a generally accepted definition of CLI that includes hemodynamic criteria.
- In clinical trials, implementing separately powered distinct trial arms in CLI trials that consider disease severity and related prognosis, i.e. tissue loss vs. rest pain, should be stimulated. We should consider adding another patient population to the spectrum of PAD patients, namely the group of subcritical limb ischemia, as suggested previously,[65] however detailed thoughts and research on how to define this population are essential.
- Appropriate endpoints should be selected in studies that focus on CLI, considering that for instance AFS only partly embraces true interventional effects, since AFS does not separate limb from life loss. Endpoints that include reinterventions and early intervention related complications may be preferable, and ideally a measure of hemodynamic success should be incorporated.
- Study populations should reflect the CLI population encountered in vascular clinics' daily practice and not a delicate selection of patients that is not generalizable to the total CLI population.
- *Transatlantic or large continental collaborative efforts*, as have been done in carotid disease, may be needed to guarantee sufficiently powered trials, with room for stratification.Treatment of cardiovascular risk factors in CLI patients should be given a

central role on international conferences. Increased recognition of data on prevalence, management, outcomes, and treatment costs of PAD and CLI by public and governmental authorities will promote further evidence-based treatment of these patients, enhance detection of PAD, and improve funding resources for essential research in this specific patient population.

If we are able to fulfill these recommendations by joining international forces, we might be able to optimize treatment of the CLI patient, hence offer a relief of the burden on health care providers and resources, but foremost the patient.

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PART ONE

Current treatment of critical limb ischemia and rationale for novel strategies

CHAPTER 3

Treatment of Critical Limb Ischaemia

Eur J Vasc Endovasc Surg. 2011 42;S2:S43-S59

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ABSTRACT

Recommendations stated in the TASC II guidelines for the treatment of peripheral arterial disease (PAD) regard a heterogeneous group of patients ranging from claudicants to critical limb ischaemia (CLI) patients. However, specific considerations apply to CLI patients. An important problem regarding the majority of currently available literature that reports on revascularisation strategies for PAD is that it does not focus on CLI patients specifically and studies them as a minor part of the complete cohort. Besides the lack of data on CLI patients, studies use a variety of endpoints, and even similar endpoints are often differentially defined. These considerations result in the fact that most recommendations in this guideline are not of the highest recommendation grade.

In the present chapter the treatment of CLI is not based on the TASC II classification of atherosclerotic lesions, since definitions of atherosclerotic lesions are changing along the fast development of endovascular techniques, and inter-individual differences in interpretation of the TASC classification are problematic. Therefore we propose a classification merely based on vascular area of the atherosclerotic disease and the lesion length, which is less complex and eases the interpretation.

Lesions and their treatment are discussed from the aorta downwards to the infrapopliteal region. For a subset of lesions, surgical revascularisation is still the gold standard, such as in extensive aorto-iliac lesions, lesions of the common femoral artery and long lesions of the superficial femoral artery (>15 cm), especially when an applicable venous conduit is present, because of higher patency and limb salvage rates, even though the risk of complications is sometimes higher than for endovascular strategies.

It is however more and more accepted that an endovascular first strategy is adapted in most iliac, superficial femoral, and in some infrapopliteal lesions. The newer endovascular techniques, i.e. drug-eluting stents and balloons, show promising results especially in infrapopliteal lesions. However, most of these results should still be confirmed in large RCTs focusing on CLI patients.

At some point when there is no possibility of an endovascular nor a surgical procedure, some alternative non-reconstructive options have been proposed such as lumbar sympathectomy and spinal cord stimulation. But their effectiveness is limited especially when assessing the results on objective criteria. The additional value of cell-based therapies has still to be proven from large RCTs and should therefore still be confined to a research setting.

Altogether this chapter summarises the best available evidence for the treatment of CLI, which is, from multiple perspectives, completely different from claudication. The latter also stresses the importance of well-designed RCTs focusing on CLI patients reporting standardised endpoints, both clinical as well as procedural.

INTRODUCTION

Recommendations stated in the TASC II guidelines for the treatment of peripheral arterial disease (PAD) regard a heterogeneous group of patients ranging from claudicants to critical limb ischaemia (CLI) patients. However, specific considerations apply to CLI patients. CLI is characterised by multi-level disease, high burden of comorbidity and limited life span. Thus decision-making in revascularisation strategies in CLI differs substantially from that in patients with claudication as wound healing, limb salvage and maintained ambulation are different treatment aims than improved walking ability and there are often considerable time constraints. Long-term patency as such is probably of less importance. The choice of endovascular treatment may be supported by presence of major comorbidities and hence high risk for open interventions.

A minority of studies specifically addresses CLI, precluding optimal decision for this specific group of patients. Moreover, different outcome measures are reported in the scarce studies that specifically focus on CLI patients. Some studies address the success of the primary intervention, exemplified by primary patency rates, while others emphasise the clinical results, such as limb salvage rates. The former is of major importance to evaluate the success of the intervention per se and the latter seems to be more important from a patient's perspective. So, in our opinion both should be reported in clinical studies and in a more standardised fashion as well.[1] An important problem related to the use of limb salvage as a measure of treatment success is that it is a composite endpoint affected by a variety of factors besides the revascularisation procedure per se. Therefore it is a valid endpoint only in randomised controlled trials. From a larger perspective, amputation-free survival has been suggested to be the most important endpoint for therapeutic studies on CLI.[2]

Most publications so far have been case series, cohort studies or case–control studies. The availability of well-conducted randomised controlled trials in this field is limited and therefore most recommendations in these guidelines are based on a low level of evidence. This underlines the need for future research that specifically addresses CLI in a prospective randomised-controlled fashion with well-circumscribed standardised reported outcomes.

AORTO-ILIAC REVASCULARISATION

Aorto-iliac arterial occlusive disease (AIOD) may lead to CLI, especially if concomitant atherosclerotic disease of infra-inguinal and/or below-the-knee (BTK) arteries is present. Surgical repair with aorto(bi)femoral bypass grafting or aortoiliac endarterectomy has proven effective in alleviating ischaemic pain and providing good long-term patency. Aorto(bi)femoral bypass is the most efficient procedure in case of diffuse aorto-iliac disease but carries substantial risk of peri-operative mortality, morbidity and delay in return to normal activities.

An alternative approach is represented by endovascular techniques that include angioplasty, stents, stent-grafts, and plaque debulking, which offer both good clinical and procedural results and have lower procedure-related morbidity and mortality.
There are no RCTs directly comparing surgical vs. endovascular treatment of AIOD. As a result, the selection of the optimal approach for a patient with aorto-iliac occlusive disease should be based on several variables, including an assessment of the patient's general condition and extension of the disease.

Surgical treatment of AIOD

Anatomical open surgical arterial reconstructions for treatment of AIOD are: aortofemoral bypass (AFB), iliofemoral bypass (IFB), aorto-iliac endarterectomy (AIE). In rare cases a further alternative is an extra-anatomic reconstruction by descending thoracic aortofemoral bypass (DTAF).
Aorto(bi)femoral bypass is generally the preferred treatment for diffuse aorto-iliac disease in patients who are acceptable surgical candidates. Proximal anastomosis is generally performed in an end-to-end or end-to-side fashion at the level of the infrarenal aorta, without important differences between the two techniques.[3] The simplest procedure that maintains adequate pelvic and colonic blood supply, according to angiographic findings, should be selected. The use of knitted gelatine-coated polyester, knitted collagen-coated polyester or stretch polytetrafluoroethylene (PTFE) has been reported with comparable results in terms of primary and secondary patency and long-term complication rates.[4-6] A MEDLINE (1970–2007) and Cochrane Library search for articles that report results of different open-surgical approaches for arterial reconstruction for AIOD was recently published. Studies reporting long-term primary patency data following open anatomical repair of AIOD were included, for a total of 5738 patients treated by AFB, 778 by IFB and 1490 by AIE.[7] The operative mortality rate for AFB, IFB and AIE was 4.1%, 2.7% and 2.7%, respectively ($p < 0.0001$), while the systemic morbidity rate was 16% for AFB, 18.9% for IFB and 12.5% for AIE ($p < 0.0001$). In a sub-analysis according to clinical presentation, the 5-year primary patency in case of CLI was 79.8%, 74.1% and 81.7% for AFB, IFB and AIE, respectively ($p = 0.06$), significantly worse in comparison to 5-year patency rates for patients with intermittent claudication ($p < 0.0001$).
All three anatomical techniques for open-surgical aorto-iliac reconstructions were equally effective in terms of primary patency rates, but AIE appears to be associated with significantly lower operative mortality and systemic and local complication rates compared to the two bypass procedures. This can probably be explained by the fact that AIE is utilised predominantly for localised aorto-iliac disease and AFB and IFB may be used for more extensive disease.
DTAF is predominantly reserved for patients in whom the aforementioned reconstructions are unsuitable and is associated with higher operative mortality and graft-related complication rates and lower 5-year patency rates than the other three techniques.[7-9]

Recommendations

Aorto(bi)femoral bypass is generally the preferred treatment for diffuse aorto-iliac disease in patients who are suitable surgical candidates. **(Level 2a; Grade B)**

Aorto-iliac endarterectomy is to be recommended for patients with suitable occlusive lesions as it appears to be associated with significantly lower operative mortality and systemic and local complication rates compared with bypass procedures. **(Level 4; Grade C)**

Descending thoracic aortofemoral bypass is to be reserved for patients that cannot be otherwise revascularised as it is associated with higher operative mortality and graft-related complication rates and lower patency rates. **(Level 5; Grade D)**

Laparoscopic repair

A variety of different techniques are encompassed in the term laparoscopic AIOD repair, including totally-laparoscopic repair, hand-assisted laparoscopic repair and robotic-assisted laparoscopic repair. These are considered together for the purposes of these guidelines. Laparoscopic repair offers patients a third option for AIOD repair that provides the durability of an "open" sutured graft with a rapid recovery and reduced length of hospital stay.[10, 11]

Currently, the role of laparoscopic repair remains limited and should be confined to centres with specific expertise in laparoscopic aortic repair. This is in part due to the requirements for advanced laparoscopic practice, and also due to the steep learning curve for this procedure. It should be noted that the cardiac risk of laparoscopic procedures should be considered to be the same as for open repair.[12] Procedures should initially only be conducted under supervision by someone experienced in laparoscopic aortic repair. Facilities to deal with emergency surgical conversion should be available at all times.

Recommendation

The role of laparoscopic repair of AIOD remains limited, but in selected patients it might represent a third option for aorto-iliac atherosclerotic disease repair. **(Level 5; Grade D)**

Extra-anatomical bypass

Extra-anatomical arterial reconstructions such as axillo-(bi)femoral bypass and crossover femoral bypass are generally reserved for patients with increased comorbidities or a hostile abdomen. For isolated unilateral iliac artery occlusive disease, for which endovascular angioplasty failed or does not seem feasible, a crossover femoro-femoral bypass can be considered as effective as an aorto-femoral or iliofemoral bypass, but with less operative morbidity. Extra-anatomical repair also allows to preserve the autonomic nerve fibres at the aortic bifurcation and has less influence on sexual function.

Recommendation

Because of the relatively low patency rates, extra-anatomical bypass should be reserved for patients who have no other alternatives for revascularisation. **(Level 4; Grade C)**

Endovascular treatment of aorto-iliac occlusive disease

There are no RCTs directly comparing surgical vs. endovascular treatment of AIOD, and therefore there is a lack of objective grounds on which the choice between the two techniques can be made.

In clinical practice, because of its minimal invasiveness, many clinicians consider endovascular therapy to be the firstline strategy, feasible and effective for the treatment of the majority of aorto-iliac atherosclerotic lesions. The technical success rate of angioplasty of iliac stenosis is nearly 100%, and the technique is also used to treat long-segment iliac occlusion. Unfortunately, CLI is seldom caused by limited aorto-iliac lesions but rather occlusive disease affecting multiple arterial segments. If focal lesions are identified, they mostly cause an inflow problem above infrainguinal occlusions in these patients.

Endovascular treatment of extensive aorto-iliac occlusive disease

A recent systematic review performed by Jongkind et al.[13] identified 19 non-randomised cohort studies reporting on 1711 patients with extensive AIOD. Although the lesions treated were described adequately, unfortunately no data on the indication were included. Technical success was achieved in 86–100% of the patients with extensive AIOD, defined as less than 30% residual diameter stenosis and/or a residual trans-lesion pressure gradient of less than 10 mmHg. Clinical symptom improvement was observed in 83–100% of the patients, and mortality ranged from 1.2% to 6.7%. Although a number of procedural or perioperative complications were reported, including distal embolisation, access-site haematomas, pseudoaneurysms, arterial ruptures, and arterial dissections, the majority could be treated using percutaneous or non-invasive techniques. Four- and 5-year primary and secondary patency after endovascular treatment of these extensive aorto-iliac lesions ranged from 60% to 86% and 80% to 98%, respectively.

In two studies retrospectively comparing endovascular therapy vs. open-surgical reconstruction for extensive AIOD, a significantly lower long-term primary patency was reported for endovascular therapy (69% vs. 93%, $p = 0.013$[14] and 74% vs. 93%, $p = 0.002$[15]), while secondary patency did not differ significantly (89% vs. 100% and 96% vs. 96%). The applicability of these data on treatment decisions for CLI is affected by the low proportion of patients with CLI in these studies, 21%14 and 40%.[15]

Recommendations

Endovascular treatment can be considered a successful primary strategy for patients with aorto-iliac lesions, most often before or in conjunction with a distal revascularisation. Its major advantage is its less invasiveness, characterised by a lower operative morbidity-mortality. **(Level 3a; Grade C)**

Even though primary patency rates after endovascular therapy for extensive AIOD are inferior to those reported after surgery, re-interventions may be performed percutaneously. **(Level 5; Grade D)**

Plain balloon angioplasty vs. primary stenting

Although primary stenting has been proposed as more effective than plain balloon angioplasty for iliac atherosclerotic lesions, the evidence described in the literature does not allow clear conclusions. The only RCT comparing the technical results and clinical outcomes of two treatment strategies (primary stenting or PTA followed by selective stenting when haemodynamic results were inadequate) concluded that patients treated with PTA and selective stent placement in the iliac artery had a better outcome for symptomatic success compared with patients treated with primary stent placement, whereas data about iliac patency, ABI, and quality of life did not support a difference between groups.[16] Notably, the trial was performed in a cohort of patients with lifestyle-limiting intermittent claudication. Nonetheless, primary stenting is now preferred in most studies for extensive aorto-iliac lesions, considering the fact that primary stenting without pre-dilatation is considered to involve less risk of causing vessel rupture and/or distal embolisation.

Recommendation

Angioplasty followed by selective stenting for PTA with inadequate result should be preferred for iliac artery occlusive disease. **(Level 2; Grade B)**

INFRAINGUINAL DISEASE

Common femoral artery (CFA)

Surgical endarterectomy of CFA lesions (CFE), isolated or within a hybrid setting, provides excellent 1- and 5-year patency rates of 93% and 91%, respectively, and secondary patency rates reaching 100%.[17, 18] Ballotta et al. confirmed the excellent long-term patency of CFE with a patch in a cohort of 117 patients (40% CLI) with 7-year primary patency rates of 96%, assisted primary patency of 100%, and 100% limb salvage.[19] An advantage of surgical treatment of atherosclerotic disease of the CFA is that it provides the potential to endarterectomise adjacent diseased segments of the deep femoral artery (DFA) and the proximal superficial femoral artery (SFA) or the opportunity for hybrid iliac or SFA recanalisation. It should be noted, however, that CFE per se can also worsen pathology of the SFA.

There are reports on treating CFA lesions with endovascular techniques as well, although with variable results.[20-24] In particular the early reports on angioplasty of the CFA without stenting have been associated with relatively poor results.[20] However, technical success rates of 100% of CFA angioplasty with primary stenting have been reported with acceptable mid-term outcome.[25] However, placing a stent in the CFA may increase risk of potential future surgical interventions and limit future access for endovascular revascularisation in this location. CFA stenting is likely to be an alternative for special indications and therefore RCTs comparing it with endarterectomy are hardly possible. It is further characterised by an increased risk of stent-strut fracture in this mobile segment of the arterial tree, due to repetitive hip flexion–extension and compression by the inguinal ligament.

Recommendation
Endarterectomy of atherosclerotic disease of the common femoral artery provides excellent results with limited morbidity and mortality and is the standard treatment in this location. **(Level 4; Grade C)**

Hybrid procedures
Concomitant disease of the external iliac artery (EIA), DFA or SFA is commonplace in CLI. In patients in whom CFE is to be performed, the direct access via the CFA can offer the opportunity to simultaneously perform endovascular treatment of the adjacent diseased EIA or SFA. These hybrid procedures have been performed with promising results and acceptable patency rates.[18] Hybrid procedures of the aorto-iliac segment will be discussed here and the infrainguinal hybrid procedures will be discussed in the section on treatment of the SFA.
Aorto-iliac hybrid procedures often combine CFA surgery or infrainguinal femoropopliteal bypass surgery with aorto-iliac recanalisation, where the surgical part provides the access for the endovascular reconstruction of the diseased aorto-iliac segment. Initial technical success rates of hybrid aorto-iliac intervention generally approach 100% and peri-operative mortality rates are low.[26-28] Reported primary patency rates after hybrid procedures for aorto-iliac occlusive disease are probably somewhat lower than for sole endovascular interventions of the aorto-iliac segment,[28, 29] with 5-year primary patency rates of 60% for hybrid procedures[28] and 4-year primary patency rates of 68% (65–71%) and 77% (72–81%) for PTA and PTA with stenting, respectively.[30] Chang et al. showed improved patency rates for stent grafts compared to bare-metal stents.[28] A recent report by Dosluoglu and co-workers[31] reports similar results of open, endovascular and hybrid techniques for patients with similar disease complexity and even better limb salvage rates in CLI patients with complex hybrid revascularisations (TASC C or D).

Recommendations
Endovascular treatment of aorto-iliac occlusive disease in a hybrid fashion offers an acceptable alternative treatment in patients with aorto-iliac disease and concomitant common femoral artery disease that requires open surgery. **(Level 3b; Grade C)**
Stent grafts probably provide better results compared to bare-metal stents in the hybrid treatment of aorto-iliac occlusive disease. This should however be confirmed by future prospective studies. **(Level 4; Grade C)**

Deep femoral artery (DFA)
Profundoplasty is of limited value in the treatment of CLI, but can be considered in patients with stenotic lesions of the DFA and where restoration of continuous blood flow from the aorto-iliac tract to the SFA or popliteal artery is not an option. Limb salvage rates of profundoplasty have been reported to be 67% after 1 year[32] and 49% and 36% after 3 and 5 years, respectively.[33-36] Profundoplasty is rarely performed as an isolated procedure and can be performed with or without a patch based on the intra-operative judgment by the

surgeon. Besides its role for potential limb salvage profundoplasty can be of value in preserving the knee joint when amputation is deemed inevitable.[33]
Studies on endovascular treatment of DFA obstructive disease have been mainly confined to relatively small case series, and long-term limb salvage rates are usually not reported.[37-42] Initial technical success rates of percutaneous DFA recanalisation range from 77% to 100%,[43] but long-term results seem less favourable.[24, 42] However, more promising results of endovascular treatment of the DFA were published recently by Donas et al. in a selected group of patients (n=15) with CLI with sufficient run-off vessels, in which 3-year primary and secondary patency rates were 80% and 86.7%, respectively, and limb salvage was 93%.[44] Stenting of the DFA has also been reported,[25] but stenting the DFA likely hampers potential future surgical interventions in this area.

Recommendations

Revascularisation of the deep femoral artery can be considered in CLI patients without options for restoration of continuous blood flow from the aorto-iliac segment to the popliteal artery in conjunction with haemodynamically significant stenosis of the DFA. Based on currently available evidence, surgical profundoplasty is preferred over endovascular recanalisation, due to a relatively high rate of late failures of the latter. **(Level 3b; Grade C)**

Profundoplasty can be of additional value in preserving the knee joint when amputation is inevitable. **(Level 4; Grade C)**

Superficial femoral artery (SFA)

In the present guideline, lesions of the SFA are not classified according to the TASC II guidelines,[45] though these are generally regarded as the standard method of classification in treating peripheral arterial disease. The definitions of atherosclerotic lesions are changing with the rapid development of mainly endovascular techniques and devices (TASC I vs. TASC II). Furthermore, the use of the TASC classification may be problematic due to considerable inter-individual differences in interpretation.[46-48] However, since the widespread use of the TASC classification system in the past decades most studies used this method to classify lesions under investigation. Therefore the TASC classification is still mentioned repeatedly in this guideline, but is eliminated from the treatment recommendations. For future use in research and for treatment recommendations we propose a classification system based on lesion length instead of complex loco-anatomic descriptions of lesions as provided by the TASC classification.

Endovascular treatment

Endovascular treatment is increasingly considered as the first-line treatment for atherosclerotic lesions of the femoro-popliteal segment. Yet, the success rate of endovascular treatment of femoropopliteal lesions depends on variables such as the presence of diabetes mellitus or chronic kidney disease, stenosis vs. occlusion, lesion length and crural run-off status,[49] factors which are often unfavourable in patients with CLI.[50, 51]

Despite excellent initial technical and clinical success rates of PTA of femoropopliteal artery stenoses in series studying the full range of peripheral arterial disease – most including less than 15% CLI patients – the data for CLI are far worse. This was illustrated by a meta-analysis of Muradin et al.,[52] which showed clearly inferior 3-year primary patency rates after recanalisation of SFA occlusions in CLI patients compared to claudicants. Technical failure of angioplasty due to dissection or recoil has been largely reduced with the introduction of the bare metal stents,[53] but restenosis has remained a major problem precluding long-term benefit of stenting. Yet, a meta-analysis reported a 3-year patency rate of 58–68% in CLI patients.[52]

The self-expanding nitinol stents have further improved endovascular treatment of the SFA and provide more durable results than stainless steel (balloon-expandable) stents.[54-59] Despite the fact that these studies mainly included claudicants (proportion of CLI patients 14–89%), results of self-expanding nitinol stenting seem beneficial in CLI patients as well. Primary nitinol stenting proved beneficial compared to PTA with provisional stenting especially for longer SFA lesions (average lesion length varying from 9.8±5.4 cm to 20.35±9.46 cm).[60-64] Limb salvage rates 36 months after stenting of the SFA in CLI patients have been reported to be 67–75%.[65, 66] For lesions <5.0cm in length the benefit of primary stenting is clearly more debatable as has been shown in a meta-analysis by Kasapis and colleagues,[67] who showed no differences in restenosis rate and target vessel recanalisation, despite a higher immediate success rate for stenting compared to angioplasty alone.

Different studies have been published supporting endovascular treatment of long femoropopliteal (TASC C and D) lesions with or without stenting. Han and co-workers published their results of endovascular treatment stratified by TASC lesion type and showed that in 243 CLI patients limb salvage rates 24 months after endovascular treatment were 81.0±12.9%, 81.1±6.8%, and 71.9±8.0% for TASC A+B, TASC C and TASC D lesions of the SFA, respectively.[68] Similar limb salvage rates were obtained by Taneja and co-workers in CLI patients with long-segment occlusions (average 23.8 cm, range 10–39 cm) treated with bare nitinol stents, however primary patency rates were rather low with 61.5% and 27% after 6 and 12 months, respectively.[69] These studies suggest that endovascular treatment of long femoropopliteal lesions can be – at least clinically – successful.

The high restenosis rates of bare nitinol stents observed mainly in long atherosclerotic lesions of the SFA and the popliteal region provide the fertile soil for further technical innovations aiming at increasing patency rates. An important and promising innovation has been the stent graft (also referred to as covered stent, endograft, endoluminal bypass or thrupass). Most stent grafts are composed of nitinol stents covered with polytetrafluoroethylene (PTFE) and were developed to prevent restenosis due to intimal hyperplasia. Primary patency rates after 1 year for lesions <10 cm treated with PTFE stent grafts have been reported to be approximately 90% in CLI.[70, 71] However, lower and considerably varying 1-year patency rates have been recorded for longer lesions of the SFA treated with PTFE stent grafts, ranging from approximately 48% to 81%,[72-77] and generally lower patency rates are observed in CLI patients and occlusive lesions. Despite a 69% primary patency rate at 3 years, Alimi et al. reported a 86% limb salvage rate in

CLI patients treated with the PTFE stent graft for lesions with a mean length of 12.4cm (range 2.6–30.2 cm).[75]

Studies directly comparing stent grafts with plain PTA or PTA with bare stents are very limited. Saxon et al. compared stent grafts (n=97, 9% CLI) with PTA alone (n = 100, 12% CLI) in a randomised fashion for treatment of SFA lesions (stenosis or occlusions) up to 13 cm. In the stent graft group a higher technical success rate and 1-year primary patency rate of 65% vs. 40% (p = 0.0003) was observed.[78] The preliminary results of the VIBRANT trial that compares angioplasty of long SFA lesions with either the PTFE stent graft or bare nitinol stenting do not show any differences regarding primary patency at 1-year follow-up; however, secondary patency at 1 year was somewhat higher in the stent graft group. The official mid-term (3 years) follow-up results are not yet available but could prove superiority of one of both treatment modalities. An FDA-approved heparin-bonded version of the stent graft has been developed to improve patency rates. Future randomised trials still have to prove the efficacy and superiority of (heparin-bonded) stent grafts over bare nitinol stents.

There is one major concern of using covered stents, namely the potential loss of pre-existent collateral vessels with acute deterioration in case the stent graft occludes; however, this hypothesis is not yet confirmed by evidence.[79]

Another proposed strategy to prevent intimal hyperplasia is represented by drug-eluting stents (DES). The first piloting trials (SIROCCO I&II) comparing sirolimus-coated stents with bare nitinol stents in the SFA failed to show important and significant differences between the two treatment groups.[80, 81] Currently there are two trials, which have not yet published their results, that study the paclitaxel-coated Zilver pTX stent (Cook Medical, Bloomington, Indiana, USA) and the everolimus-eluting Dynalink-E (Abbott Vascular, Abbott Park, Illinois, USA), the Zilver pTX trial and the Strides study, respectively. In the Zilver pTX trial patients with moderate to severe symptomatic femoropopliteal artery disease (lesions up to 14 cm; average lesion length 6.6 cm) were randomised to undergo either traditional PTA or PTA plus Zilver pTX stent deployment (n=479). In the PTA group non-optimal (>30% residual stenosis or >5 mmHg pressure gradient) PTAs were again randomised to either subsequent deployment of a bare Zilver stent or the pTX version of the stent. The preliminary short-term results of the Zilver pTX trial are promising, with 12-month patency rates in the provisional stent group (after suboptimal PTA) of 89.9% and 73% for the Zilver pTX and bare Zilver stent, respectively (p = 0.01). These results seem to be consistent and are confirmed by the 2-year follow-up data, where primary patency rates in the provisional stent group are 81.2% (n=56) and 62.7% (n=56) for the Zilver pTX and the bare-metal Zilver stent, respectively. On the other hand the Strides study (n=106; mean lesion length 9.0±4.3 cm; 17% CLI patients) did not show any benefit of the everolimus-eluting Dynalink-E stent compared to historical controls treated with a similar non-everolimus-eluting stent, both showing a primary patency of between 60% and 70% at 12 months, despite a promising 94±2.3% primary patency rate of the former.[82]

Since long complex lesions are usually present in CLI patients, successful endovascular recanalisation of the SFA can sometimes only be performed with subintimal angioplasty

(SIA). SIA has been associated with high limb salvage rates between 85% and 90% at 1 year, even despite a low 50% 1-year primary patency rate.[83] These results were recently confirmed by Bolia et al. and Setacci et al. with primary success rates of 80% and 83.5% and limb salvage rates of 85% and 88% at 1 year, respectively.[84, 85]

A major concern of the popularity of endovascular interventions, especially in complex lesions, is the potential alteration of the level for subsequent open procedures after failed endovascular intervention. Joels et al. have reported that the problem of alteration of the level of a subsequent open procedure after failed endovascular intervention is acceptable and even when the level alters it does not necessarily change clinical outcome.[86] They showed that only 23 out of the 276 patients subjected to endovascular recanalisation of the SFA presented with early failure of the procedure and that this altered the level of the subsequent open intervention in one third of the patients. Amputation due to early failure was necessary in only one patient (0.4%). However, they did not include TASC D lesions. In another study, by Gur et al.,[87] of the 192 patients who underwent PTA with primary stenting of the SFA, [69] stented arteries lost primary patency (over a 5-year period). In 10 patients open bypass was eventually required and the bypass level was changed in two of them. The risk of stent failure, loss of run-off vessels and necessity for open procedures was higher in the TASC C and D lesions. The fact that CLI patients are amenable to subsequent intervention (both open and endovascular) for limb salvage even after failed endovascular intervention is further supported by Ryer and colleagues.[88]

Surgery

Bypass surgery has long been and still is the gold standard therapy in the treatment of long SFA lesions. The great saphenous vein is the best performing conduit for infra-inguinal bypass surgery. Above-the-knee femoropopliteal bypass has a patency rate of 77.2% at 5 years in claudicants and 69.4% in CLI patients, when saphenous vein is used as a conduit. Autologous great saphenous vein bypass below the knee has similar long-term patency rates compared to above the knee bypass.[89] Limb salvage rates of 86.9% (±7.6%) 2 years after non-reversed vein grafts in above-the-knee femoropopliteal bypasses have been reported for CLI.[90] Venous conduits outperform prosthetic conduits irrespective of the material used (Dacron or PTFE). This also applies for arm vein conduits compared to prosthetic bypasses in CLI patients.[91]

The BASIL trial is the only randomised trial comparing a PTA-first vs. a bypass surgery-first strategy in patients with severe limb ischaemia; it showed no differences in amputation-free survival between bypass surgery and PTA. However, for patients with a more than 2-year survival after the initial intervention, patients randomised to bypass surgery showed higher overall and amputation-free survival.[92-94] The superiority of femoropopliteal bypass procedures compared to femoropopliteal PTA in CLI patients, especially in the long-term, is supported by a retrospective study by Korhonen et al. which used a propensity score analysis to minimise bias.[95] They showed considerable differences in favour of the bypass group (80.5% vein and 19.5% prosthetic graft) with 5-year limb salvage rates of 78.2% vs. 91.8% and survival rates of 49.2% vs. 57.1% for the PTA and bypass group, respectively.

These results were still significant in the propensity score-matched pairs, with 5-year limb salvage rates of 74.3% vs. 88.2% (p = 0.031) for the PTA vs. the bypass group, respectively. Bypass surgery has also been randomly compared with stent graft procedures. Kedora et al.[74] reported on 100 patients with SFA occlusive disease and symptoms ranging from claudication to rest pain, with or without tissue loss, who were randomised to PTA with one or more self-expandable stent grafts (n = 50) or prosthetic femoral-popliteal above-the-knee bypass (n = 50). The mean total length of artery stented was 25.6±15 cm. Both 1-year primary and 1-year secondary patency rates – based on life-table analysis – were not significantly different between the two groups, with primary patency rates of 73.5% vs. 74.2% and secondary patency rates of 83.9% vs. 83.7% for the stent graft and bypass group, respectively. Neither did limb salvage between the two groups differ significantly. Severity of limb ischaemia (Rutherford classification) did not differ between the two groups; however, ischaemia showed a non-significant tendency to be more severe in the bypass group.[74] Later results from the same patient group showed a trend to lower patency rates for the stent graft group in the higher TASC II lesions (TASC C and D).[77] Less favourable results for stent graft procedures were reported by Lepäntalo and co-workers in a prematurely terminated (due to disadvantageous outcome in the stent graft group) randomised multicentre trial comparing stent graft procedures and prosthetic bypass surgery for occlusions (TASC II B and C occlusions) of the SFA.[72] In contrast to the two other studies, which mainly included patients with intermittent claudication and did not clearly reveal data on concealment of treatment allocation, Lepäntalo et al. only included CLI patients based on rigorous inclusion criteria and properly reported concealment. They reported substantial lower primary and secondary 1-year patency rates for the stent graft group vs. the bypass group, 46% vs. 84% and 63% vs. 100%, respectively. Overall the results of these studies still favour the use of femoropopliteal bypass vs. stent graft procedures in CLI patients with long SFA lesions, especially long occlusions. Future RCTs comparing these treatment modalities (and the heparin-bonded endograft) in specific subgroups of CLI patients are necessary to allow definitive conclusions on these therapies.

Hybrid procedures

Hybrid procedures combining CFA surgery or distal origin bypass surgery with angioplasty of the SFA is another possibility to treat lesions of the SFA. Hybrid procedures studied are highly heterogeneous, therefore no exact numbers can be provided on patency rates and limb salvage rates. Patency rates after hybrid procedures vary considerably, with 3-year primary patency rates as high as 84% and primary patency rates as low as 58% after 41 months follow-up.[31, 96, 97] However, reported limb salvage rates 3 years after the intervention are over 80%.[31, 96, 98]

In a hybrid procedure it is also possible to perform remote superficial femoral artery endarterectomy (RSFAE). Patency rates of retrospective studies are promising so far, with patency rates of 61–69% at 18–33 months.[99] In the REVAS trial, RSFAE compared with above-knee bypass surgery has been studied for the treatment of TASC C and D lesions

of the SFA. Primary patency rates after 1 year were 61% for RSFAE and 73% for bypass surgery, with similar secondary patency rates of 79%.[100]

Drug-eluting balloon, cryoplasty, cutting balloon, excimer laser

Drug-eluting balloons, successfully applied for angioplasty of coronary arteries, have not been widely studied in the femoropopliteal arteries. The available short-term data on the use of drug-eluting balloons for PTA of the femoropopliteal region are encouraging, however these studies have mainly focused on relatively short lesions and almost invariably address claudicants.[101, 102] Longer-term follow-up and larger randomised trials are needed to clarify whether drug-eluting balloons can be beneficial in the long term and should include sufficient numbers of CLI patients to draw reasonable conclusions in this subset of patients.

No data exist on the direct comparison of cryoplasty, cutting balloons and excimer laser with conventional endovascular treatment in patients with CLI. Cryoplasty seems not beneficial in the femoropopliteal area.[103, 104] Cutting balloons have a short design thereby limiting their use in long lesions of the SFA. Use of the excimer laser has been shown to be effective in CLI,[105, 106] however there is no evidence of superiority compared to conventional angioplasty or subintimal angioplasty in CLI.

Recommendations

A new and simplified classification system for peripheral arterial disease lesions is needed to improve interindividual interpretation as this is problematic for the TASC classification. Therefore we would recommend a system based on lesion length to classify lesions for research applications and clinical management (EUSC classification; Figure 1). Future research should prove the applicability and reproducibility of the classification and the additional value of a potential subdivision of stenotic vs. occlusive lesions. **(Level 5; Grade D)**

Short SFA lesions (<5 cm) are preferably treated with angioplasty. Stenting of short lesions should only be performed when suboptimal results are obtained with PTA alone. **(Level 1a; Grade B)**

The preferred treatment of intermediate SFA lesions (5–15 cm) is PTA with primary bare nitinol stenting. **(Level 1b; Grade B)**

The additional value of drug-eluting and PTFE stent grafts still has to be confirmed in CLI patients. When autologous vein is available as a conduit, bypass surgery is the preferred treatment for long SFA lesions (>15 cm), especially in younger patients. Although the outcome after an endovascular first approach is equal in the short term to prosthetic bypass surgery for long SFA lesions in CLI patients, bypass gives better results in more fit patients and should be preferred in patients with estimated longevity >2 years. **(Level 1b; Grade B)**

In long SFA lesions (>15 cm) endovascular treatment (intraluminal or subintimal) with a stent graft seems acceptable when the patient's condition precludes an open procedure. The value of the heparin-bonded stent-graft has still to be confirmed. **(Level 3b; Grade C)**

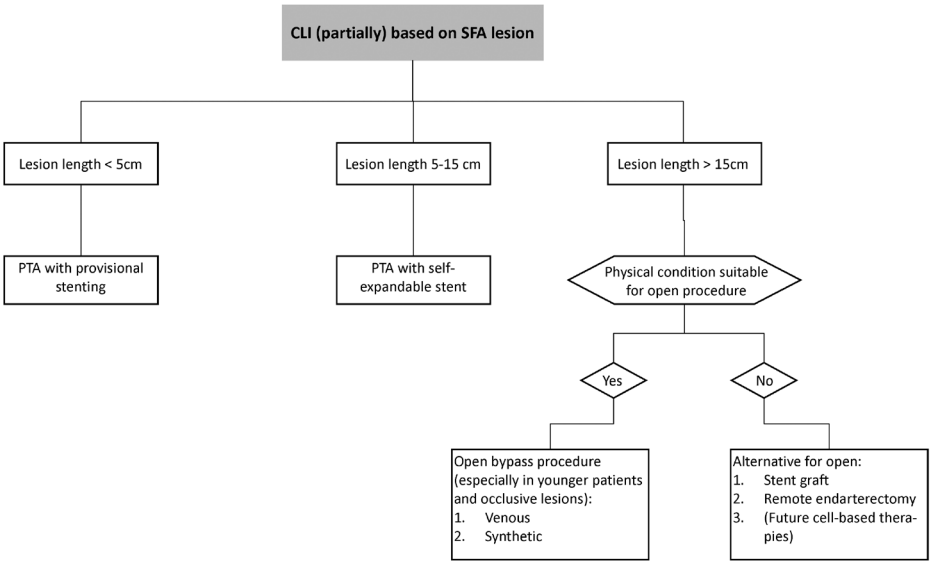

Figure 1 EUSC classification for SFA lesions and treatment advice. In view of problems with the use of TASC guideline classification for atherosclerotic lesions – mainly poor inter-individual interpretation and hence problematic interpretation of treatment results published in the literature – we propose a simplified classification based on lesion length rather than based on complex loco-anatomic descriptions. Future research using this method has to prove its applicability and need for subclassifications, e.g., occlusive vs. stenotic lesions.

Hybrid procedures are the preferred treatment modality irrespective of lesion length in high-risk patients not suitable for open bypass surgery or when no suitable vein is available if minimally open revascularisation is mandated, such as CFE. **(Level 2b; Grade B)**

INFRAPOPLITEAL DISEASE

Despite the magnitude of the problem – currently greater than ever due to the increasing diabetic and ageing population – unexpectedly little high-quality evidence exists in the literature to support a strategy paradigm in patients with CLI and infrapopliteal disease. Several studies have demonstrated that surgical revas-cularisation is the standard treatment for limb salvage in patients with CLI due to atherosclerotic disease of infrapopliteal arteries, but endovascular interventions of infrapopliteal lesions represent a far less invasive option and are now considered a valid alternative to surgical bypass in many cases.

Surgical revascularisation for infrapopliteal lesions

Unfortunately, a detailed anatomical description of the disease and relative localisation of the treatment is rarely reported in RCTs and observational studies comparing different treatments for CLI, and no specific conclusion can be drawn for patients with isolated infrapopliteal disease.

The PREVENT III study[107] was a prospective, randomised, double-blinded, multi-centre phase III trial of a novel molecular therapy (edifoligide; E2F decoy) to prevent vein graft failure in patients undergoing infrainguinal revascularisation for CLI, reporting a peri-operative mortality rate of 2.7%, primary and secondary graft patency rates of 61% and 80%, and 1-year limb salvage and survival rates of 88% and 84%, respectively. In the majority of the 1404 patients who underwent surgical bypass procedures (n=914, 65%) the bypass was anastomosed at the tibial or pedal/plantar vessels distally, but no separate analysis of this subgroup was provided.

In the BASIL trial,[92-94] only 10% of distal bypass anastomoses were located distally to the popliteal artery. Moreover, the design of the trial included only patients considered suitable for both surgical and endovascular treatment, which means that very complex cases have been treated outside the scope of the trial (probably by surgical approach).

A set of suggested objective performance goals (OPG) for evaluating the results of new catheter-based treatments in CLI has recently been elaborated, based on evidence from RCTs of patients treated by surgical vein bypass. The patient-level data from three RCTs identified 838 patients with autogenous vein bypass. The primary efficacy endpoint, defined as freedom from perioperative (30-day) death or any major adverse limb event (amputation or major re-intervention) occurring within 1 year was 76.9%, and the primary amputation-free survival at 1 year was 76.5%. The authors suggest that these data should be considered the most suitable current framework for non-randomised comparisons, especially for evaluating outcomes after endovascular treatments for CLI. They also stress that risk stratification should be incorporated in design and reporting of studies since the CLI population is heterogeneous and the OPG thresholds differ substantially between the lower- and higher-risk procedures (based on clinical, anatomical and conduit characteristics).[108]

Although no RCTs have selectively studied the outcomes of different graft materials for the construction of bypasses to the infrapopliteal arteries, there is a large body of evidence that vein offers better results in comparison to other graft material. Both immediate and long-term patency benefit from the use of autologous great saphenous vein, whether in situ or reversed. Proximal (CFA, SFA or popliteal artery) and distal anastomoses (tibial and pedal arteries) of infrainguinal bypasses may vary, depending on the extent of the atherosclerotic disease. Since the proposal of the "short bypass principle" by Veith[109] in 1981, the use of more distal sites for the origin of the bypass have been recommended (popliteal-to-distal bypasses). The advantages include the reduction of groin dissection, the use of shorter graft material, and the decrease in operative time.

A meta-analysis of popliteal-to-distal vein bypass grafts reported a 5-year primary graft patency rate of 63±4%, a secondary patency rate of 70±5%, and a foot salvage rate of 78±4%.[110]

When the great saphenous vein is unavailable or unsuitable, alternative graft materials include autologous vein, allograft material (cryopreserved vein and arterial allograft) and synthetic material such as PTFE. A meta-analysis of allograft bypass grafting to infrapopliteal arteries revealed a poor patency rate at 5 years (ranging from 19% to 30% for different grafts: cold-storage vein, cryopreserved arteries, umbilical-cord veins, and cryopreserved veins) and reduced rate of foot preservation (ranging from 55% to 60%).[111] Better results in cases of great saphenous vein absence come from the use of alternative autologous vein. Although this approach carries disadvantages such as the need to harvest vein from distal sites and construction of composite grafts, the meta-analysis of alternative autologous vein bypass grafts to infrapopliteal arteries reported results far superior to those reported for non-autologous grafts.[112] The 5-year pooled estimates were 46.9% for primary patency, 66.5% for secondary patency, and 76.4% for foot preservation. The radial artery (or arteries) can be used for limb-salvage revascularisation when no other valuable autologous veins are available. Only short case series have been reported in the literature, almost all in diabetic patients.[113, 114]

The use of PTFE bypass to infrapopliteal arteries is associated with poor long-term outcomes. Random-effects meta-analysis[115] yielded 5-year pooled estimates of 31% for primary graft patency, 40% for secondary graft patency, and 56% for foot preservation. Outcomes were slightly higher for a series of PTFE grafts with adjunctive procedures at distal anastomoses (composite PTFE-vein grafts, patches, cuffs and arteriovenous fistulas), compared with a series of PTFE grafts only.

Combinations of popliteal-to-distal bypass and endovascular treatment of SFA lesions have also been reported to improve technical success and patency rate.[98, 116]

Recommendations

The great saphenous vein is superior to other materials and should be preferred in bypass grafting to infrapopliteal arteries. **(Level 3b; Grade B)**

When the great saphenous vein is unavailable or unsuitable, the use of alternative autologous vein grafts (single-segment or composite) is preferable to that of allograft bypass and PTFE bypass graft. **(Level 4; Grade C)**

Endovascular revascularisation

Angioplasty

The primary aims of infrapopliteal angioplasty in CLI are to restore at least one straight line of blood flow to the ischaemic foot and to maintain the patency of the treated artery for as long as possible or at least as long as necessary to allow ulcer healing, pain relief and to avoid recurrence of CLI.

In the past, infrapopliteal angioplasty has been reserved for patients with short stenotic lesions or for patients who are poor candidates for bypass surgery, but in the last 5–10 years this technique has been used with increasing frequency, also for more complex lesions. Due to the evolution of techniques and the availability of dedicated materials, the endovascular first-line approach to below-the-knee (BTK) vessels should be preferred over

bypass according to some authors.[117-119]

Different endovascular approaches have been proposed, including ipsilateral, antegrade or contralateral retrograde femoral puncture, or more recently, retrograde anterior or posterior tibial puncture[120, 121] or retrograde crossing through the pedal arch (pedal-plantar loop technique).[122, 123]

Unfortunately, the level of evidence for endovascular treatment of BTK vessels is still low. Considering the absence of RCTs comparing surgical vs. endovascular techniques in patients with infrapopliteal disease, the most relevant data in the literature come from extrapolations of RCTs comparing the outcome of bypass and balloon angioplasty at different levels in patients with CLI and from the meta-analysis of retrospective case series where no biases were detected.

Another way to try to decrease bias in comparisons is to adjust differences by using propensity score analysis in large patient cohorts.[124, 125] Recently, in a study cohort comprising 1023 patients treated for CLI with 262 endovascular and 761 surgical revascularisation procedures to their crural or pedal arteries were compared. In the overall series, PTA and bypass surgery achieved similar 5-year limb salvage (75.3% vs. 76.0%), survival (47.5% vs. 43.3%), and amputation-free survival (37.7% vs. 37.3%), indicating that when feasible, infrapopliteal PTA as a first-line strategy is expected to achieve similar long-term results to bypass surgery in CLI when redo surgery is actively utilised. In a subgroup of patients who underwent isolated infrapopliteal revascularisation, PTA was associated with better limb salvage (75.5% vs. 68.0%, p = 0.042).[126] Additionally, in 584 consecutive patients aged at least 80 years treated with either PTA (n=277) or bypass surgery (n = 307) for CLI irrespective of the level of infrainguinal revascularisation, PTA achieved better results than bypass surgery after 2 years (leg salvage: 85.4% vs. 78.7%, p = 0.039; survival: 57.7% vs. 52.3%, p = 0.014; amputation-free survival (AFS): 53.0% vs. 44.9%, p = 0.005). Cox regression analysis showed that increased age [relative risk (RR) 1.05, 95% confidence interval [CI] 1.02–1.08], decreased estimated glomerular filtration rate (RR 0.99, 0.99–1.00), diabetes (RR 1.30, 1.04–1.62), coronary artery disease (RR 1.36, 1.05–1.75) and bypass surgery (RR 1.55, 1.24–1.93) were associated with decreased AFS. In 95 propensity score-matched pairs, limb salvage at 2 years (88% vs. 75%; p = 0.01) and AFS (53% vs. 45%; p = 0.033) were significantly better after PTA. Classification and regression tree analysis suggested that PTA was associated with better 1-year AFS, especially in patients with coronary artery disease (63.8% vs. 48.9%; p = 0.008). When feasible, a strategy of PTA first appears to achieve better results than infrainguinal bypass surgery in patients aged 80 years and older.[127]

Regrettably, the only major randomised trial comparing PTA vs. surgery for peripheral arterial occlusive disease (BASIL) included patients with infrainguinal rather than isolated infrapopliteal lesions and did not report details of the anatomic segments treated and relative outcomes. Consequently, no extrapolation of data is possible, which limits analysis of the results of angioplasty vs. surgery for patients with isolated crural disease.

In a recent meta-analysis of infrapopliteal angioplasty for CLI including a large number of case series, the pooled estimate of success was 89.0±2.2% for immediate technical

results, and the early mortality rate was 1.8%. The mid-term estimates of primary patency, secondary patency and limb salvage were assessed reliably until 36 months.[128] When compared to the results of the meta-analysis of popliteal-to-distal bypass graft, the durability of infrapopliteal angioplasty is limited, but the clinical benefit is acceptable because the limb salvage rate of 82% at 3 years is not inferior to that of surgical revascularisation, which underlines that limb salvage does not only depend on patency rates. Hence both patency rates and clinical success should be assessed when evaluating a treatment in CLI patients. Secondary interventions are much more frequent after endovascular treatment of infrapopliteal arteries. Repeated angioplasty attempts, which are not always innocuous, have some advantages over repeat bypass grafting, which is troublesome and not always feasible.

Recommendations

Endovascular treatment of infrapopliteal arteries has the potential to achieve similar limb salvage rates with less procedural morbidity and mortality than surgical bypass. Angioplasty as the first-line therapeutic modality for patients with CLI and infrapopliteal lesion is reasonable in the majority of cases, considering that the interventional procedure should not preclude future surgical intervention. **(Level 4; Grade C)**

Surgical treatment should be considered for more complex anatomical lesions of BTK vessels or in case of endovascular failure and persisting clinical symptoms of CLI. **(Level 4; Grade C)**

Stenting

New endovascular techniques have been proposed to improve the results of plain angioplasty, including the use of bare metal stent (balloon-expandable and self-expanding stents), drug-eluting balloon and stent, cryoplasty, laser and atherectomy. The data for these new technologies still derive predominantly from a few small RCTs and from retrospective case series, with a limited number of patients and a relatively short clinical and instrumental follow-up.

Although the first use of stents for infrapopliteal lesions was reported more than 15 years ago,[129] several concerns have been raised regarding their utilisation with respect to the risks of stent fracture, restenosis, thrombosis, and the possibly limited role of a focally acting endoprothesis in a diffusely diseased vessel.

Balloon-expandable stent

Fering et al.[130] were the first to demonstrate the safety and utility of primary stenting of infrapopliteal lesions using coronary stents, in a large retrospective series.

The first RCT on the topic was the InPeria trial published by Rand et al.[131] The trial was a European multi-centre randomised study that investigated carbon-coated stents (a 0.014-inch coronary balloon-expandable stent with a thin coating of 0.5nm of polycrystalline carbon film to prevent thrombus formation) vs. balloon angioplasty in the infrapopliteal arteries. A total of 51 patients, with 95 lesions, were enrolled (PTA: 53 lesions in 27

patients; stent: 42 lesions in 24 patients). Inclusion criteria were isolated stenosis greater than 70% or occlusion of the tibial arteries, up to three lesions; and lesions up to 3 cm with a cumulative lesion length of 9 cm. Follow-up evaluation was performed with intra-arterial and/or CT angiography at 6 months by two double-blind observers. For the stent group, the cumulative primary patency at 6 months was 83.7% (70% restenosis threshold) and 79.7% (50% restenosis threshold). For PTA, the primary patency at 6 months was 61.1% (70% restenosis threshold) and 45.6% (50% restenosis threshold) ($p < 0.05$). Although the results at 6 months were superior in the stent group, 9-month clinical follow-up of an extended 88-patient group (InPeria II; 45 limbs with 69 lesions treated with PTA vs. 44 limbs with 62 lesions treated with Carbostents) showed similar levels of clinical improvement and limb salvage (96% vs. 91%, respectively) and similar 1-year patency rates of about 60%.[132] Until now, longer follow-up data have not been available.

Other authors reported positive results of primary stenting using coronary balloon-expandable drug-eluting stents for infrapopliteal disease in small, non-randomised, single-centre studies.[133-137] These studies show favourable clinical results for drug-eluting stents in the early follow-up period, with significantly higher angiographic patency and less clinically driven re-interventions compared to simple angioplasty or bare-metal stent. However, these results have to be interpreted with caution because these studies were small in size and had limited follow-up. Notably most of these studies were industry-sponsored.

More recently, data from three RCTs comparing new solutions for infrapopliteal atherosclerotic disease have been presented at international meetings[138] (although manuscript publications are still awaited). These trials have found that drug-eluting stents are superior to angioplasty, or bare metal stents, in below-the-knee revascularisation. In particular, the ACHILLES trial has shown better primary patency for sirolimus-eluting balloon-expandable stents in the infrapopliteal region compared to balloon angioplasty, and the YUKON and DESTINY trials have shown a similar benefit for drug-eluting stents below the knee (respectively, a sirolimus-eluting stent and an everolimus-eluting stent) as compared to a bare metal stent. However, no significant difference in limb salvage rate was observed.

Despite the encouraging results from these RCTs for drug-eluting devices in infrapopliteal vessels, it should be noted that several inclusion criteria present in the protocols restricted study enrolment to patients with limited manifestations of tibial atherosclerotic disease, including patients with claudication (Rutherford 3) and excluding patients with severe tissue loss (Rutherford 6).

Complete longer follow-up data including clinical endpoints and wound healing assessments are expected to be published in the near future.

Self-expanding nitinol stents

The main restrictions of currently available bare or drug-eluting balloon-expandable stent platforms for BTK vessels are the small lengths available and the vulnerability to external compression (especially in the distal third of the anterior and posterior tibial artery). This is the reason why the majority of available studies are limited to short focal infrapopliteal

lesions up to 3 cm, which are not representative of typical long BTK lesions.
Long, thin-strut, low-profile, self-expanding nitinol stents designed and engineered specifically for the infrapopliteal arteries are now commercially available, but clinical data are still limited to small non-randomised studies.[139-141]
One RCT designed to compare PTA vs. self-expanding stent (The XXS – Balloon Angioplasty Versus Xpert Stent in CLI Patients) in patients with infrapopliteal lesions has recently completed the recruitment (180 CLI patients and a maximum of 2 arteries with a maximum lesion length of 150 mm). Interesting data about subjects with very long BTK lesions (which might better reflect real-world cases) are expected shortly.
A collaborative systematic review and meta-analysis[142] of clinical studies focusing on BTK stenting in patients with CLI identified 18 non-randomised studies including more than 600 patients. Data showed that bailout stenting of BTK vessels, performed with either balloon-expandable or self-expanding stents for suboptimal balloon dilation, was associated with satisfactory results up to a median of 12 months after treatment: binary in-stent restenosis occurred in 25.7% (95%CI 11.6–40.0%), primary patency in 78.9% (95%CI 71.8–86.0%), improvement in Rutherford class in 91.3% (95%CI 85.5–97.1%), target vessel revascularisation in 10.1% (95%CI 6.2–13.9%), and limb salvage in 96.4% (95% CI 94.7–98.1%). Subanalyses focusing on device type showed that balloon-expandable and self-expanding stents avoiding joint segments or pedal vessels perform similarly at early and midterm follow-up. In addition, the available data suggest superiority of sirolimus-eluting stents in comparison to bare metal stents in terms of primary patency and need for re-revascularisations.

Recommendation
Short, focal infrapopliteal lesions can be treated by drug-coated or drug-eluting stents, with improved patency rate. **(Level 2b; Grade B)**

Bioabsorbable stent
The possibility of not having a permanent metallic implant (bioabsorbable stent scaffold technology) has emerged as an exciting technology to combine mechanical prevention of vessel recoil with the advantages of long-term perspective. The bioabsorbable stent could permit the occurrence of positive remodelling with lumen enlargement to compensate for the development of intimal hyperplasia or new lesions.
The first published data with coronary application of an absorbable polymeric everolimus-eluting stent were very promising,[143] revealing a nearly complete elimination of both intimal hyperplasia and the need for re-interventions at 1 year.
Unfortunately, the same promising results have not been validated for BTK vessels. The prospective multi-centre randomised trial investigating infrapopliteal absorbable magnesium stents (AMS) vs. angioplasty (AMS-INSIGHT 1 trial)[144] indicated that the AMS technology can be safely applied, but it did not demonstrate efficacy regarding long-term patency over standard PTA in the infrapopliteal vessels. Data from 117 patients (147 CLI limbs) showed significantly higher binary restenosis rate at 6 months (68% vs. 42%,

p = 0.01) with a rate of lumen loss that was nearly doubled (1.4 vs. 0.7 mm, p = 0.001). It should be noted that the AMS stent was not drug-eluting.

Recommendation
The current-generation absorbable metal stent does not show superiority in long-term patency over standard PTA in infrapopliteal vessels. Reliable stent design modifications are required, and further clinical trials should be performed before potential widespread application of the technology. **(Level 1b; Grade B)**

Drug-eluting balloon
The concept of using a balloon catheter to directly deliver an antirestenotic drug at the site of arterial disease is of paramount interest. The plan to reduce the risk of restenosis without irreversibly modifying the structure of the vessel is a new interesting perspective, but limited clinical data are available.
Two different paclitaxel-coated balloon catheter systems are currently being compared to standard uncoated PTA balloon catheter for treatment of infrapopliteal lesion in a randomised fashion (INPACT-DEEP trial, PICCOLO trial, EURO CANAL trial).
Various angiographic and clinical efficacy measures will be evaluated to study whether paclitaxel-coated PTA balloons effectively inhibit restenosis of BTK arteries. Additionally, safety and tolerance of the drug-eluting device will be evaluated. No preliminary data are available.
It is likely that in the near future, the extent of the use of drug-coated balloons for BTK vessels in daily practice will be driven by the proof of their efficacy in reducing the restenosis rate and by the limitations of other available techniques. However, the clinical effectiveness of the drug-eluting balloons should be of crucial importance in deciding whether or not to opt for the device.

Recommendation
Drug-eluting balloon angioplasty is a promising technology for patients with CLI and infrapopliteal vessel lesions. However, prior to widespread clinical implementation, the results of pilot studies should be confirmed by RCTs with short- and long-term follow-up. **(Level 4; Grade D)**

NON-RECONSTRUCTIVE OPTION IN CLI

Lumbar sympathectomy
Lumbar sympathectomy can be performed both chemically and surgically. Studies directly comparing lumbar sympathectomy to a conservative treatment in CLI patients are limited. The small number of randomised trials that have been conducted failed to show beneficial effects on hard endpoints like amputation rate, mortality or ankle-brachial pressure index.[145, 146] However, lumbar sympathectomy has a beneficial effect on subjective endpoints, such

as relief of rest pain. The latter has consistently been confirmed in multiple cohort studies. These studies also suggest enhanced ulcer healing. Chemical and surgical lumbar sympathectomy seem to perform equally well[147] and can also be beneficial in diabetic patients.[148]

Recommendation
Lumbar sympathectomy, either surgical or chemical, should not be considered an option to prevent amputation. However, chemical lumbar sympathectomy can be considered in CLI patients not amenable to revascularisation in order to relieve symptoms. **(Level 2a; Grade B)**

Spinal cord stimulation (SCS)

Spinal cord stimulation involves a technique where an implanted pacemaker activates the dorsal columns of the spinal cord with an epidural lead. Early studies have reported a potential role for the device in limb salvage in patients with CLI. However, a recent meta-analysis of randomised trials that have studied the effects of SCS failed to show a beneficial effect on amputation rate or mortality.[149] It has been suggested that some subgroups could potentially benefit from SCS, but this could not be confirmed in the meta-analysis. Most randomised studies showed pain relief in the group treated with SCS compared to standard care.[150] However, complication rates are considerable (12%) and treatment costs are high.[149, 151]

Recommendation
Evidence is insufficient to recommend spinal cord stimulation in the treatment of CLI. **(Level 1a; Grade A)**

Gene and cell therapy

Regenerative medicine has raised much interest as a potential therapeutic strategy in patients with peripheral arterial disease, especially critical limb ischaemia. Both angiogenic gene and cell therapy have been studied in clinical context after promising results in animal experiments. Early piloting trials have been carried out for different gene-based therapies involving vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF), and showed promising results. The subsequently performed larger trials have generally failed to confirm the promising findings of the pilot trials, therefore gene therapy is still confined to research settings.[152] For example, the large TAMARIS trial randomised 525 patients with CLI unsuitable for revascularisation to treatment with non-viral FGF1 or placebo (8 intramuscular injections in the ischaemic leg, four times with 2-week intervals).[153] The trial could not prove that FGF is effective in reducing major amputation or death and amputation in these patients.

Studies that investigate the potential use of cell-based therapies in CLI are very heterogeneous, with varying amounts of cells administered, different administration routes, different cell sources and cell types used. Recently, Fadini et al.[154] performed a meta-analysis of clinical studies using cell-based therapies in patients with peripheral arterial

disease. These studies almost invariably show improvement of both objective and subjective endpoints; however, conclusions based on these studies are largely limited by the small size and mainly non-randomised design of these studies. Large randomised placebo-controlled trials focusing on clinically relevant endpoints are needed to confirm the promising results and to clarify the remaining questions surrounding cell therapy, such as preferred administration route and cell source.

Recommendation

There are data to suggest promising potential of cell-based therapies in patients with CLI. However, prior to widespread clinical implementation, the results of pilot studies should be confirmed by large-scale randomised placebo-controlled trials. Until then, both cell and gene therapy should be confined to the research setting. **(Level 5; Grade D)**

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PART ONE

Current treatment of critical limb ischemia and rationale for novel strategies

CHAPTER 4

Quality of life in patients with no-option critical limb ischemia underlines the need for new effective treatment

Journal of Vascular Surgery. 2010 Oct;52(4):843-9

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ABSTRACT

Objective

To provide a solid baseline reference for quality of life (QoL) in no-option critical limb ischemia (CLI) patients.

Summary Background Data

CLI is associated with surgery, endovascular interventions, hospitalization, and a poor prognosis. An increasing number of clinical trials is therefore investigating new treatment strategies (eg, therapeutic neovascularization) in CLI patients. QoL serves as an important secondary endpoint in many of these trials, but solid reference QoL data for no-option CLI patients are lacking.

Methods

The SF-36 and EQ-5D questionnaires were used to obtain baseline QoL scores from 47 no-option CLI patients participating in a therapeutic neovascularization trial. To allow for easy comparability a norm-based scoring (NBS) method was used to report the results of the SF-36. Scores of CLI patients were furthermore compared with scores of patients with milder forms of PAD and with patients with cardiovascular risk factors only. Determinants of Qol in PAD patients were identified using multiple linear regression methods.

Results

No-option CLI patients reported QoL scores below the general population mean on every health dimension of the SF-36. Physical Functioning, Role Physical Functioning, and Bodily Pain were affected most intensively. These poor physical QoL scores were further underlined when compared with other patients with milder forms of PAD or patients with cardiovascular risk factors only. CLI patients scored poorly on the pain/discomfort and the usual activities domains of the EQ-5D. Diabetes, female sex, body mass index, and the ankle-brachial index at rest were significant determinants of the QoL in PAD on multivariate analysis.

Conclusions

The QoL data of no-option CLI patients using NBS methods for the SF-36 provide a baseline reference for ongoing clinical trials on new treatment strategies. Our data stress the need for new revascularization therapies in no-option CLI patients.

INTRODUCTION

Critical limb ischemia (CLI), defined as chronic rest pain or tissue necrosis as a result of progressing peripheral arterial disease (PAD), is an important health issue. Yearly, CLI will develop in 500 to 1000 patients in a Western population of 1 million people.[1] It is associated with surgery, hospitalization,[2] and death, with 5-year survival rates of 50% or less being reported.[3, 4] Despite the rapid development of interventional techniques, therapeutic options for CLI patients remain limited: approximately 40% of CLI patients are not eligible for surgical or endovascular revascularization.[5, 6] No pharmacologic therapy has proven to be effective,[7] and ultimately, amputation is often the only option left. New therapies are therefore urgently needed. Angiogenic therapies by administration of growth factors or bone marrow-derived stem or progenitor cells appear to be promising treatment strategies and are currently being evaluated in clinical trials in no-option CLI patients.[8]

Besides the improvement in clinical status, improving the quality of life (QoL) of CLI patients has become an important treatment goal.[1] Qol is therefore considered to be an important secondary end point in these trials. This is particularly true because the assessment of vascular growth as an end point for angiogenic studies remains difficult.[9] To enable optimal evaluation of these trials and comparisons between patient populations and treatment strategies, standardized QoL assessments are important.

Between 1997 and 2008, 14 studies [10-23] reported reduced QoL of CLI patients using the Medical Outcomes Study Short Form 36 (SF-36) Health Survey. This QoL questionnaire is a widely accepted, reliable, and commonly used QoL assessment tool for various diseases [24] and is considered the most appropriate generic QoL assessment tool in patients with limb ischemia.[10] Comparisons of these studies of CLI patients with QoL data obtained in other disease states are hampered, however, by differences in patient populations and inconsistent reporting of the results.

To improve comparability with the same underlying condition as well as with other diseases, a norm-based scoring (NBS) method to evaluate SF-36 questions has been developed.[24] The NBS method provides scores that are standardized in a way that mean general population scores are the same (50) for every dimension, thereby serving as a reference. Furthermore, the NBS method considers two additional summary scores, the physical component score (PCS) and the mental component score (MCS), which allow independent assessment of the effect of a condition on the physical and mental aspects of QoL.

To date, two studies have used the NBS method to report the QoL in CLI patients. One study reported separate baseline NBS scores in 18 CLI patients who underwent angioplasty.[18] The second study only reported the summary scores of the NBS method in 452 patients with CLI who were randomized to bypass surgery or balloon angioplasty.[17] Other studies have reported QoL scores using conventional, untransformed SF-36 scores. Equally sparse are studies reporting the QoL in PAD and CLI patients measured by the EuroQol-5D (EQ-5D), another widely accepted QoL questionnaire. No studies addressing QoL in CLI patients have focused on CLI patients without any options for revascularization. The aim of this study was to present QoL scores in no-option CLI patients using two well-

established QoL questionnaires, the SF-36 and the EQ-5D. The SF-36 was scored using the NBS method, thereby introducing the easily interpretable PCS and MCS. With the increasing number of studies investigating new experimental therapies in these no-option patients, NBS scores in this population could serve as a useful baseline reference. In addition, we placed the QoL scores obtained in the no-option CLI patients in perspective with scores obtained in patients with milder forms of PAD and cardiovascular risk factors only. We also identified patient characteristics associated with QoL in PAD.

METHODS

QoL data were analyzed from (1) 47 patients with CLI, with no surgical or endovascular options for revascularization, who were included in the JUVENTAS trial; (2) 313 patients with milder PAD, defined as intermittent claudication (grade II of the Fontaine classification), participating in the SMART cohort; and (3) 1182 patients without manifest cardiovascular disease, but referred for the treatment of cardiovascular risk factors (RF), and for that reason included in the SMART cohort. Both studies are conducted in the Netherlands and patients originate from the same geographic area.

The JUVENTAS trial is an ongoing randomized, placebo-controlled, double-blind clinical trial (registered on clinicaltrials.gov under NCT00371371) that studies the effects of repeated intra-arterial infusion of bone marrow (BM) mononuclear cells in patients with unreconstructable CLI. Patients with a Fontaine classification of IIb, III, or IV, and an ankle-brachial index (ABI) of less than 0.6 or unreliable (noncompressible or not in proportion to the Fontaine classification), who are not suitable for conventional revascularization, are eligible for participation in this trial. Exclusion criteria are neoplasm or malignancy in the past 10 years, concomitant disease with life expectancy of less than 1 year, inability to obtain sufficient BM aspirate, known infection with human immunodeficiency virus or hepatitis B or C virus, and inability to complete follow-up. The present study used baseline QoL data obtained at inclusion of patients with CLI (Fontaine III or IV) included in this trial in the period between September 2006 and July 2009.

The SMART study is an ongoing, prospective, single-center cohort study of patients with clinically manifest vascular disease or cardiovascular risk factors only. The rationale and design of the SMART study have been described in detail elsewhere.[25] The SMART study population comprises patients newly referred because of manifest arterial disease or cardiovascular risk factors. All patients undergo a detailed examination at baseline, are followed up with regard to their cardiovascular risk, and complete QoL questionnaires at inclusion. Our study used baseline data from patients included in the SMART study in the period between September 1996 and March 2007.

Questionnaires

The SF-36 consists of 35 items covering eight health dimensions and an additional item that assesses the change in general health status over time. The scores on each health

dimension are summed and transformed on a scale from 0 (worst health) to 100 (best health).[24] The SF-36 questions were additionally scored using the NBS method.[24] NBS scores for each of the eight health dimensions were obtained by first standardizing the score on each health dimension by subtraction of the population mean of the score for that specific dimension and dividing the difference by the population's standard deviation (Z-score). A norm-based transformation was conducted by multiplying the product of the former calculation by 10 and adding 50 to this product.

Next, the Physical Component Summary (PCS) and Mental Component Summary (MCS) scores were calculated. Both scores are obtained by adding the Z-scores of the eight health dimensions, each multiplied by a specific weight factor.[24] This specific weight factor for each dimension is based on the impact of the dimension on either the PCS or MCS and is corrected for interactions between individual dimensions. The PCS and MCS thus take into account the correlations between the eight SF-36 dimensions and therefore reflect the effect of a condition on either the physical or mental component of health. To calculate the NBS score, the 1998 United States population norms were used, which have been used in a wide variety of conditions.[24]

Research has shown the EQ-5D is acceptable, valid, and reliable in a wide variety of diseases.[10, 26] The EQ-5D is composed of five items covering five domains of QoL, comprising mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. Problem severity on each of the five domains is rated as having "no problems" (level 1), "some problems" (level 2), or "extreme problems" (level 3). Combining the level of health problems on each domain results into 243 (3^5) possible health states (excluding death). Each health state was converted to a scale ranging from −1 to + 1, using the Preference-Based EuroQol Tariff reported by Dolan.[27]

We used a visual analogue scale (VAS) to measure the experienced pain across a continuum of values. The VAS was anchored between 0 (no pain at all) and 10 (unbearable pain).

Data analysis

Data were analyzed using SPSS 15.0.1 software (SPSS Inc, Chicago, Ill). For the SF-36, missing items (1% of the total items) were imputed if at least 50% of the items had been completed for each health dimension. The missing items were replaced by the mathematic mean of the residual items of that specific health dimension.[24] One-way analysis of variance was used in conjunction with a Bonferroni post hoc analysis to test for differences between continuous baseline characteristics, QoL dimensions, and summary scores for CLI, PAD, and cardiovascular risk factor patients. The Fisher exact test was used for categoric differences. Correspondence of different dimensions of the SF-36 and EQ-5D, and of QoL dimensions considering pain and the VAS pain score, were tested using bivariate correlation and reported as the Pearson correlation coefficient.

To identify factors associated with QoL in PAD, CLI and PAD patients were analyzed as one patient group. We also performed stratified analyses for age and gender. Therefore patients were age stratified into two groups (above and below the median) and additionally stratified by gender. Patient characteristics potentially associated with QoL dimensions

were selected based on clinical judgement and previous reports.[28, 29] Relationships between these factors and the PCS and MCS were tested for normality, linearity, and homoscedasticity before multivariate linear regression (MLR) was conducted. Factors with a value of P <.10 on univariate analysis were selected for the MLR analysis. Statistical significance was set at P <.05. Mean data are presented with the 95% confidence interval (CI).

RESULTS

Patient demographics

The CLI group (JUVENTAS participants) had a mean age of 65.2 ± 11.4 years and 70% were men. By Fontaine grade, 47% were at grade III and the rest were at grade IV. Cardiovascular risk factors were abundantly present: 92% smoked or were currently smoking, 59% had a history of hypertension, 75% had hypercholesterolemia, and diabetes was present in 33%. SF-36 data were available for 46 of the 47 patients and EQ-5D data for all patients.

Table 1 reports the baseline characteristics of all patients. JUVENTAS patients were significantly older, underwent more revascularizations, had sustained more myocardial infarction, and had a lower resting ankle-brachial index (ABI). All patients with milder PAD (SMART patients) were at Fontaine grade II, whereas none of the SMART patients with risk factors only were at Fontaine grade II, III or IV.

Patients with CLI have poor physical QoL

Patients with CLI demonstrated low scores for every dimension of the SF-36 (Figure 1 and Table 2). Mean NBS scores were well below 50 (general population mean). Worst scores were obtained for the physical health domains, including Physical Functioning, Role Physical Functioning, and Bodily Pain. This poor physical health is also reflected by the low mean PCS score of 30.8 (95% CI, 28.8-32.9). The Physical Functioning score of CLI patients was two standard deviations below the general population mean, indicating very severe problems. No differences for SF-36 NBS scores were observed between patients with CLI at Fontaine grade III or IV (data not shown).

Table 1 Patient characteristics.

Data are number (%) in case of categorical variables and mean (SD) in case of continuous variables. BP, Blood pressure, CABG, coronary artery bypass graft, ABI, ankle-brachial index, HDL, high-density lipoprotein; LDL, low-density lipoprotein; VAS, visual analog scale; RF, risk factors . [a] Significant difference with JUVENTAS (P<.05). [b] Significant difference between PAD and RF (P<.05). [c] LDL-cholesterol calculated using Friedewald calculation. [d] Data not available for SMART-patients.

Table 1 Patient characteristics.

	JUVENTAS (n=47)	PAD (n=313)	RF (n=1182)
Male sex	33 (70%)	206 (66%)	632 (54%) [b]
Age (mean [SD])	65.2 (11.4)	58.5 (10.7)[a]	47.6 (13.3)[a,b]
Smoking status:			
Never smoked	4 (8%)	29 (9%)	438 (37%)[a,b]
Current smoker	13 (28%)	127 (41%)[a]	274 (23%) [b]
Former smoker	30 (64%)	157 (50%)[a]	468 (40%)[a,b]
Diabetes	15 (33%)	77 (25%)	338 (29%)
History of hypertension	26 (59%)	184 (59%)	737 (62%)
Systolic BP (mmHg, mean [SD])	143.0 (22.1)	146.8 (21.3)	145.9 (22.3)
History of hypercholesterolemia	33 (75%)	189 (60%)	782 (68%)
Lipids (mmol/l, mean [SD]):			
Total cholesterol	4.5 (1.3)	5.3 (1.2)[a]	5.8 (1.6)[a,b]
HDL-cholesterol	1.3 (0.5)	1.3 (0.4)	1.4 (0.5) [b]
LDL-cholesterol c	2.5 (1.0)	3.2 (1.0)[a]	3.5 (1.4)[a,b]
Triglycerides	1.7 (1.1)	1.9 (1.3)	2.2 (3.0)
Body Mass-Index (kg/m2, mean [SD])	25.2 (4.0)	26.5 (4.0)	27.2 (5.3)[a]
Hyperhomocysteinemia	14 (30%)	36 (11.5%)[a]	93 (8%)[a]
Homocysteine (µmol/l, mean [SD])	16.1 (6.1)	13.0 (4.8)	12.2 (11.0)[a]
Creatinin (µmol/l, mean [SD])	103.0 (42.9)	94.6 (63.0)	84.2 (20.5)[a,b]
Medical history:			
Previous revascularization	39 (83%)	26 (8.3%)[a]	0 (0%)[a,b]
Myocardial infarction or angina	19 (40%)	46 (14.7%)[a]	0 (0%)[a,b]
CABG	10 (21%)	[d]	[d]
Stroke	5 (11%)	14 (4.5%)	0 (0%)[a,b]
Transient ischemic attacks	5 (11%)	[d]	[d]
End Stage Renal Disease	1 (2%)	[d]	[d]
Fontaine classification:			
Stage IIA/B	0 (0%)	313 (100%)[a]	0 (0%)[a]
Stage III	22 (47%)	0 (0%)[a]	0 (0%)[a]
Stage IV	25 (53%)	0 (0%)[a]	0 (0%)[a]
ABI at rest (mean [SD])	0.47 (0.33)	0.75 (0.22)a	1.14 (0.12)[a,b]
Use of analgesics:		[d]	[d]
None	14 (30.4%)		
Paracetamol	6 (13.0%)		
Non steroidal anti-inflammatory drugs	2 (4.3%)		
Opioids	24 (52.2%)		
Pain free walking distance (m, mean [SD])	55.6 (52.2)	treadmill test not performed	
Maximum walking distance (m, mean [SD])	146.5 (73.0)	treadmill test not performed	

On the EQ-5D, CLI patients scored worst on the subscales Pain/Discomfort (0.0%, 63.8%, and 36.2% scoring level 1, 2, and 3, respectively) and Usual Activities (10.6%, 61.7%, and 27.7% scoring level 1, 2, and 3, respectively). The subscales Bodily Pain and Mental Health of the SF-36 and Pain/Discomfort and Anxiety/Depression of the EQ-5D were highly corresponding ($r^2 = 0.549$ and $r^2 = 0.729$, respectively; $P < .05$). The subscales Bodily Pain of the SF-36 and Pain/Discomfort of the EQ-5D both correlated with the VAS pain score (mean, 5.1; 95% CI, 4.4-5.8) at $r^2 = 0.454$ and $r^2 = 0.398$, respectively ($P <.05$).

QoL in CLI patients is worse than in PAD and RF

No-option CLI patients showed significantly lower QoL scores than PAD patients, especially with regard to the physical domains (Figure 2, Table 3). This was confirmed by the significantly lower PCS (mean difference, –6.8; 95% CI, –9.9 to –3.7) for CLI patients. The PCS also showed a decreasing trend with increasing disease severity: the means for PCS were 30.8 (95% CI, 28.8-32.9), 37.6 (95% CI, 36.5-38.8), and 48.4 (95% CI, 47.8-49.0) in patients with CLI, PAD, and cardiovascular risk factors, respectively. The MCS for these three groups were 45.9 (95% CI 41.4-50.4), 51.4 (95% CI, 50.2-52.7), and 47.8 (95% CI, 47.1-48.4), respectively.

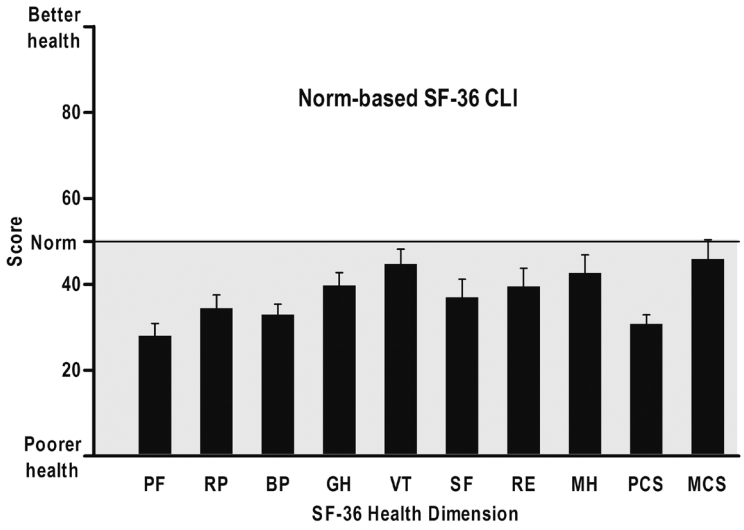

Figure 1 Mean values of quality of life indices and 95% confidence intervals (error bars) for 46 patients with critical limb ischemia (CLI) using the norm-based scoring (NBS) method. The general population norm is 50, as indicated, for every dimension. PF, Physical Functioning; RP, Role-Physical; BP, Bodily Pain; GH, General Health; VT, Vitality; SF, Social Functioning; RE, Role-Emotional; MH, Mental Health; PCS, Physical Component Summary Score; MCS, Mental Component Summary Score

Table 2 Mean SF-36 (n=46) and EQ-5D (n=47) values for CLI patients.

	Scoring method		EQ-5D			
SF-36 Health				**Frequency of scores (%)**		
Dimension	**SF-36 NBS**	**SF-36 CS**	**Dimension**	**1**	**2**	**3**
Physical Functioning	28.0 (25.2-30.9)	30.6 (23.8-37.5)	Mobility	0.0%	95.7%	4.3%
Role Physical	34.4 (31.3-37.5)	22.8 (11.9-33.8)	Self-Care	68.2%	27.3%	4.5%
Bodily Pain	32.9 (30.5-35.4)	30.4 (24.7-36.1)	Usual Activities	10.6%	61.7%	27.7%
General Health	39.7 (36.7-42.7)	48.1 (41.7-54.6)	Pain/Discomfort	0.0%	63.8%	36.2%
Vitality	44.8 (41.4-48.2)	46.0 (38.8-53.1)	Anxiety/Depression	50.0%	34.8%	15.2%
Social Functioning	36.9 (32.7-41.2)	53.5 (43.6-63.3)				
Role Emotional	39.5 (35.2-43.7)	49.8 (36.3-63.2)				
Mental Health	42.6 (38.4-46.8)	62.3 (54.9-69.6)				
PCS	30.8 (28.8-32.9)	[a]				
MCS	45.9 (41.4-50.4)	[a]	Overall Score	0.34 (0.24-0.44) [b]		

Values are mean (95% confidence interval). EQ, Euro-Qol; SF-36, Short Form 36; NBS, norm-based scoring; CS, conventional scoring; PCS, Physical Component Score; MCS, Mental Component Score. [a] PCS and MCS only calculable with NBS. [b] Tariff score by Dolan. Conventional scoring of SF-36 are untransformed scores and provided to facilitate comparison with studies not using norm-based scoring. Conventional scores range from 0 to 100.

Table 3 SF-36 scores PAD versus CLI.

SF-36 Health Dimension	**SF-36 NBS**	
	PAD (n=313)	**CLI (n=46)**
Physical Functioning	36.4 (35.3-37.4)	28.0 (25.2-30.9)[a]
Role Physical	41.4 (40.1-42.8)	34.4 (31.3-37.5)[a]
Bodily Pain	43.1 (41.9-44.3)	32.9 (30.5-35.4)[a]
General Health	41.5 (40.5-42.5)	39.7 (36.7-42.7)
Vitality	48.9 (47.8-50.0)	44.8 (41.4-48.2)[a]
Social Functioning	43.7 (42.4-45.0)	36.9 (32.7-41.2)[a]
Role Emotional	48.4 (47.1-49.7)	39.5 (35.2-43.7)[a]
Mental Health	48.1 (46.9-49.2)	42.6 (38.4-46.8)[a]
PCS	37.6 (36.5-38.8)	30.8 (28.8-32.9)[a]
MCS	51.4 (50.2-52.7)	45.9 (41.4-50.4)[a]

Values are mean (95%CI). NBS Norm-based scoring; PCS, Physical Component Score; MCS, Mental Component Score. [a] Significant difference between Fontaine II and III/IV

QoL in CLI and PAD correlates with nonmodifiable risk factors

Analyzing the 47 CLI and 313 SMART-PAD patients together resulted in 360 patients with any degree of PAD. When stratified by age and gender no significant differences were found for the PCS. The MCS was significantly lower for the older females. Univariate

analysis showed that older age, female sex, previous revascularization, history of myocardial infarction, history of stroke, higher BMI, previous or current smoking, hyperhomocysteinemia, hypertension, hypercholesterolemia, diabetes, higher serum creatinine level, lower ABI at rest, and higher PAD staging by Fontaine classification were associated with lower PCS scores. On multivariate linear regression analysis, a history of diabetes, female sex, a higher BMI, and a lower ABI remained as significant determinants of a reduced PCS score (adjusted model $R^2 = 0.137$). No relation was found between patient characteristics and the MCS score.

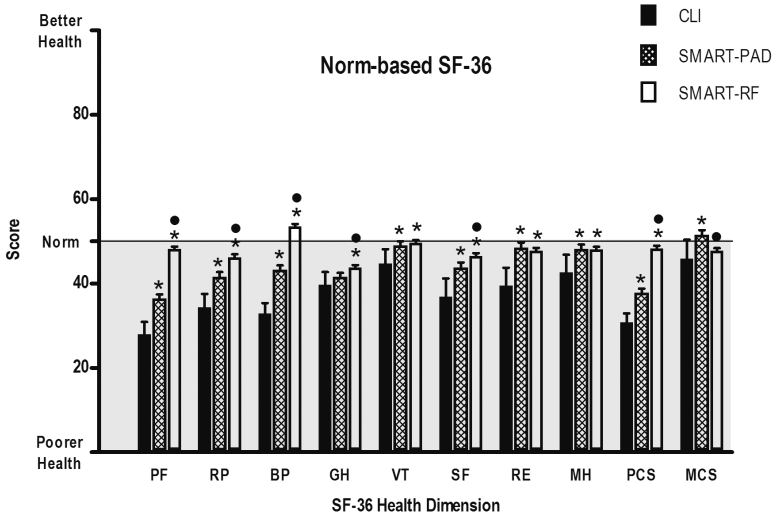

Figure 2 Mean values of quality of life indices and 95% confidence intervals (error bars) for 46 critical limb ischemia (CLI) patients compared with patients with peripheral arterial disease (PAD) or cardiovascular risk factors (RF) only. The general population norm is 50, as indicated, for every dimension. PF, Physical Functioning; RP, Role-Physical; BP, Bodily Pain; GH, General Health; VT, Vitality; SF, Social Functioning; RE, Role-Emotional; MH, Mental Health; PCS, Physical Component Summary score; MCS. Mental Component Summary score. * Significant difference JUVENTAS vs PAD and RF ($P < .05$), • Significant difference PAD versus RF ($P < .05$)

DISCUSSION

Using the NBS method, we show that no-option patients with CLI have worse outcomes in essentially every SF-36 domain of QoL compared with patients with milder forms of PAD (Fontaine II) and cardiovascular risk factors only. Multivariate linear regression showed that the QoL in patients with PAD mainly depends on gender and other factors such as diabetes, BMI, and resting ABI that cannot be easily modified by therapeutic interventions.

Poor QoL of patients with CLI has been reported in several publications in the past decade using the SF-36.[10-23] We used the norm-based scoring (NBS) method for the SF-36. The norm-based transformation of the scores to general population means allows comparison of the physical and mental components of the health of patients with similar as well as other underlying conditions. The NBS method has been used in a wide variety of physical and mental conditions,[24, 30, 31] but its use in PAD or CLI patient populations has been limited. To our knowledge, our study provides the first NBS data on QoL in no-option patients with CLI, which thereby may provide a baseline reference for this patient group. Using this standardized methodology may improve evaluation of the many ongoing clinical studies on angiogenic therapies by enabling comparisons between patient populations. Furthermore, our data show that the PCS in our population of no-option patients with CLI (score, 30.8) is significantly worse than scores previously obtained in patients with cancer (score, 41.1), chronic heart disease (score, 39.4), and chronic kidney disease (score, 36.8),[24] underlining the need for improved treatment of these patients.

We identified gender, diabetes, BMI, and resting ABI as factors associated with QoL (i.e., PCS) in PAD, of which gender cannot be modified and the others are not easily modified. Although BMI may be influenced to some extent by therapeutic intervention, long-term weight reduction seems hard to achieve,[32] and functional limitations in patients with CLI make this particularly difficult in these patients. The association between QoL and resting ABI again underscores the importance of revascularization and thus the need for development of new treatment strategies in these no-option patients.

Despite analgesia use in 70% of the CLI patients, they had a particularly low PCS score, with Bodily Pain as one of the physical QoL factors that is strikingly poor (mean, 32.9; 95% CI, 30.5-35.4). This is consistent with the relatively high mean VAS pain score of 5.1 (95% CI, 4.4-5.8) in our patients and in line with reports indicating that ischemic pain is hard to suppress.[33, 34] It shows that pain management is essential in improving QoL, which may require a multidisciplinary approach in patients who do not have the option of revascularization.

In conclusion, this study provides QoL data in no-option patients with CLI using the NBS method for scoring the SF-36, hence providing a baseline reference for the increasing number of clinical trials in this patient group. The results of this study show that the physical component of health is exceptionally poor in these patients, with pain as important determinant. Our data stress the need for development of management strategies to avoid patients to reach a no option status. Moreover it underlines the need for new revascularizing treatment options in patients with CLI who currently have no options for surgical or endovascular revascularization.

APPENDIX

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PART TWO

Bone marrow-derived cell therapy in critical limb ischemia

CHAPTER 5

Autologous bone marrow derived cell therapy in patients with critical limb ischemia: A meta-analysis of randomized controlled clinical trials

Ann Surg. 2013; 19 Feb Epub ahead of print

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ABSTRACT

Background

Critical Limb Ischemia (CLI) is the most advanced stage of peripheral arterial disease (PAD) and is usually treated with bypass surgery or endovascular revascularization. However, a considerable proportion of CLI patients is not eligible to these treatment strategies and amputation is often the only option left. In the past decade research has focused on bone marrow (BM) derived cell-based strategies that aim at neovascularization to improve limb perfusion. Individual studies did not convincingly prove efficacy of BM derived cell therapy in CLI patients thus far.

Objectives

Perform a meta-analysis of all randomized controlled trials (RCTs) available that studied BM derived cell therapy compared to standard care with or without placebo in CLI patients and provide summary efficacy data on this approach.

Methods

A systematic search in the electronic databases of Medline, Embase, and the Cochrane Controlled Trials Register was performed. All studies were critically appraised and data were extracted and meta-analyzed using a random-effects model. Major amputation and Amputation Free Survival (AFS) were considered as the primary endpoints.

Results

A total of 12 RCTs jointly including 510 CLI patients were identified and analyzed. The meta-analysis showed beneficial effects of BM derived cell therapy on both subjective and surrogate objective endpoints, i.e. pain score, pain-free walking distance, ankle-brachial index and transcutaneous oxygen measurements (all $P < 0.00001$). Overall the RCTs showed reduced amputation rates in the therapeutic arms of the included trials with an RR on major amputation of 0.58 (95% CI, 0.40 to 0.84; $P = 0.004$). However, when only the placebo-controlled RCTs were considered the beneficial effect on major amputation rates was considerably reduced and non-significant (RR 0.78; 95% CI, 0.40 to 1.51; $P = 0.46$). AFS did not significantly differ between the BM treated and the control group (RR 1.16; 95% CI, 0.92 to 1.48; $P = 0.22$).

Conclusions

This meta-analysis underlines the promising potential of BM derived cell therapy in CLI patients. Importantly, the results of placebo-controlled and non-placebo-controlled RCTs seem to diverge, which stresses the necessity to use placebo in the control arms of these trials. Future well-designed larger placebo-controlled RCTs are needed and should include long-term follow-up data in order to assess durability of treatment effects.

INTRODUCTION

Critical Limb Ischemia (CLI) is usually caused by atherosclerosis obliterans (ASO) or thrombangiitis obliterans (TAO). CLI is the most advanced stage of peripheral arterial disease (PAD) and is characterized by ischemic rest pain or tissue loss. Current treatment options for CLI consist of bypass surgery or endovascular revascularization. However, 25-40% of these patients are not eligible to such interventions because of anatomical reasons, such as poor outflow vessels, lack of suitable autogenous conduit, extensive comorbidities or previously failed revascularization attempts.[1,2] These so-called 'no-option CLI (NO-CLI) patients' have a poor prognosis for both life and limb, a poor quality of life and their treatment is associated with very high costs.[1-4] Although a recent study shows that amputation free survival (AFS) and mortality rates in NO-CLI have improved over the past 2 decades, current one year AFS rates still remain in the range of 60-80%.[5] With an incidence of 500-1,000 new cases per million individuals in Western society CLI poses a considerable burden on patients, doctors and health care resources.[1]

In the past decade much research as well as commercial activity has focused on cell-based therapies for CLI that aim to improve limb perfusion by enhancing neovascularization using cells obtained from the bone marrow (BM). Since 2002, when Tateishi-Yuyama and colleagues[6] published the outcomes of the TACT trial that showed for the first time that autologous BM derived cell therapy could be safe and effective for achievement of therapeutic neovascularization in CLI, several small, non-controlled and non-blinded case series have reported positive effects of cell therapy on both subjective and objective surrogate endpoints, such as pain scores, pain-free walking distance, ankle-brachial index (ABI), and transcutaneous oxygen measurements (tcO2).[7,8] However, the lack of adequate, randomized controls precludes definitive conclusions on the efficacy of BM derived cell therapy in these studies. Recently, several larger randomized controlled trials (RCTs) have been published that evaluated hard and clinically relevant endpoints, especially amputation rates and amputation-free survival (AFS).[9-11] In this meta-analysis we critically appraise the quality of the available RCTs that studied BM derived cell therapy in CLI and summarize the overall clinical efficacy of this treatment modality in CLI patients.

METHODS

Search Strategy

The present meta-analysis was performed according to PRISMA statements.[12,13] On February 24, 2012 we performed a search in the electronic databases of Medline, Embase, and the Cochrane Controlled Trials Register. A broad search strategy was adopted without applying particular search filters to identify all potential relevant articles (Box 1).

The eligibility criteria were clearly defined prior to performing the literature search (Figure 1). RCTs were included exclusively in order to provide sound conclusions regarding the therapeutic effect of BM derived cell therapy in comparison with standard care with or

BOX 1. Search Syntaxes

Syntax "Medline"

("Peripheral arterial disease"[TIAB] OR PAD[TIAB] OR "peripheral arterial occlusive disease"[TIAB] OR PAOD[TIAB] OR "Critical limb ischemia"[TIAB] OR "Critical limb ischaemia"[TIAB] OR CLI[TIAB] OR "Severe limb ischemia"[TIAB] OR "Severe limb ischaemia"[TIAB] OR "arteriosclerosis obliterans"[TIAB] OR "thromboangitis obliterans"[TIAB] OR Buerger[TIAB] OR "Fontaine 3"[TIAB] OR "Fontaine 4"[TIAB] OR "Fontaine III"[TIAB] OR "Fontaine IV"[TIAB] OR "Rutherford 4"[TIAB] OR "Rutherford 5"[TIAB] OR "Rutherford 6"[TIAB] OR Amputation[TIAB] OR "Ischemic ulcer*"[TIAB] OR "Ischaemic ulcer*"[TIAB] OR gangrene[TIAB] OR necrosis[TIAB] OR "diabetic foot"[TIAB] OR "diabetic ulcer"[TIAB]) OR (BM[TIAB] OR "Bone marrow" [TIAB] OR BM-MNC[TIAB] OR BMMNC[TIAB] OR BMMC[TIAB] OR "Bone marrow mononuclear cell*"[TIAB] OR "bone marrow derived mononuclear cell"[TIAB] OR PB-MNC[TIAB] OR PBMNC[TIAB] OR PB-MC[TIAB] OR PBMC[TIAB] OR "Peripheral blood mononuclear cell*"[TIAB] OR "peripheral blood derived mononuclear cell*"[TIAB] OR "Stem cell*"[TIAB] OR "Progenitor cell*"[TIAB] OR cell[TIAB] OR cellular[TIAB] OR "Cell-based" [TIAB] OR "Cell based" [TIAB] OR "Neovascular*"[TIAB] OR angiogen*[TIAB] OR "Mesenchymal stem cell*"[TIAB] OR "Mesenchymal stromal cell*"[TIAB] OR MSC[TIAB]) AND (therapy[TIAB] OR therapies[TIAB] OR therapeutic*[TIAB] OR intervention[TIAB] OR infusion[TIAB] OR administration[TIAB] OR injection[TIAB] OR application[TIAB] OR treatment[TIAB] OR transplantation[TIAB]) AND (RCT[TIAB] OR randomis*[TIAB] OR randomiz*[TIAB] OR trial[TIAB] OR "placebo controlled"[TIAB] OR placebo-controlled[TIAB] OR placebo[TIAB] OR "clinical trial"[TIAB] OR "prospective study"[TIAB] OR "double-blind"[TIAB] OR "double blind"[TIAB] OR blinded[TIAB])

Syntax "Embase"

("Peripheral arterial disease":ti,ab OR PAD:ti,ab OR "peripheral arterial occlusive disease":ti,ab OR PAOD:ti,ab OR "Critical limb ischemia":ti,ab OR "Critical limb ischaemia":ti,ab OR CLI:ti,ab OR "Severe limb ischemia":ti,ab OR "Severe limb ischaemia":ti,ab OR "arteriosclerosis obliterans":ti,ab OR "thromboangitis obliterans":ti,ab OR Buerger:ti,ab OR "Fontaine 3":ti,ab OR "Fontaine 4":ti,ab OR "Fontaine III":ti,ab OR "Fontaine IV":ti,ab OR "Rutherford 4":ti,ab OR "Rutherford 5":ti,ab OR "Rutherford 6":ti,ab OR Amputation:ti,ab OR "Ischemic (ulcer OR ulcers)":ti,ab OR "Ischaemic (ulcer OR ulcers)":ti,ab OR gangrene:ti,ab OR necrosis:ti,ab OR "diabetic foot":ti,ab OR "diabetic (ulcer OR ulcers)":ti,ab) OR (BM:ti,ab OR "Bone marrow":ti,ab OR BM-MNC:ti,ab OR BMMNC:ti,ab OR BMMC:ti,ab OR "Bone marrow mononuclear (cell OR cells)":ti,ab OR "bone marrow derived mononuclear cell":ti,ab OR PB-MNC:ti,ab OR PBMNC:ti,ab OR PB-MC:ti,ab OR PBMC:ti,ab OR "Peripheral blood mononuclear (cell OR cells)":ti,ab OR "peripheral blood derived mononuclear (cell OR cells)":ti,ab OR "Stem (cell OR cells)":ti,ab OR "Progenitor (cell OR cells)":ti,ab OR cell:ti,ab OR cellular:ti,ab OR "Cell-based":ti,ab OR "Cell based":ti,ab OR Neovascular*:ti,ab OR angiogen*:ti,ab OR "Mesenchymal stem (cell OR cells)":ti,ab OR "Mesenchymal stromal (cell OR cells)":ti,ab OR MSC:ti,ab) AND (therapy:ti,ab OR therapies:ti,ab OR therapeutic*:ti,ab OR intervention:ti,ab OR infusion:ti,ab OR administration:ti,ab OR injection:ti,ab OR application:ti,ab OR treatment:ti,ab OR transplantation:ti,ab) AND (RCT:ti,ab OR randomis*:ti,ab OR randomiz*:ti,ab OR trial:ti,ab OR "placebo controlled":ti,ab OR placebo-controlled:ti,ab OR placebo:ti,ab OR "clinical trial":ti,ab OR "prospective study":ti,ab OR "double-blind":ti,ab OR "double blind":ti,ab OR blinded:ti,ab)

Syntax "Cochrane"

("Peripheral arterial disease" OR PAD OR "peripheral arterial occlusive disease" OR PAOD OR "Critical limb ischemia" OR "Critical limb ischaemia" OR CLI OR "Severe limb ischemia" OR "Severe limb ischaemia" OR "arteriosclerosis obliterans" OR "thromboangitis obliterans" OR Buerger OR "Fontaine 3" OR "Fontaine 4" OR "Fontaine III" OR "Fontaine IV" OR "Rutherford 4" OR "Rutherford 5" OR "Rutherford 6" OR Amputation OR "Ischemic ulcer*" OR "Ischaemic ulcer*" OR gangrene OR necrosis OR "diabetic foot" OR "diabetic ulcer*") OR (BM OR "Bone marrow" OR BM-MNC OR BMMNC OR BMMC OR "Bone marrow mononuclear cell*" OR "bone marrow derived mononuclear cell" OR PB-MNC OR PBMNC OR PB-MC OR PBMC OR "Peripheral blood mononuclear cell*" OR "peripheral blood derived mononuclear cell*" OR "Stem cell*" OR "Progenitor cell*" OR cell OR cellular OR "Cell-based" OR "Cell based" OR "Neovascular*" OR angiogen* OR "Mesenchymal stem cell*" OR "Mesenchymal stromal cell*" OR MSC) AND (therapy OR therapies OR therapeutic* OR intervention OR infusion OR administration OR injection OR application OR treatment OR transplantation) AND (RCT OR randomis* OR randomiz* OR trial OR "placebo controlled" OR placebo-controlled OR placebo OR "clinical trial" OR "prospective study" OR "double-blind" OR "double blind" OR blinded)

without sham injections. Each article retrieved was screened by two independent reviewers (M.T. and C.P.) to assess whether the article fulfilled the eligibility criteria. Divergence was solved by consensus. Cross-referencing was conducted to identify studies potentially missed in the initial search.

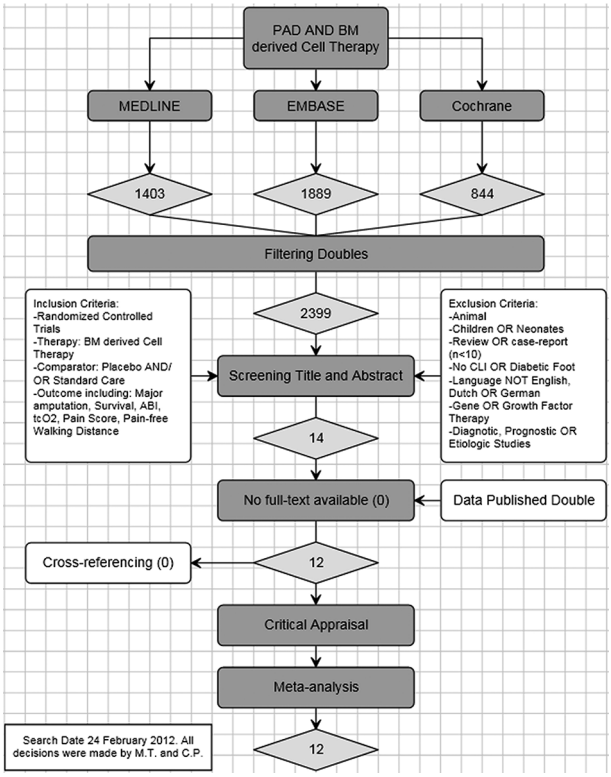

Figure 1 Flow Chart.

Critical Appraisal, Data Extraction and Data Management

Critical appraisal and quality assessment of included trials was performed by two reviewers independently (M.T. and C.P.) according to modified standardized guidelines for interventional research (Table 1). [14]

Primary outcomes of interest were incidence of major amputation and AFS. ABI, tcO_2, pain-free walking distance, pain score and ulcer healing, were considered secondary endpoints. Data available at last follow-up were extracted from the studies. If raw data on a specific endpoint were not specified but implemented in a graph or figure, data were extracted using GetData Graph Digitizer 2.24 (S. Fedorov). If studies reported standard error of the means (SE) instead of standard deviations (SD), SD was calculated with the assumption that data were distributed parametrically. If medians and interquartile range

were provided in the original publication means and SD were estimated using previous published methods.[15,16] Pain scales were transformed to a scale ranging from 0-4.

Statistical Analysis

All statistics in this meta-analysis were performed using Review Manager software version 5.1 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). To calculate overall treatment effects statistical and methodological heterogeneity among the selected studies was assumed and therefore a random-effects model was applied. Weighted mean difference (WMD) or relative risk (RR) with their respective 95% CIs, whichever applicable, were calculated to express the treatment effects. The absolute risk reduction (ARR) on amputation and number needed to treat (NNT) to prevent one amputation with their respective SD were estimated from the difference in amputation rates between

Table 1 Critical Appraisal.

Study	Method of randomization [1]	Method of allocation concealment [1]	Blinding of outcome assessors [2]	Loss to follow-up [3]	Patients treated in the assigned group [4]	Placebo-controlled [5]
Arai et al. 2006	○	○	●	●	●	○
Barć et al. 2006	○	○		●	●	○
Benoit et al. 2011	●	●	●	●	●	●
Dash et al. 2009	●	●		●	●	○
Lu et al. 2011	●	○	●	○	●	●
Lu Debin et al. 2008	○	○		●	●	○
Huang et al. 2005	○	○		●	●	○
Ozturk et al. 2012	●	○		●	●	○
Powell et al. 2012	●	●	●	●	●	●
Procházka et al. 2010	○	○		●	●	○
Tateishi-Yuyama et al. 2002	●	●	●	○	●	●
Walter et al. 2011	○	○	●	●	●	●

1) ● Clearly defined; ○ Inadequate/ Not reported
2) ● Double-blind; ○ Single-blind; Blank: Not blinded
3) ● <5%; ○ 5-10%; Blank>10%
4) ● All; ○ Cross-over
5) ● Placebo-controlled; ○ Standard Care

treatment and control group. We performed sensitivity analyses by repeating the main computations using a fixed-effect method. Funnel plots were visually inspected for small study effects or publication bias. A prespecified subgroup analysis for placebo-controlled trials with and without a separate control group was performed. Heterogeneity amongst the studies included in the analyses was explored using the X^2 test. Subsequently the inconsistency was quantified with the I_2 statistic, where I_2 values less than 25% representing mild inconsistency, between 25-50% moderate inconsistency and values over 50% are indicative of severe heterogeneity between the studies. *P*-values below 0.05 were considered to represent statistical difference.

RESULTS

Search Results and Study Characteristics

The intitial search retrieved 2399 articles after correction for citations appearing in more than one database (Figure 1). After screening based on exclusion criteria 142 articles were reviewed according to the inclusion criteria, which ultimately resulted in 14 studies that fulfilled both the in- and exclusion criteria. Of these 14 studies two [17,18] were excluded since they published interim data from patient populations also reported on in two other separate publications included in the meta-analysis. [9,10] Eventually twelve RCTs were included in the meta-analysis. [6,9-11,19-26]

Quality of trial design and potential risk for bias was assessed in the included studies (Table 1). There was a striking difference between trials regarding how rigorous they implemented methods to prevent potential bias, especially blinding of outcome assessors and use of placebo as a control was not frequently applied.

The 12 RCTs jointly included a total of 510 subjects, of which 301 were treated within the therapeutic arms of the trials (Table 2). Sample size in the individual RCTs was generally small with a maximum of 96 patients. Age, number of patients with Fontaine grade IV and patients with diabetes were equally distributed among the cell therapy and control group within the studies, however the amount of Fontaine grade IV and percentage of diabetic patients varied widely (25-100%) between the individual RCTs. Follow-up duration was 3 months or less in six of the included studies [11,19,21-24] and in only one study follow-up exceeded 6 months. [10] Importantly, only five out of the twelve included RCTs incorporated the application of placebo injections in their study protocol (jointly including 223 patients), [6,9-11,26] whilst the others compared the therapeutic effects with standard care.

The administered cell type and quantity as well as the administration route varied between the studies, 11 studies applied intramuscular infusions (IM) [6,9,10,19-26] whereas only one used the intra-arterial (IA) route routinely. 11 Six out of 12 studies used BM Mononuclear Cells (BMMNC) in the therapeutic arm, [6,9,11,19,20,25] two studies used BM Mesenchymal Stromal Cells (BMMSC), [21,22] two used G-CSF mobilized Peripheral Blood Mononuclear Cells (M-PBMNC) [23,24] and one RCT used Ixmyelocel-T, a commercial pre-expanded cell product obtained from BM. [10] Additionally, the RCT of Lu et al. applied a 3-armed trial design,

Table 2 Study Characteristics.

Study	Sample size*	Mean age*	%Fontaine IV *	% Diabetes*	Follow-up	Therapy	Control	Number of cells	Administration Route
Arai et al. 2006	13/12	62/68	38/58	38/42	1 Month	BMMNC	Standard Care	1-3 x 109	IM
Barć et al. 2006	14/15	NA	NA	NA	6 Months	BMMNC	Standard Care	NA	IM (+IA in 4)
Benoit et al. 2011	34/14	72.5/65.7	68/50	53/43	6 Months	BMMNC	Placebo	240mL BM	IM
Dash et al. 2009	12/12	40±10	100/100	25/25	12 Weeks	BMMSC	Standard Care	>1x106/cm2	IM + topical
Lu et al. 2011	21/41 limbs	63±8	100/100	100/100	24 Weeks	BMMNC	Placebo	9.3x108	IM
Lu et al. 2011	20/41 limbs	65±10	100/100	100/100	24 Weeks	BMMSC	Placebo	9.6x108	IM
Lu Debin et al. 2008	22/23	66.6/65.5	NA	100/100	12 Weeks	BMMSC	Standard Care	7.32 – 56.1x108	IM + SC
Huang et al. 2005	14/14	71.1/70.9	78/75	100/100	3 Months	M-PBMNC	Standard Care + IV prostaglandin E1	3x109	IM
Ozturk et al. 2012	20/20	71.9/70.8	45/40	100/100	12 Weeks	M-PBMNC	Standard Care	NA	IM
Powell et al. 2012	48/24	69.2/67.3	60/67	44/63	12 Months	Ixmyelocel-T	Placebo	35-295x106	IM
Procházka et al. 2010	42/54	66.2/64.1	100/100	88/98	120 Days	BMMNC	Standard Care	240mL BM	IM
Tateishi-Yuyama et al. 2002	22/22 limbs	69±11	70	65	24 Weeks	BMMNC	Placebo	0.88 – 2.8x109	IM
Walter et al. 2011	19/21	64.4/64.5	79/71	53/48	3 Months	BMMNC	Placebo	153±78x106	IA

* Therapeutic arm/Control Arm, *NA* Not Available, *BMMNC* Bone Marrow derived Mononuclear Cells, *BMMSC* Bone Marrow derived Mesenchymal Stromal Cells, *M-PBMNC* Mobilized Peripheral Blood Mononuclear Cells, *Ixmyelocel-T* Expanded cells obtained from *BMMNC, IM* Intramuscular, *IA* Intra-arterial, SC Subcutaneous

including a BMMNC, a BMMSC, and a placebo-arm.[26] In this trial patients with bilateral CLI were included and randomized to receive either BMMNC or BMMSC in one leg and normal saline in the other, which served as an internal control. The original publication did not report separate results for the placebo treated contralateral limbs of the BMMNC and the BMMSC groups, but used the contralateral saline treated limbs of both the BMMNC and BMMSC treated patients as a merged control group. To include the results of this RCT in the meta-analysis the study groups were stratified in a BMMNC, BMMSC, and the corresponding control group including the contralateral limbs of both groups, which resulted in double-counting of the control limbs. To evaluate potential influence of this double-counting on the ultimate results a re-analysis was performed wherein the number of observations in the control group were divided by the number of strata, which resulted in only minor changes of the summarized results. Therefore we opted to adhere as tight as possible to the original trial data, hence summary measures are presented with double-counting of the control group as has been done in former meta-analyses,[27,28] however in the detailed description on number of amputated limbs and ulcer healing the contralateral limbs were counted only once.

Meta-analysis and Overall Efficacy

Major amputation and AFS: Nine RCTs reported on major amputation rates in such a way that they could be incorporated in the meta-analysis (primary endpoint) (Figure 2A). Overall 38 of the 259 limbs treated within the therapeutic arm were amputated compared to 62 of the 232 limbs treated in the control group (single-counting of control limbs in RCT of Lu et al.). Overall BM derived cell therapy significantly reduced major amputation rates compared to patients included in the control groups with a RR of 0.58 (95% CI, 0.40 to 0.84; $P = 0.004$). No substantial heterogeneity was detected between the included studies ($P = 0.46$; $I^2 = 0\%$). The corresponding ARR was 12.1% (95% CI, 4.9 to 19.2) and the NNT to prevent one major amputation was 8 (95% CI, 5 to 20). AFS did not differ significantly between groups (Figure 2B), however only six of the 9 studies analyzed in the overall efficacy analysis on major amputation rates could be included in the AFS analysis since Huang et al, Lu et al, and Procházka et al. did not report on AFS specifically nor on mortality, hence extraction of AFS data and subsequent inclusion in the AFS analysis were not feasible.

Healing of pre-existent ulcers: Complete ulcer healing data could be analyzed from 8 of the 12 included RCTs in a total of 237 limbs, which showed beneficial effects of BM derived cell therapy in these patients with an overall RR of complete ulcer healing of 1.87 (95% CI, 1.49 to 2.36; $P < 0.00001$; Figure 2C).

ABI and tcO_2: Significant overall improvement of ABI was observed in the therapeutic arm compared to the control group (Figure 2D). In the BM derived cell treated limbs post-treatment ABI was significantly higher with 0.12 (95% CI, 0.09 to 0.15; $P < 0.00001$) in the seven RCTs including ABI as an outcome measure. TcO2 also significantly improved in the therapeutic arm compared to the control limbs with 14.28mmHg (95% CI, 8.54 to 20.02; $P < 0.00001$; Figure 2E).

Pain score and pain-free walking distance: These subjective endpoints improved more prominent in the therapeutic arm. Mean difference in pain score was 1.10 (95% CI, 1.37 to 0.83; $P < 0.00001$; Figure 2F) in the eight RCTs that could be analyzed. Pain-free walking distance was assessed in only 3 non-placebo-controlled RCTs, which showed substantial longer pain-free walking distance in the therapeutic arm (Figure 2G). Both Tateishi-Yuyama et al. and Lu et al. reported pain-free walking time, which also showed improvement in the treated limbs.

Sensitivity and Subgroup Analyses

The sensitivity analysis based on the fixed-effect methods did not substantially change the observed effects, however the beneficial effects of BM derived cell therapy on amputation rate and AFS were slightly stronger. Visual inspections of the funnel plots were not indicative of small study effects or publication bias.

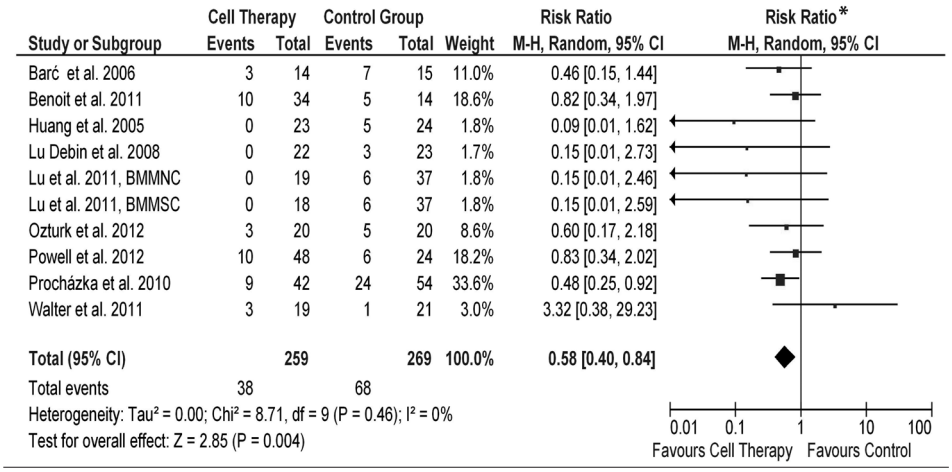

Figure 2A Major amputation.
* Results favoring cell therapy are depicted on the left-hand side of the forrest plot.

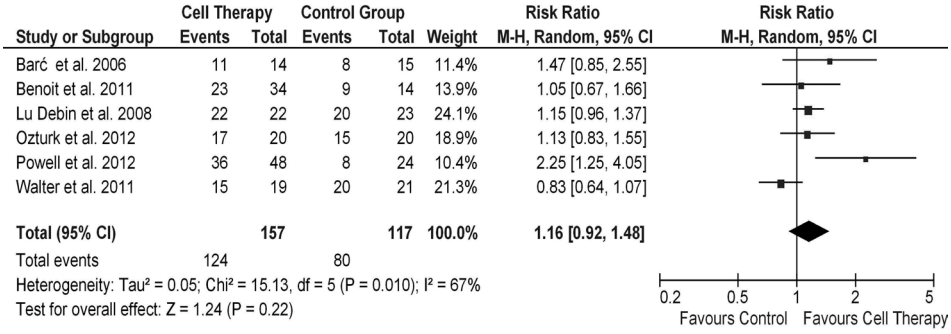

Figure 2B Amputation-free survival.

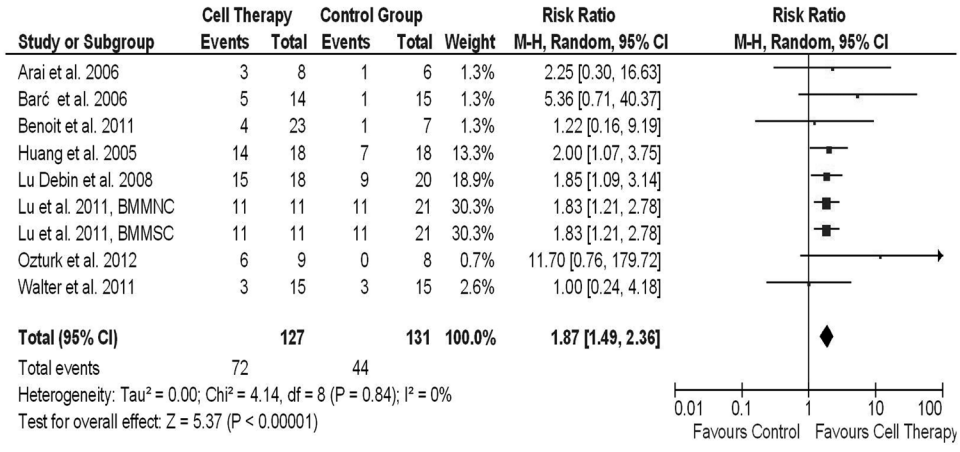

Figure 2C Ulcer healing (completely healed pre-existent ulcers).

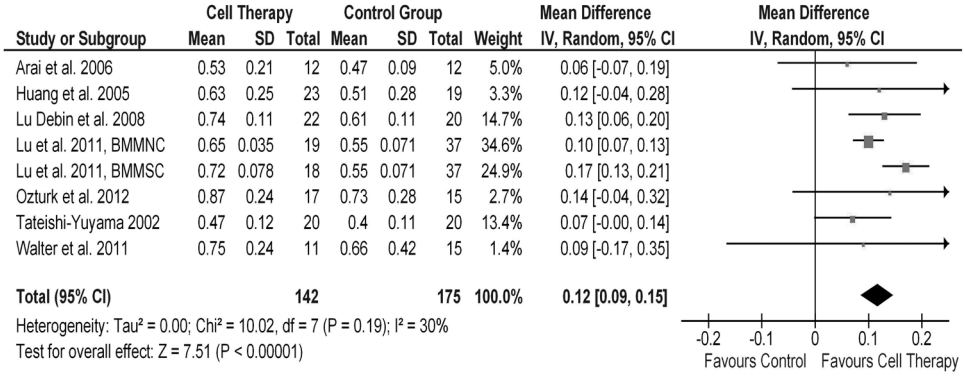

Figure 2D Ankle-brachial index.

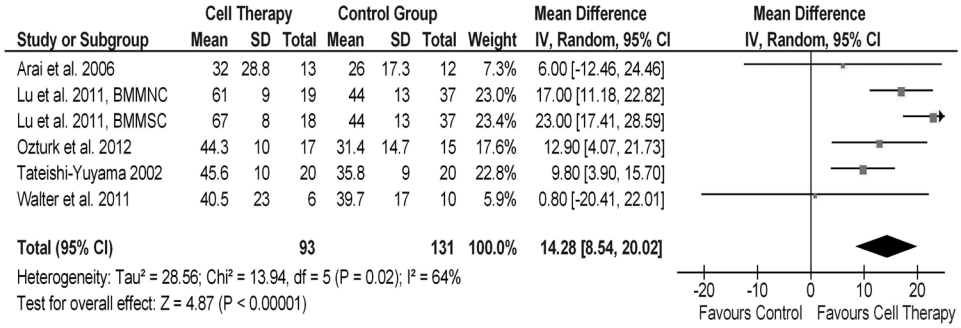

Figure 2E Transcutaneous oxygen measurements.

In the summary effect analysis for ABI, tcO_2, and pain score the I^2 statistic was indicative for substantial heterogeneity among the included studies. By visual inspection of the forrest plots the RCTs that studied BMMSC seem to report more pronounced effects on these endpoints compared to the other BM derived cell products, therefore re-analyses were performed excluding these studies. The overall effect of BM derived cell therapy remained significant in the re-analyses and did indeed show substantial reduction of heterogeneity when the BMMSC studies were excluded.

In the overall meta-analysis the reduction in the incidence of major amputation appeared to be modest in the placebo-controlled RCTs (Figure 2A), which was confirmed by the prespecified subgroup analysis excluding the non-placebo-controlled RCTs and placebo-controlled RCTs without a separate control group (Figure 3). In the placebo-controlled RCTs including a separate control group the observed beneficial effect on amputation rates in the cell therapy group compared to the group treated with placebo was considerably reduced with a RR of 0.92 (95% CI, 0.51 to 1.67; $P = 0.78$). Re-inclusion of the placebo-controlled results of Lu et al. using the contralateral limbs as controls did not result in substantial changes in the overall effect on major amputation rates with a RR of 0.78

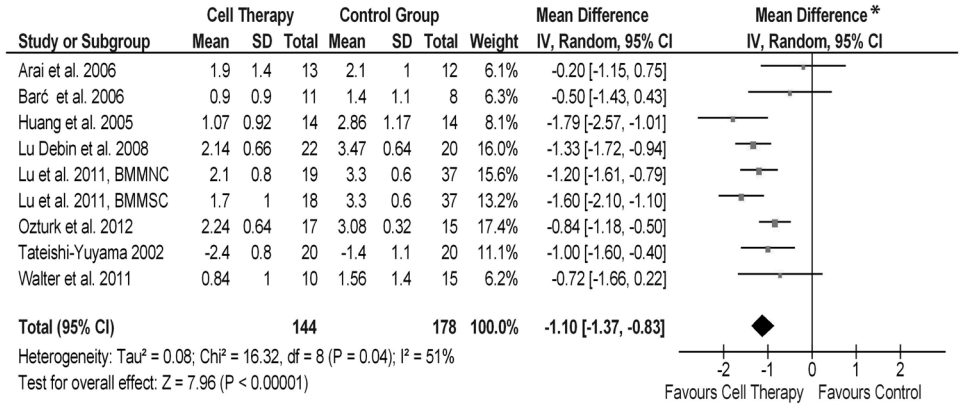

Figure 2F Pain score.
* Results favoring cell therapy are depicted on the left-hand side of the forrest plot.

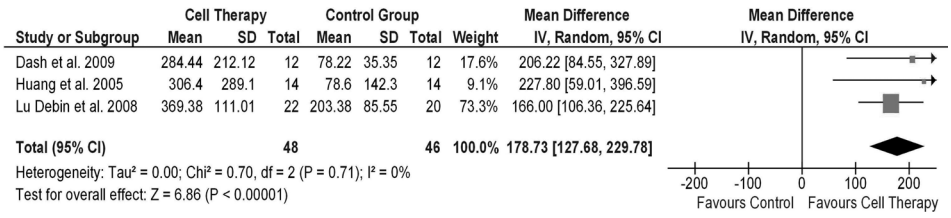

Figure 2G Pain-free walking distance (m).

(95% CI, 0.40 to 1.51; $P = 0.46$). While inclusion of the non-placebo-controlled RCTs only led to substantial amplification of the treatment effect with RR for major amputation of 0.45 (95% CI, 0.27 to 0.75; $P = 0.002$) in favour of the BM cell treated group.

Safety issues

In the considered RCTs BM derived cell therapies appeared relatively safe and side effects were generally mild and transient. Side effects consisted of pain and tenderness at the site of BM aspiration. Decrease of haematocrit has been reported following the aspiration procedures, but was transient and well-tolerated.[9] No adverse effects on kidney function, progression of retinopathy or muscle damage after IM infusions were reported.[9] Powell et al. showed no difference in mortality, and the amount of adverse events and side effects in the cell therapy compared to the placebo group.[10] Occurrence of malignancies were reported in two trials, one after the first month[11] and the other more than one year after treatment.[9] Furthermore, Walter et al. reported three procedure-related adverse events, i.e. stent thrombosis, a haematoma in the groin and a pseudoaneurysm, following the IA catheter-based infusions.[11]

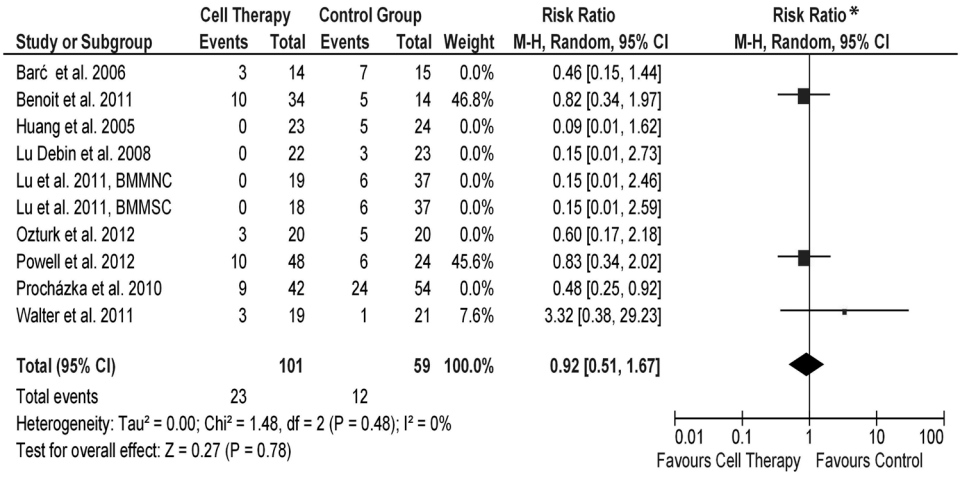

Figure 3 Subgroup analysis for major amputation in studies with a placebo-controlled design and including a separate control group.

* Results favoring cell therapy are depicted on the left-hand side of the forrest plot.

DISCUSSION

The systematic search for RCTs that study BM derived cell therapy in CLI resulted in the identification of 12 RCTs, jointly including 510 subjects of which 301 were treated in the therapeutic arms of these trials, with considerable divergence in trial design and quality. The results of the current meta-analysis which summarizes the efficacy data of these RCTs underline the promising potential of these therapies in CLI patients. Besides significant improvement of subjective and surrogate objective endpoints, the results show that major amputation rates are potentially reduced with the application of BM derived cell therapy in CLI. Importantly, the subgroup analysis of placebo-controlled RCTs showed more modest non-significant treatment effects on major amputation rates, which stresses the need for proper designed placebo-controlled RCTs to definitely prove the effectiveness of BM derived cell therapy to prevent major amputation.

Study design of trials that investigate BM derived cell therapy widely diverges. An important source of difference between these studies regards the specific population used as a control, whereas some trials opted for separate control groups, [9-11,19-25] others used the contralateral leg as a control. [6,26,29,30] In this meta-analysis we decided to include all RCTs that reported on the treatment effects of BM derived cell therapy irrespective of their choice for the specific control group, however it should kept in mind that the difference in control group can be a source of divergent results in cell therapy trials in CLI for two main reasons. First, in some studies that used the contralateral limb as the "control group" the most ischemic leg was treated with cell therapy, while the less ischemic limb served as the control. [29,30] These studies were not included in this meta-analysis since they were not randomized and could therefore not influence the results of the present study. Second, although the exact mechanisms whereby BM derived cell therapy exerts its effects are not completely unraveled, paracrine stimulation of neovascularization is probably one of the major mechanistic pathways via which BM derived cell therapy acts to increase perfusion. [31,32] These angiogenic factors would not be confined to the compartment where these cells are administered and could induce a paracrine neovascularization response in the contralateral limb as well. In order to exclude these potential blurring effects a subgroup analysis was performed wherein only placebo-controlled RCTs with a separate control group were considered, which showed modest non-significant effects on major amputation rates.

Noteworthy, in the twelve RCTs that were included in this meta-analysis four different BM derived cell types were used, including BMMNC, BMMSC, M-PBMNC and Ixmyelocel-T, which differ in respect of exact isolation or culture procedure. Whereas treatment with BMMNC or M-PBMNC is related with a relatively short interval between harvesting and the ultimate treatment, both BMMSC and Ixmyelocel-T require a delicate expansion process, which prolongs the interval between cell harvest and treatment. This meta-analysis does not indicate substantial differences regarding the clinical effectiveness of the different cell types, however studies that focus on BMMSC seemed more effective and introduced heterogeneity in the analyses for some endpoints, i.e. ABI, tcO_2 and rest

pain score. This could be a sign of a differential effect of BMMSC compared to the other cell types, but data on direct comparisons are very limited. Lu et al. concluded in their RCT that BMMSC are probably more potent than BMMNC to restore limb perfusion. [26] A similar conclusion for M-PBMNC was drawn in a RCT comparing M-PBMNC with BMMNC in a group of CLI patients due to TAO, [33] however this potential superiority of M-PBMNC was not observed on the long term and the amount of CD34-positive cells present in the cell product administered appeared to be more important. [34] Altogether the available studies that compare the effectiveness of different BM derived cell products are too limited to draw definitive conclusions and additional studies are warranted to get informed about the superiority of a specific cellular product for the treatment of CLI.

The majority of the included RCTs opted for the IM route to administer the cells and only one trial used an IA approach. Both routes to administer the cell product appear safe and effective, but studies directly comparing either IM, IA or a combination of the two routes to administer the cells are very limited and in general fail to show superiority of either route, possibly due to small sample sizes in these studies. [35-37]

BM cell therapy has been suggested to be effective in CLI either caused by ASO or TAO, however the effectiveness in TAO is probably more pronounced and likely more durable as well. [8,38] This could explain the more modest treatment response observed in Caucasian compared to Asian patients and might be related to the in general older age of ASO patients and to the higher prevalence of cardiovascular risk factors and associated endothelial progenitor cell dysfunction. [36,39,40] Moreover, ASO tends to have a progressive course and the newly formed vasculature could be impacted by the disease that originally led to CLI causing gradual loss of neovessels and relapse of the disease. [38] With the exception of Dash et al. where the majority patients suffered from CLI caused by TAO, [21] the RCTs included in this meta-analysis did consider mainly ASO induced CLI. Therefore this meta-analysis does not allow conclusions on differential effects of BM derived cell therapy in ASO vs. TAO induced CLI. Importantly, this meta-analysis shows that BM derived cell therapy is effective in the treatment of CLI, even despite its almost exclusive inclusion of ASO-patients.

When autologous BM derived cell therapy found its way to the clinic much concern about safety aspects of the treatment existed, especially tumorigenesis. Nowadays with over a thousand CLI patients treated in cell therapy trials ample evidence is available on – at least short-term – safety of autologous BM derived cell therapy. Based on the clinical results of many small non-controlled trials no critical safety concerns appear to exist in relation to these treatment regimens and no evidence for dedifferentiation and tumorigenesis related to BM derived cell therapy exists. [8] The RCTs considered in this meta-analysis further support these safety data. However, most trials were not specifically designed to prove safety of the treatment and follow-up data are generally short. In respect to safety as well as efficacy long-term follow-up data are warranted.

As RCTs are the preferred study design to evaluate therapeutic efficacy we included only RCTs in our meta-analysis. However, as the decision for amputation often depends on opinions and experiences of both patient and doctor, it has been proposed that trials

evaluating therapies in CLI patients that use amputation rate or AFS as outcome measure, as advised by TASCII,[1] should be double-blinded to eliminate bias.[9] Notably, the results of the placebo-controlled RCTs are more modest and if only these trials were analyzed no clear benefit of BM derived cell therapy on amputation rates compared to placebo injections was observed. This observation does not preclude beneficial effects of BM derived cell therapy on amputation rates in CLI patients, since lack of statistical power could be a reasonable explanation, but clearly underlines the need for placebo use in the control arms of comparable trials to exclude potential bias.

All in all this meta-analysis of twelve RCTs convincingly underlines the promising potential of BM derived cell therapy in CLI patients. The relative consistency of the results between the studies, the stability across the sensitivity analyses, and similar findings in non-randomized studies lend strong support to the reliability of the findings in this meta-analysis. Importantly, the results of placebo-controlled and non-placebo-controlled RCTs seem to diverge, which stresses the necessity to use placebo in the control arms of these trials. Future well-designed larger placebo-controlled RCTs are warranted to confirm these results and should include long-term follow-up data in order to assess whether the observed treatment effects are durable.

ACKNOWLEDGEMENTS

The reported work was supported by the “Stichting Vrienden UMC Utrecht” on behalf of the Dirkzwager-Assink foundation (The Netherlands, grant CS 06.007), the Dutch Heart Foundation (grant 2008B094), The Netherlands Organisation for Scientific Research (ZonMw-TAS grant 116001026) and foundation “De Drie Lichten” (The Netherlands, grant 10/06). M.C. Verhaar is supported by the Netherlands Organisation for Scientific Research (NWO) (The Netherlands, Vidi grant 016.096.359).

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PART TWO

Bone marrow-derived cell therapy in critical limb ischemia

CHAPTER 6

Bone marrow-derived cell therapy in no-option critical limb ischemia*

Submitted

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* The Juventas-trial is only published in an abstract version in this thesis in order to adhere to embargo-rules of certain journals, since the article is not published yet. At time of publication a direct link to the article will be placed at the Juventas-trial's website at www.juventas-trial.nl.

ABSTRACT

Background

Patients with critical limb ischemia (CLI) may not be eligible for conventional therapeutic interventions. Pioneering clinical trials suggest that bone marrow (BM)-derived cell therapy enhances neovascularization, improves tissue perfusion, and prevents amputation.

Methods

160 patients with no-option CLI were included in this double-blind, placebo-controlled RCT (NCT00371371). Patients were randomized to repetitive (3x; 3-weeks interval) intra-arterial infusion of BM mononuclear cells (MNC) or placebo into the common femoral artery (CFA). Primary outcome was major amputation at 6 months. The primary and safety outcome (all-cause mortality, occurrence of malignancy or hospitalization due to infection) were monitored sequentially. Secondary outcomes were combined occurrence of major amputation or death, minor amputations, change in clinical status, ulcer area, rest pain, ankle-brachial index (ABI), transcutaneous oxygen pressure (tcO2), and quality of life (QoL).

Results

No differences were observed for the primary outcome with rates of 19% in the BMMNC vs. 13% in the placebo group at 6 months (Relative risk [RR] 1.46; 95% confidence interval [CI] 0.62 to 3.42). The safety outcome was not different between the groups (RR 1.46; 95%CI 0.63 to 3.38), as was all-cause mortality at six months with 5% vs. 6% (RR 0.78; 95%CI 0.22 to 2.80). QoL, rest pain, ABI, and tcO2 improved during follow-up, but this did not differ between the groups.

Conclusions

Repetitive intra-arterial infusion of autologous BMMNC into the CFA did not reduce major amputation rate in patients with no-option CLI. The general improvement in secondary outcomes in both groups during follow-up underlines the need for placebo-controlled design of future trials.

PART TWO

Bone marrow-derived cell therapy in critical limb ischemia

CHAPTER 7

Core diameter of bone marrow aspiration devices influences cell-density of the bone marrow aspirate in patients with severe peripheral artery disease

Submitted

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ABSTRACT

Background

Bone marrow (BM) transplantations are an accepted therapeutic strategy for hematologic conditions. In the past decades interest for bone marrow derived cell therapy has extended towards the field of regenerative medicine. Irrespective of the treatment strategy its success depends on the amount of cells available for transplantation. Both patient and procedural factors have been shown to influence the cell-density of the BM aspirate. In the present study the influence of core diameter of the BM aspiration device on cell-density of the BM aspirate is studied.

Materials & Methods

BM harvesting procedures performed in a clinical trial investigating the effect of BM cell therapy in patients with severe peripheral artery disease were retrospectively studied (clinicaltrials.gov NCT00371371). Patients underwent BM harvesting using a 15G (n=85) or a 8G (n=75) needle. The numbers of harvested white blood cells (WBC) and $CD34^+$ hematopoietic cells (HPC) were quantified.

Results

The amount of WBC per mL of BM aspirate was significantly higher when a 8G needle ($27.8x10^6$ WBC/mL [95%CI $25.4\text{-}30.5x10^6$]) was used compared to the smaller 15G core needle ($20.1x10^6$ WBC/mL [95%CI $18.7\text{-}21.7x10^6$], $p<0.001$). For the amount of $CD34^+$ HPC a similar pattern was observed ($185x10^3$ HPC/mL [95%CI $161\text{-}213x10^3$]; $114x10^3$ HPC/mL [95%CI $96\text{-}134x10^3$]; $p<0.001$).

Conclusion

The application of a BM aspiration device with a larger core diameter is associated with an increased cell-density of the BM aspiration product in patients with severe peripheral artery disease.

INTRODUCTION

Bone marrow (BM) aspirations have been performed for several decades to obtain stem and progenitor cells for BM transplantations (BMT) to treat malignant and nonmalignant hematologic disorders. More recently, stem and progenitor cell therapy has gained interest as potential regenerative medicine (RM) strategy for cardiovascular and orthopedic diseases. Successful allogeneic hematopoietic BMT depends on the total number of nucleated donor cells transplanted.[1, 2] The number of administered BM cells has also been suggested to be an important determinant of clinical effectiveness in RM therapies.[3-6]
As higher BM aspiration volumes and more puncture sites are related with increased risk for complications,[7, 8] strategies to optimize cell density of the harvested BM are of large clinical relevance. Patient related factors, such as body weight (BW) and peripheral white blood cell (WBC) count, have been reported to influence the cell density of the obtained BM. [9] However, these patient characteristics cannot easily be influenced in contrast to procedural factors. Limiting the aspiration volume per site or using an aspiration needle with additional side-holes [10-12] are procedural factors that have been associated with an increased density of the BM product.
In a retrospective study of 160 BM harvests for the Juventas-trial (clinicaltrials.gov NCT00371371), [13] a randomized, double-blind, placebo-controlled trial that investigates the efficacy of repeated intra-arterial infusion of BM mononuclear cells (BM-MNC) in patients with no-option critical limb ischemia (CLI), we evaluated the influence of patient characteristics and the core diameter of the BM aspiration needle on the cell density of the harvested BM product.

MATERIALS AND METHODS

Study population

Data on 160 BM harvestings of patients participating in the Juventas-study in the period from September 2006 to July 2012 were analyzed. The Juventas-study is a clinical trial evaluating the clinical effects of intra-arterial infusion of BM-MNC in CLI (clinicaltrials.gov NCT00371371).[13] Patients with chronic CLI, an ankle-brachial index (ABI) of 0.6 or less, or an unreliable index (non-compressible or not in proportion to the Fontaine classification), and who were not candidate for conventional revascularization are included in this trial. 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 impossibility to complete follow-up.
The study was approved by the local Internal Review Board of the University Medical Center Utrecht and written informed consent was obtained from all patients.

Bone marrow aspiration procedure

Patients were administered fentanyl and midazolam to induce conscious sedation. Xylocain 2% was used for additional local anesthesia at the location where the BM collection was performed. BM aspiration was performed by an experienced hematologist at the right iliac crest, which was identified by manual palpation. The BM needle was inserted into the BM compartment and 8-10mL of BM was collected and after advancing the needle somewhat deeper into the compartment again 8-10mL of BM was obtained. Thereafter the needle was relocated to another part of the iliac crest and the identical procedure was repeated until approximately 100mL of BM was obtained. The BM was collected in bottles containing 50mL of saline with 100IU/mL sodium heparin. Subsequently BM-MNC were isolated using density gradient separation (DGS) with Ficoll-Paque (GE Healthcare, Waukesha, WI, USA) in the Cell Therapy Facility of the University Medical Center Utrecht, according to GMP-graded protocols.

From September 2006 to February 2011 a 15G BM aspiration needle (Lettix B.V., Apeldoorn, The Netherlands) was used, unless the length of the device was insufficient to reach the BM compartment. From March 2011 routine practice shifted towards the use of a larger 8G needle (Angiotech Pharmaceuticals Inc., Vancouver, BC, Canada).

Cell quantification and characterization

The number of WBC per mL was counted using an automatic cell counter (Coulter Ac*T 8, Beckman Coulter, Fullerton, CA, USA) and the total number of WBC was calculated by multiplying by the total volume of collected BM.

$CD34^+$ hematopoietic progenitor cells (HPC) were quantified with flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA). A BM volume containing $1x10^6$ WBC was incubated with monoclonal anti-human $CD^{45PerCP}$, $CD34^{PE}$, $CD14^{FITC}$ and $CD66^{FITC}$ antibodies. Erythrocytes were lysed with standard lysis solution. Cells in the lymphocytic range that were CD34/CD45 double-positive and negative for both CD14 and CD66 were characterized as HPC. The number of HPC was expressed as percentage of the total number of WBC counted. To calculate the total number of HPC the percentage obtained by flow cytometry was multiplied by the total number of WBC assessed with the automatic cell counter.

Statistical analyses

Normality of continuous variables was explored using histograms and Q-Q-plots. Equality of variances was tested using the Levene's test for variances. In case of non-normality, data were Log-transformed and again tested for their normality characteristics and equality of variances. Data are expressed as means and 95% confidence intervals (95%CI) for normally distributed data or medians and interquartile ranges (IQR) for data that retained a non-normal distribution even after transformation. Log-transformed data were reconverted to a linear scale to express their geometric means and confidence intervals. Group differences for continuous variables were tested using the independent t-test for data with a normal distribution and equal variances. In all other cases, non-parametric testing was

performed using the Mann-Whitney U-test. Group differences of categorical baseline variables were analyzed using the X^2-test. Spearman-rank tests were performed to identify univariate correlation between cell numbers and patient characteristics. P-values <0.05 were considered statistically significant. All analyses were performed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, Il, USA).

RESULTS

Patient characteristics

Patient characteristics of 160 patients who underwent a BM aspiration procedure according to the Juventas-study protocol in the period from September 2006 to July 2012 are shown in Table 1. The 15G BM device was used in 85 and the 8G device in 75 CLI patients. Age, gender, body weight, severity of PAD and presence of diabetes were not statistically different between groups.

Table 1 Subject characteristics.

	15G (n=85)	8G (n=75)	p-value
Age (years)	65.4±18.7	68.0±23.0	0.369
Male gender	59 (69%)	49 (65%)	0.583
Diabetes Mellitus	32 (38%)	29 (39%)	0.941
Body Weight (kg)	77.7 (74.4-81.2)	79.5 (75.6-83.6)	0.508
Fontaine classification			0.095
IIB	6 (7%)	4 (5%)	
III	33 (39%)	18 (24%)	
IV	46 (54%)	53 (71%)	

Values represent absolute numbers with percentage (n[%]) for categorical variables and medians ± IQR or means (95%CI) for continuous values.

Influence of patient characteristics on aspirate composition

A weak inverse correlation was observed between age and the total number of $CD34^+$ HPC as well as the number of $CD34^+$ HPC per mL (rho=-0.191, p=0.019; rho=-0.206, p=0.011; respectively). For body weight, a factor shown to influence cell density of BM aspirates in previous studies, we found no significant correlation, however a trend towards a positive correlation was observed for both the number of WBC as well as $CD34^+$ HPC per mL (rho=0.134, p=0.095; rho=0.140, p=0.090; respectively). We found no associations between the other patient characteristics and BM aspirate composition.

Influence of bone marrow needle on aspirate composition

Overall the median amount of BM aspirated per procedure was 100.0mL (IQR 5.8mL).

Total WBC count prior to DGS was 2390x10^6 (95%CI 2241x10^6-2550x10^6), whereas the CD34$^+$ HPC yield prior to DGS was 14.6x10^6 (95%CI 13.1x10^6-16.3x10^6). Mean number of WBC per mL of crude BM was 23.5x10^6 (95%CI 22.0x10^6-25.0x10^6). Overall 26.3% (IQR 12.0%) and 68.0% (95%CI 64.7-71.3%) of respectively the WBC and HPC were recovered after DGS.

Table 2 shows the outcome parameters according to needle type. BM harvesting yielded a total of 2072x10^6 (95%CI 1915-2242x10^6) and 2805x10^6 (95%CI 2554-3080x10^6) WBC using the 15G and the 8G device respectively (mean difference 739x10^6 [95%CI 655-834x10^6]; p<0.001), which resulted in 20.1x10^6 (95%CI 18.7-21.7x10^6) WBC/mL for the 15G and 27.8x106 (95%CI 25.4-30.5x10^6) WBC/mL of harvested BM for the 8G needle (mean difference 7.2x10^6 WBC/mL [95%CI 6.4-8.1x10^6]; p<0.001; Figure 1). A similar pattern was observed for the CD34$^+$ HPC population where the 15G needle led to cell counts of 114x10^3 (95%CI 96-134x10^3) and the 8G device to 185x10^3 (95%CI 161-213x10^3) CD34$^+$ HPC/mL of crude BM (mean difference 61x10^3 HPC/mL [95%CI 49-76x10^3]; p<0.001; Figure 2). Both the recovery of WBC and CD34$^+$ HPC after DGS were not influenced by the choice of the needle used for BM harvesting, also leading to significant higher CD34$^+$ HPC yields post DGS using the 8G device (8.4x10^6 [IQR 7.9x10^6] and 12.6x10^6 [IQR 9.8x10^6] for the 15G and 8G respectively [p<0.001]).

Table 2 Results of BM harvesting.

	15G (n=85)	**8G (n=75)**	**p-value**
Prior to DGS			
Aspirate volume (mL)	100.0±6.5	100.0±5.0	0.629
Total WBC prior to DGS (x10^6)	2072 (1915-2242)	2805 (2554-3080)	<0.001
WBC density per mL of aspirate (x10^6)	20.1 (18.7-21.7)	27.8 (25.4-30.5)	<0.001
Total CD34+ HPC prior to DGS (x10^6)	11.6 (9.9-13.6)	18.6 (16.3-21.4)	<0.001
CD34+ HPC density per mL of aspirate (x10^3)	114 (96-134)	185 (161-213)	<0.001
Post DGS			
Percentage WBC recovery after DGS	26.2±9.8	27.2±14.9	0.689
Total WBC after DGS (x10^6)	524 (469-586)	690 (600-794)	0.002
Percentage CD34$^+$ HPC recovery after DGS	70.4 (65.3-75.5)	65.5 (61.3-69.8)	0.149
Total CD34$^+$ HPC after DGS (x10^6)	8.4±7.9	12.6±9.8	<0.001

Values represent medians ± IQR or means (95%CI). *DGS* Density Gradient Separation, *WBC* White Blood Cell Count, *HPC* Hematopoietic progenitor cells.

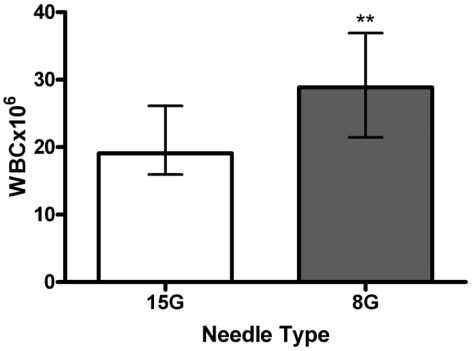

Figure 1 WBC density prior to DGS. The number of WBC per mL obtained during BM harvesting procedures was significantly higher when a BM device with a 8G core was used. **p<0.001

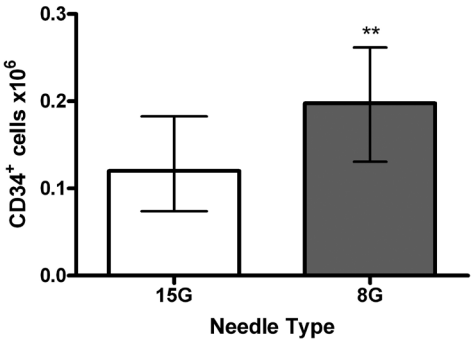

Figure 2 HPC density prior to DGS. The number of CD34+ HPC per mL obtained during BM harvesting procedures was significantly higher when a BM device with a 8G core was used. **p<0.001

DISCUSSION

This study shows that the composition of BM harvested during BM aspiration procedures in patients with severe PAD is influenced by the core diameter of the device used for the procedure. Both the total WBC yield as well as the number of HPC are substantially higher when a 8G device is used compared to a smaller 15G device. As the number of transplanted BM cells both in hematology as well as in the RM field has been shown to determine treatment success,[1-6] our results suggest that application of a needle with a larger core diameter provides a strategy to optimize BM cell yield for hematologic and RM strategies. Since increasing BM cell yield by increasing the total volume of collected BM requires more puncture sites and leads to higher risk of blood loss and prolonged duration of the procedure,[8] increasing the amount of cells per volume is preferable. This may be even more important in patients with cardiovascular conditions, an important target population for new RM strategies. Previous studies have reported that limiting the amount of BM aspirated per puncture site,[12, 14] using a BM device with additional side-holes,[10] and priming the patient with G-CSF prior to BM collection[15, 16] may increase cellular density of the collected BM. To our best knowledge this study is the first to report that the diameter of the BM aspiration needle determines cell density of the harvested BM. This may be due to a simple increase in surface area where the BM is collected from, similar to the needles with additional side-holes. Alternatively, also similar to what has been observed for multiple-side-hole needles,[17] our experience is that the procedure of BM collection using the larger diameter needles is faster, and may hence lead to a reduced time-window of bleeding into the compartment where BM is collected from and therefore less hemodilution. Importantly, we did not observe any puncture-site related complications, bleeding and post-procedural hematoma in particular.

The effects observed for the larger diameter needles in our study are likely based on similar mechanisms as what has been observed for the multiple-side-hole needles. However, the reported differences between the single-hole needles and multiple-side-hole needles are not as striking as the influence of diameter observed in this study,[10, 17, 18] for instance Wang et al observed a slight but significant increase of cell density of approximately 8%, while Lannert et al and Tanikawa et al did not observe significant differences between single-hole and multiple-side-hole needles in relatively small study populations. The more pronounced effect observed for the larger core needles in the current study could be a result of the smaller BM volumes obtained in this study, a different patient population, and the larger core of the 8G needles in our study compared to the needles used in the other studies.

It has been suggested that the multiple-side-hole needles are less strong[18] and therefore maybe not applicable for every patient. Additional potential benefits of opting for a larger diameter needle with a single hole instead of a multiple-side-hole needle are that the same needle can be used to obtain a BM biopsy during the same procedure and single-hole needles are in general less expensive.[19] However, we think that the choice of the device that is used for the procedure should be carefully considered in each patient and depend on the experience of the physician performing the procedure.

A potential limitation of this study is that it was not specifically designed to study the influence of core diameter of the BM needle used to harvest BM. In the initial phase of the Juventas-trial the 15G needle was routinely used for the BM collections. Only if the 15G needle was of insufficient length, due to excessive subcutaneous fat, a longer and larger 8G needle was used. It was striking that the cell yield during these procedures seemed higher which led us to shift to routine use of the 8G needle to potentially increase the cell yield and to evaluate whether this observation was merely due to differences in body weight, since body weight is a factor that has been related to cell density of BM aspirate.[10] However, in the present study only a weak trend was observed towards increased cell density in patients with higher body weight. Moreover, body weight did not differ between the groups and therefore it is unlikely that the results are influenced by selection bias.

Furthermore, we did not study whether there is a potential synergistic effect of additional side-holes and larger core diameter of the device. It could be that this strategy, by further enlargement of the BM harvest area and enhanced harvesting speed, improves cell yield even further.

Our study shows that in our patient population with severe PAD the amount of both WBC and $CD34^+$ HPC obtained during BM harvesting are over 50% higher when a 8G BM device is used instead of a 15G needle. Whether these data can be extrapolated to healthy BM donors and larger BM volumes relevant to hematologic BMT mandates further research. The results of this study emphasize that procedural factors for collecting BM can significantly influence BM cell yield and should be carefully considered in order to improve clinical efficacy of BM cell-based therapeutic strategies.

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PART TWO

Bone marrow-derived cell therapy in critical limb ischemia

APPENDIX

Angiographic demonstration of neoangiogenesis after intra-arterial infusion of autologous bone marrow mononuclear cells in diabetic patients with critical limb ischemia. Commentary

Cell Transplant. 2012; 21(8): 1803-4

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Dear editor

With interest we read the article by Ruiz-Salmeron and colleagues in your journal.[7] Although the reported results are clearly interesting and do support the earlier findings regarding bone marrow cell therapy in patients with critical limb ischemia, the article and especially the part on increased collateralization as quantified on digital substraction angiographies (DSA) using a semi-computerized method raises some questions.

We agree with Ruiz-Salmeron et al. that therapeutic neovascularization by administration of bone marrow cells is a promising new strategy for treatment of peripheral arterial disease, and critical limb ischemia in particular. Clinical benefit has been reported, however mainly through assessment of surrogate measures for improved perfusion, such as Ankle-Brachial Index and transcutaneous oxygen measurements, techniques that have their specific limitations, especially in diabetics.[10] Clinical studies thus far have not provided reliable and reproducible assessment of neovascularization.

Ruiz-Salmeron et al. show that intra-arterial administration of bone marrow mononuclear cells (BM-MNC) in 20 diabetic patients with severe critical limb ischemia resulted in a notable improvement in the Rutherford-Becker classification, the University of Texas diabetic wound scale and the Ankle-Brachial Index in the target limb after 12 months of follow-up as compared to baseline. Importantly, they also report increased vascularization after cell therapy, which was assessed at 3 months using conventional intra-arterial digital subtraction angiography (DSA) quantified by MetaMorph software.

We laud the attempt of the authors to quantify the neo-vessels induced with intra-arterial administration of BM-MNC. However, the authors present a novel method to quantify vascularization without providing essential technical data and background information with regard to reliability and reproducibility. Thus far, studies on the use of DSA to evaluate neovascularization in the lower limb have almost invariably stated that the angiographic resolution is too low to identify the newly formed vessels.[1,6,8,9] Although several studies have used MetaMorph software to quantify microvasculature on photographic and microscopic images,[2,3,4] no reports seem to be available on application of this computerized method to analyze vascularization on DSA in humans. No information is provided by the authors on inter- and intra-examiner reproducibility, as well as test-retest reliability regarding the use of this method for quantification of collaterals on DSA. Furthermore, as acquisition and procedural parameters e.g. the ambient temperature, patient's heart rate, medication use (i.e. vasodilating drugs), interval between contrast injection and image acquisition and venous overprojection potentially obscure the definition and quantification of collaterals on DSA when a comparison is made between timepoints,[5] these factors should have been reported in more detail.

We agree with Ruiz-Salmeron et al. that quantification of vascularization is essential for optimal evaluation of the effects of cellular therapy in patients with peripheral vascular disease. Thus far, methods to quantify neovascularization on DSA have not been established. Although the method proposed in this paper is interesting, further research is necessary for its validation.

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PART TWO

Bone marrow-derived cell therapy in critical limb ischemia

APPENDIX II

Comment on 'Stem-cell therapy for peripheral arterial occlusive disease'

Eur J Vasc Endovasc Surg. 2012; 43(4): 486

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Dear editor

With great interest we have read the article by Kim and colleagues in this journal,[1] demonstrating that intramuscular administration of human umbilical cord blood mononuclear cells (HCB-MNCs) enhances the number of capillaries, angiogenic gene expression and angiogenic factors in a canine hind limb ischemia model. We laud the attempt of the authors to translate their results into clinical practice, administering HCB-MNCs in patients with peripheral arterial occlusive disease (PAOD). However, this study raises some important questions.

The authors put much emphasis on the potential development of graft-versus-host disease (GVHD). GVHD is known to occur after allogeneic stem cell transplantations, where donor lymphocytes encounter and offend the (immunodeficient) host's tissues, and has been reported as unusual adverse event after solid organ transplantations and blood transfusions,[2, 3] always in states with impairment of the recipient's immune system. The article by Kim et al. however reports on intramuscular allogeneic HCB-MNC administration in fully immunocompetent individuals. The likelihood of developing a GVHD-like phenomenon in this setting is low, since donor derived allogeneic cells are delivered in a hostile environment with an active immune system. Induction of a widespread immunogenic reaction comparable to GVHD is therefore nearly impossible in our view. Rather, the recipient's immune response may lead to an inflammatory response and rapid removal of the infused cells, which may limit the potential therapeutic effect of allogeneic HCB-MNC. In that respect it is unfortunate that the authors do not provide any efficacy data from their clinical study. Although we agree that, to assess the ultimate efficacy of stem cell therapy, objective criteria and larger series are mandatory, evaluation of previous studies on autologous progenitor cell safety and efficacy in small series of patients with thromboangiitis obliterans[4, 5] were able to show significant improvement of clinical parameters, such as transcutaneous oxygen pressure, pain, skin temperature and ulcer healing. Presentation of such efficacy data for allogeneic HCB-MNC would be a valuable addition to this paper.

Altogether the authors propose an interesting therapeutic strategy for PAOD patients, however, as autologous approaches have already been shown promising and circumvent immunogenic complexities, we advocate the autologous approach and further improvement of these strategies.

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PART THREE

Role of endothelial progenitor cells and platelet function in cardiovascular disease

CHAPTER 8

Reduced endothelial progenitor cell numbers in diabetes: Insights and underlying mechanisms

In preparation

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ABSTRACT

Diabetes Mellitus (DM) is associated with markedly increased all-cause mortality, primarily due to an increased risk of cardiovascular diseases (CVD). The presence of DM is associated with premature atherosclerosis and impaired neovascularization. A reduced vasculoregenerative capacity, a process that involves endothelial progenitor cells (EPC), is one of the mechanisms that contributes to the increased cardiovascular risk in DM.
Studies that investigated EPC numbers in states of disturbed glucose metabolism almost invariably showed reduced circulating EPC numbers under diabetic conditions. Multiple pathways are involved in the reduction of EPC numbers in DM, such as disturbance of the bone marrow (BM) microenvironment along with a reduction of EPC mobilization from and reduced progenitor cell pools in the BM, reduced proliferation and differentiation of EPCs, decreased EPC survival, and probably increased peripheral recruitment of EPCs due to increased endothelial turnover. Strategies aimed to enhance EPC numbers and improve EPC function thus stimulating vasculoregenerative capacity may reduce the cardiovascular risk in diabetic patients.

INTRODUCTION

Diabetes mellitus (DM) is a major, growing health care problem, not only in the Western world, but in developing countries as well. Worldwide, the number of people with DM is reaching epidemic proportions and expected to increase to an estimated 380 million persons in the year 2025.[1,2] DM is associated with premature atherosclerosis and a high risk of micro- and macrovascular complications, such as ischemic heart disease, stroke and peripheral artery disease (PAD). Risk of cardiovascular disease (CVD), as well as all-cause mortality are over two-times higher in diabetic compared to non-diabetic individuals.[3-5] Moreover, DM is associated with an impaired neovascularization response leading to reduced revascularization of ischemic tissue, impaired wound healing, placental vasculopathy, and increased organ transplant rejection in diabetic patients.[6,7]

Glucotoxicity and lipotoxicity associated with DM negatively affect the vessel wall and contribute to the increased cardiovascular risk in diabetic patients. Endothelial injury and dysfunction, initial steps in atherosclerotic plaque formation, are an early phenomenon in diabetes.[8,9] The production of superoxides by endothelial cells during hyperglycemia plays a pivotal role in the etiology of diabetic cardiovascular complications via the induction of pathways that ultimately lead to vascular damage, such as increased polyol pathway flux, increased formation of advanced glycosylation endproducts (AGEs), activation of protein kinase C, and increased hexosamine pathway flux.[10]

It is becoming increasingly apparent that vascular health is not only determined by the extent of vascular injury, but by the balance between endothelial damage and endothelial regeneration. In 1997, Asahara and co-workers first reported on the existence of bone marrow-derived endothelial progenitor cells (BM-EPC),[11] which contribute to postnatal neovascularization and endothelial maintenance and repair. Impaired vasculoregenerative capacity due to reduced numbers of circulating EPCs or functional impairment of EPCs can thus increase cardiovascular risk.[12,13]

Besides its negative effects on the resident endothelium, DM has been reported to influence EPC-dependent vasculoregenerative capacity and neovascularization. In the present article we will give a short overview of the current understandings on EPC biology, summarize the available studies on EPC numbers in DM and highlight potential mechanisms that contribute to reduced EPC numbers under diabetic conditions.

EPC Subtypes: What's in a name!

The identification of a BM-derived cell type that contributes to neovascularization in the adult has sparked great interest in the mechanisms and potential therapeutic applications of BM-derived progenitor cells in vascular biology.[11] The term 'Endothelial Progenitor Cell' or 'EPC' is commonly used to refer to these progenitor cells. However, it has become clear that the exact definition of progenitor cells under investigation in a particular study deserves closer scrutiny,[14] as different subsets of circulating blood cells can be induced to display EPC-like characteristics, all with subtype specific features and functions.

An important distinction has to be made between circulating hemangioblastic (referring to their similarity with hematopoietic stem cells) EPCs and culture expanded/ modified EPCs. Hemangioblastic EPCs represent a particular subset of the hematopoietic stem cell pool that contributes to neovessel formation and re-endothelization of denuded endothelium.[15,16] Hemangioblastic EPCs are characterized and quantified using flow cytometry in peripheral blood samples directly after withdrawal without *ex vivo* modification and are defined by the presence of a marker protein associated with undifferentiated hematopoietic progenitor cells, such as CD34[11] or CD133[17] in combination with a marker for endothelial cells (e.g. vascular endothelial growth factor receptor 2,VEGFR-2, KDR) (for an overview see Timmermans et al.).[18] The most likely mechanism whereby these cells exert their proangiogenic and vasculoregenerative effect is via temporary adherence to damaged endothelium and paracrine effects on resident endothelium by secretion of growth factors and remodelling of the extracellular matrix.[19-21]

Cultured EPCs are derived by plating out peripheral blood mononuclear cells (PB-MNCs) in specific cell culture media.[22] Three major phenotypes are commonly distinguished: colony-forming unit endothelial cells (CFU-ECs), circulating angiogenic cells (CACs) – previously called early outgrowth EPCs – and endothelial colony forming cells (ECFCs) – previously called late EPCs.[23] It has been shown that the majority of the cells in these primary cultures are cells of hematopoietic origin.[24]

The frequently studied 'early EPCs' or CACs, originate from the myeloid lineage25 and show similarities to monocytes.[26,27] They seem to be derived from more differentiated cells that are early in the myeloid lineage and positive for the markers CD14 and CD45.[26,28] Similarly to hemangioblastic EPCs, CACs produce proangiogenic factors to stimulate regeneration of the local endothelium and facilitate sprouting of new vessels.[26,29,30]

ECFCs are thought to be veritable endothelial progenitor cells.[31] These cells have genuine endothelial characteristics, proliferate rapidly in culture, and are very similar to mature endothelial cells in terms of morphology and gene expression.[32] These cells are also able to promote neovascularization[33] and have the capacity to permanently integrate into the neovasculature.[34-36] However, the precursor cells that yield ECFC colonies in culture, which are CD14 and CD45 negative,[28,31] are exceedingly rare in PB and therefore difficult to culture consistently.

Endothelial progenitor cells in diabetes

Several studies have reported on numbers of circulating and cultured EPCs and low numbers have been observed over the full spectrum of deranged glucose metabolism, from the metabolic syndrome to long-term established diabetes, for essentially every EPC subtype. In Figure 1 we provide a comprehensive overview of published studies that report numbers of EPCs in diabetic patients compared to controls (See Data Supplement for Methods).[37-68] On average, circulating progenitor cell numbers are approximately 30% lower in diabetic patients (95%CI 0.65 – 0.75, $p=1.1*10^{-20}$). Interestingly, in patients with proliferative diabetic retinopathy (PDR), circulating progenitors are markedly higher, by a factor 1.6 (1.31 – 2.06, $p=2*10^{-5}$; Figure 1).[43-47] Variation in the individual study findings

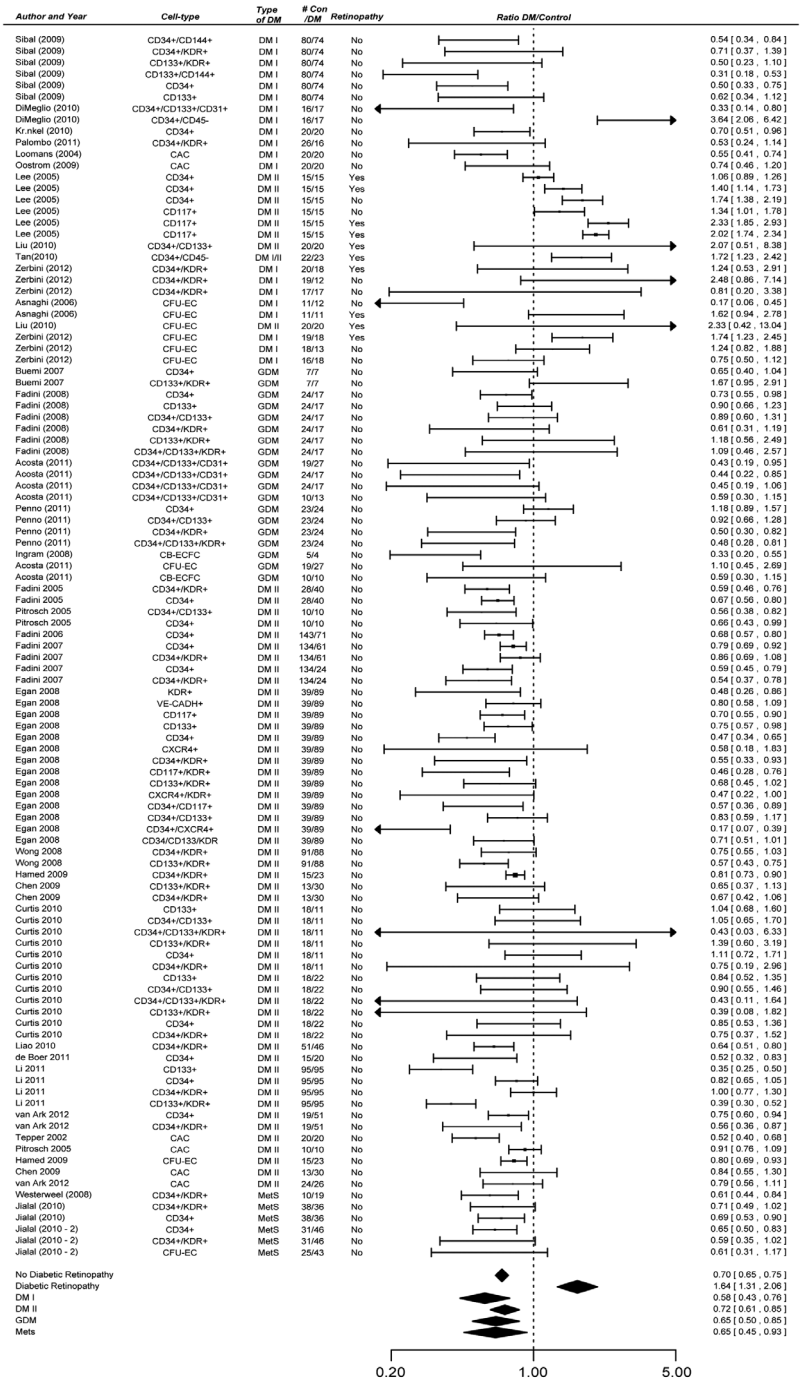

Figure 1

is approximately symmetrical around the combined estimate, indicating little publication bias (Figure S1).

No significant differences were observed between diabetes type or cell population used for quantification (See Figure 2), with the exception of a single study by DiMeglio et al.[38] In this study the authors investigated $CD34^+/CD45^-$ cells amongst others, which are not of hematopoietic origin but might indicate a progenitor type of vascular origin. This circulating cell type was found to be increased in diabetic patients, in contrast to the $CD34^+/CD133^+/CD31^+$ hemangioblastic progenitors measured in the same study, which were decreased in diabetic patients.

Several studies have reported that lower EPC numbers relate with the presence of endothelial dysfunction[37,62] and cardiovascular complications in diabetes.[53,55] Furthermore, cultured EPCs in diabetic patients have been shown to be dysfunctional, displaying decreased proliferative, adhesive, regenerative and vasculogenic potential.[41,66,69,70]

Mechanisms leading to decreased EPC numbers in diabetes

Diabetes-related EPC reduction may be due to processes such as decreased progenitor cell mobilization from and declining progenitor cell pools in the BM, reduced EPC survival, impaired EPC proliferation and differentiation, and increased peripheral recruitment to sites of endothelial damage or ischemia (Figure 3).

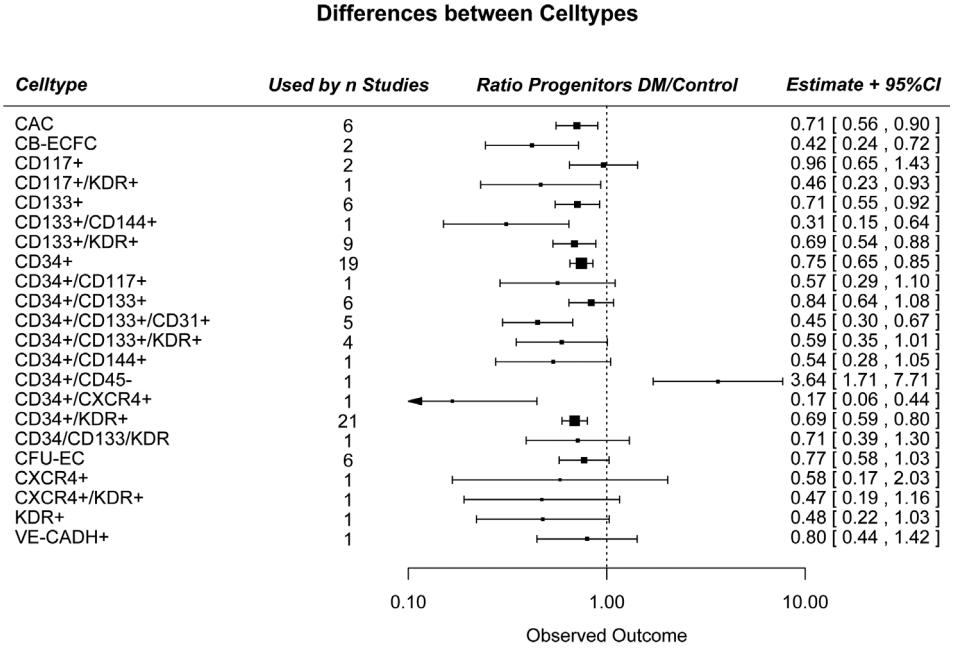

Figure 2

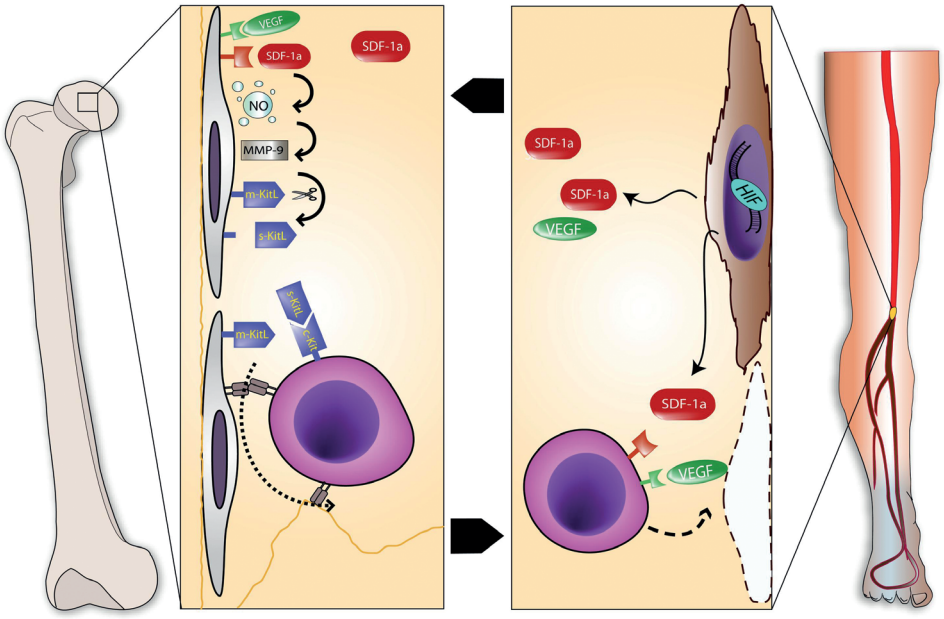

Figure 3 EPC mobilization. The induction of EPC mobilizing factors produced in ischemic tissues under the regulation of hypoxia inducible factor 1α (HIF-1α), such as stromal derived factor 1α (SDF-1α) and vascular endothelial growth factor (VEGF), leads to cascade of events ultimately leading to EPC mobilization from the BM. Important intermediates in this cascade are nitric oxide (NO), matrix metalloproteinase 9 (MMP-9), membrane-bound Kit-ligand (m-Kitl), soluble Kit-ligand (s-Kitl), and c-Kit receptor (c-Kit).

Disturbed BM microenvironment and impaired EPC mobilization from the BM

Quiescent EPCs reside in the BM in a common stem cell niche, firmly anchored to the BM stroma, together with hematopoietic stem cells and BM stromal cells.[71] Upon stimulation by an external stimulus EPCs will migrate to the vascular niche where they will expand and enter circulation under the orchestration of the sinusoid endothelium.[72] Stromal-cell derived factor 1α (SDF-1α), a quick-acting chemokine, is considered one of the most potent mediators involved in EPC mobilization and homing.[73,74] Upon ischemia, endothelial cells increase SDF-1α expression,[75] which will mobilize EPCs from the BM in a dose-dependent fashion.[76,77] Once EPCs have entered the circulation, SDF-1α acts as a very strong chemoattractant, directing EPCs to sites of ischemia.[78] Several other growth factors promote EPC recruitment, the most important of which is vascular endothelial growth factor (VEGF). Similar to SDF-1α, VEGF mobilizes progenitor cells from the BM[79,80] and thus aids in neovascularization. There seems to be substantial interplay between VEGF and SDF-1α signalling, leading to a synergistic effect.[81,82] While SDF-1α and VEGF are important factors in the homeostatic response to hypoxia via hypoxia inducible factor-1α (HIF-1α) upregulation, a great number of other factors have been shown to influence the

release of EPCs from the BM.[83] Many of these stimuli activate intracellular second messengers that converge on the PI3K/Akt-pathway. This subsequently starts a common cascade, leading to the production of nitric oxide (NO).[84] Upon binding of NO to stromal cells of the stem cell niche, the expression of matrix metalloproteinase 9 (MMP-9) increases, which in turn cleaves membrane-bound Kit Ligand (m-KitL).[85,86] The now soluble Kit ligand (s-KitL) promotes mobilization of EPCs from the BM niche. The exact mechanisms underlying the action of s-KitL are incompletely understood but may involve competitive displacement at the c-Kit receptor, or expression of proteases in response to s-KitL binding (Figure 3).

Animal experiments have shown that EPC mobilization from the BM to the circulation is disturbed in diabetes, which could – in part – explain the observed decline of EPCs in circulation. In diabetic animal models, HIF-1α upregulation as well as the release of VEGF and SDF-1α in response to ischemia is impaired resulting in reduced EPC mobilization and recruitment to ischemic tissues.[87-90] Moreover, diabetic BM was found to be relatively resistant to EPC mobilizing agents, such as G-CSF and SCF.[87] In line, diabetic BM in humans was found to be resistant to progenitor cell mobilization in response to exogenous administered mobilizing agents, i.e. G-CSF.[91,92] On top endogenous release of these factors was found reduced in subjects with the metabolic syndrome,[67] potentially further aggravating the disturbed mobilization in subjects with deranged glucose metabolism.

Diabetes is associated with endothelial nitric oxide synthase (eNOS) dysfunction and reduced NO bioavailability.[70,93-95] Gallagher et al. showed that phosphorylation of BM eNOS was reduced in diabetic mice and that eNOS phosphorylation could be restored with hyperoxia, which increased circulating EPC levels via a NO-dependent mechanism.[89] The eNOS dysfunction in the BM of diabetic mice was confirmed by Dong et al., who additionally showed that MMP-9 expression in diabetic BM was repressed and could be restored by insulin treatment via a NO-mediated pathway, leading to almost complete restoration of EPC mobilization in response to ischemia.[95] Moreover, $CD34^+$ cells obtained from diabetic patients normalized their – initially reduced – migratory potential to VEGF and SDF-1α after NO supplementation,[96] further underlining the importance of NO in EPC mobilization and its disturbed function in diabetes.

Diabetes is associated with neuropathy and vasculopathy, a process that has been shown to extend to the BM in diabetic animals[91,97] as well as in humans.[98] We recently showed that a similar process occurs in patients with critical limb ischemia (unpublished data). Neuronal impulses play a role in progenitor cell mobilization[99,100] and diabetes induced derangement of sympathetic innervation of the bone marrow is related to disturbed progenitor cell mobilization.[91] Diabetes is also associated with BM microangiopathy, functional alterations in BM endothelial cells and impaired BM perfusion, which relates to reduced progenitor cell pools in the BM and impaired EPC mobilization.[97] Diabetes-induced changes of the BM microenvironment have also been reported to reduce progenitor cell pools,[97,101] suggesting progenitor cell exhaustion as another potential mechanism leading to reduced circulating EPCs.

Decreased proliferation and differentiation

Several reports describe decreased blood-derived CACs, CFU-ECs and ECFCs in DM, which is an actual reflection of mobilization of the cells from the BM, and the adhesion, differentiation, survival and proliferative capacity of the culture-expanded isolated cells (Figure 1). Multiple pathways, including increased oxidative stress, reduced NO bioavailability and production of AGEs, contribute to the deranged proliferation and differentiation of EPCs in DM. Limited data exist on the differences in proliferation between diabetic EPCs and EPCs isolated from healthy subjects. Although reduced proliferative capacity of cells isolated from patients with DM type 2 has been reported,[59,65] the majority of studies investigating the influence of DM on proliferation and differentiation have been performed under *in vitro* hyperglycemic conditions using non-diabetic progenitor cells.[59,93,102,103] The diabetes-induced disturbances can be partially reversed *in vitro* with antioxidant treatment,[59] blocking the receptor for the AGEs[104] or increasing NO bioavailability.[103]

Besides differences in proliferation, a deranged differentiation could be important in diabetes induced reduction of EPC numbers in the circulation. Indeed, Lombardo et al. provided evidence that the ultimate differentiation of circulating EPCs is impaired in diabetes,[105] whereas Loomans et al. showed that BM progenitor cells from hyperglycemic mice have less angiogenic potential and a more pro-inflammatory phenotype.[106] Furthermore, specific subsets of BM-derived progenitor cell numbers that promote atherogenesis are increased in diabetes, i.e. smooth muscle progenitor cells and progenitor cells with a 'procalcific drift'.[107-109]

Decreased EPC survival

DM and hyperglycemia have been suggested to negatively affect EPC survival, via mechanisms associated with enhanced senescence and increased apoptosis. Increased activation of the Akt/p53/p21-pathway,[110] p38 mitogen activated protein kinase (MAPK)[111], ERK 1/2[112], and activation of nuclear factor κB[112,113] have been shown to increase cell senescence and apoptosis under hyperglycemic conditions. Inhibition of the receptor for the AGEs and ameliorating oxidative stress lead to decreased activation of these pathways, hence senescence and apoptosis are alleviated.[104,110,111]

Some reports describe a potential role for impaired progenitor cell self-renewal in DM. Ingram et al. reported that cord blood-derived ECFCs from subjects with gestational diabetes mellitus (GDM) did not differ in the extent of apoptosis, but exhibited enhanced senescence and reduced self-renewal capacity.[52] A similar effect of long-term diabetes on hematopoietic progenitor cells (HPC) in the BM of mice has been observed. Orlandi et al. showed that long-term diabetes reduced the number of HPC and their repopulating potential in mice. The expression in the BM of Bmi-1, a protein involved in prevention of cellular senescence and retainment of self-renewing capacity, was reduced probably explaining the reduced HPC in diabetic mice.[101] Since EPCs are phylogenetically tightly related to HPCs, a parallel mechanism could be involved in EPC self-renewal and its disturbance in DM.

Increased peripheral recruitment of EPCs

Whether increased peripheral recruitment of the progenitor cells to repair the endothelial damage imposed by the diabetic environment contributes to the lower circulating EPC levels is not known. Diabetes is associated with increased endothelial damage which may induce EPC recruitment and endothelial regeneration,[88] which may suggest "consumptive loss" as contributor to reduced EPC levels. However, EPC recruitment at sites of endothelial injury has also been reported to be impaired in diabetes,[114,115] challenging this concept.

Therapeutic implications

Since low circulating EPC numbers are related to high cardiovascular risk[12,13] and diabetic vascular complications,[53,55] restoration of EPC numbers could offer a strategy to attenuate the cardiovascular risk in diabetes. In this review we focused on mechanisms leading to reduced EPC numbers in the circulation, however dysfunction of EPCs is another important determinant of impaired vasculoregenerative potential in diabetes and advanced cardiovascular diseases.[116] Several therapeutic interventions have been shown to modulate pathways that are involved both in quantitative as well as functional impairment of EPCs. An important pathway that is deregulated in diabetes is the PI3/Akt-pathway, which leads to decreased responsiveness to several growth factors and reduced NO-bioavailability. Several clinically available pharmacological strategies such as statins,[84,117,118] peroxisome proliferators-activated receptor-γ agonists,[119,120] erythropoietin,[121,122] angiotensin converting enzyme inhibitors and angiotensin-II receptor blockers,[123,124] and insulin[125] have been demonstrated to enhance EPC availability and improve EPC function, which seems at least in part mediated via the PI3/Akt-pathway. Such beneficial effect on EPC availability and function may contribute to the benefit of these medications with regard to cardiovascular risk.

Cell-based therapies that aim at neovascularization as a treatment of ischemic cardiovascular disease, such as myocardial infarction and PAD have gained interest over the past decades.[126-128] Since progenitor cells in diabetes are dysfunctional, this may hamper therapeutic efficacy of cell therapy strategies in diabetes.[129,130] Pre-treatment of cells may improve cell function and thus the efficacy of the therapy. In diabetes there are still some other issues that should be kept in mind regarding cellular therapy. For instance, diabetes is sometimes associated with aberrant neovascularization in the retina, i.e. PDR, which should be carefully monitored, since cellular therapies could theoretically aggravate and augment PDR. The unique features of cell-based therapies in diabetes are increasingly recognized, which forms the rationale for the initiation of several clinical studies focusing on cell-based therapy in patients with CVD, such myocardial infarction (NCT01307371) and PAD (NCT01232673, NCT00872326), in the presence of diabetes.

Concluding remarks

Diabetes or impaired glucose regulation is associated with reduced numbers of circulating EPCs with impaired vasculoregenerative function, due changes in the BM microenvironment, decreased EPC mobilization from and availability of progenitor cell

pools in the BM, decreased proliferation and differentiation, decreased EPC survival, and increased peripheral consumption of EPCs. Interventions to restore the number and function of EPCs could contribute to reducing cardiovascular events in diabetes.

SOURCES OF SUPPORT

MC Verhaar is supported by a grant from the Netherlands Organisation for Scientific Research (NWO Vidi grant number 016.096.359). JO Fledderus is supported by a grant from the Dutch Diabetes Research Foundation (project number 2010.11.1414). Studies in the Laboratory of Renal and Vascular Biology, Dept. of Nephrology and Hypertension, are supported in part by research grants from the Netherlands Foundation for Cardiovascular Excellence (104/06), Dutch Heart Foundation (2008B094), Catharijne Stichting (CS 06.007), ZonMw (40-41400-98-9026) and the Regenerative Medicine Strategic Programme UMCU.

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SUPPLEMENTAL DATA

Methods

Search Strategy

A PubMed search was performed (1997 – July 2012) using the following search terms (EPC OR CPC OR CAC OR ECFC OR (Circulating AND Progenitor) OR "Circulating Angiogenic Cell" OR "Endothelial Colony Forming Cell" OR "Outgrowth Endothelial Cell") AND (Diabetes OR IDDM OR NIDDM OR "Metabolic Syndrome"). Only published/ peer reviewed reports written in English were included.

Data Extraction

All studies that compared numbers of circulating progenitors in diabetic or metabolic syndrome patients relative to a control population were included. Measures of central tendency and spread were copied if reported or extracted from graphs using digitization software (GraphClick, Arizona Software). No quality assessment with reference to clinical study guidelines (i.e. assessor blinding, random inclusion etc.) was performed due to the nature of the included studies.

Data Analysis

Quantification of circulating (endothelial) progenitors is performed in various different ways in literature. Variations include gradient density centrifugation, cell culture steps (see

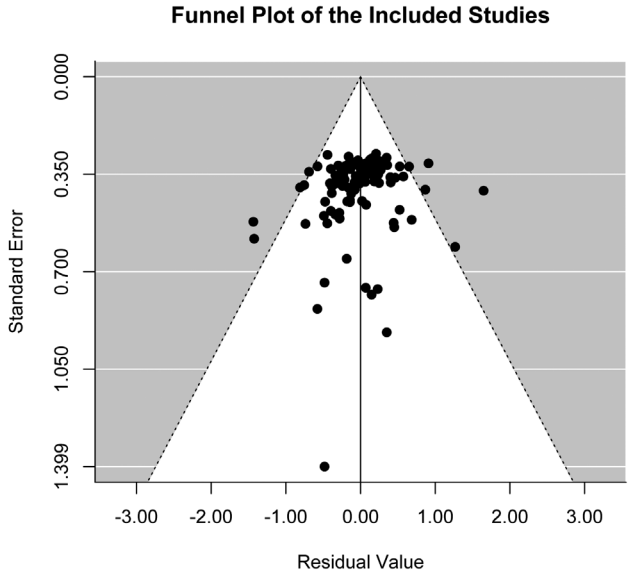

Figure S1

also body text) and different flow cytometry strategies with variations in 1 or 2 platform methods, volumetric methods, methods with counting beads and also variations in marker selection to identify progenitor cells. As a result classical effect sizes used in meta-analyses, such as (standardized) mean difference, are not suitable. In this overview we therefore present the data as the ratio of progenitor cell counts of diabetics divided by controls.
Studies varyingly report progenitor cell numbers as normally distributed or rightly skewed. In the former case the ratio diabetics/ controls is the ratio of means (ROM) of both populations with a 95% confidence interval [1]. In studies that observed right-skewed populations either the geometric mean (geomean) or the median are reported. For this review we assumed log-normality in these studies, taking the geomeans as measure of centrality. As measure of dispersion we derived the geometric standard deviation from the reported interquartile range (IQR). Subsequently the Ratio of Geometric Means (RoGM)[2] in diabetics/ controls was taken as the ratio measure.
A forest and a funnel plot were created using the metafor package in R [3]. Aside from individual study data we also provide tentative aggregate effect estimates derived from a mixed model using Restricted Maximum Likelihood (REML) estimators and moderators as reported. In studies reporting several celltypes ($CD34^+$, $CD34^+/KDR^+$ etc.) we only took one population (the $CD34^+$) for the aggregate estimate.

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PART THREE

Role of endothelial progenitor cells and platelet function in cardiovascular disease

CHAPTER 9

Impaired endothelial progenitor cell mobilization and dysfunctional bone marrow stroma in diabetes mellitus

Plos One. 2013; 8(3): e60357

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ABSTRACT

Background

Circulating Endothelial Progenitor Cell (EPC) levels are reduced in diabetes mellitus. This may be a consequence of impaired mobilization of EPC from the bone marrow. We hypothesized that under diabetic conditions, mobilization of EPC from the bone marrow to the circulation is impaired –at least partly– due to dysfunction of the bone marrow stromal compartment.

Methods

Diabetes was induced in mice by streptozotocin injection. Circulating Sca-1$^+$Flk-1$^+$ EPC were characterized and quantified by flow cytometry at baseline and after mobilization with G-CSF/ SCF injections. *In vivo* hemangiogenic recovery was tested by 5-FU challenge. Interaction within the bone marrow environment between CD34$^+$ hematopoietic progenitor cells (HPC) and supporting stroma was assessed by co-cultures. To study progenitor cell – endothelial cell interaction under normoglycemic and hyperglycemic conditions, a co-culture model using E4Orf1-transfected human endothelial cells was employed.

Results

In diabetic mice, bone marrow EPC levels were unaffected. However, circulating EPC levels in blood were lower at baseline and mobilization was attenuated. Diabetic mice failed to recover and repopulate from 5-FU injection. *In vitro*, primary cultured bone marrow stroma from diabetic mice was impaired in its capacity to support human CFU-forming HPC. Finally, hyperglycemia hampered the HPC supportive function of endothelial cells *in vitro*.

Conclusion

EPC mobilization is impaired under experimental diabetic conditions and our data suggest that diabetes induces alterations in the progenitor cell supportive capacity of the bone marrow stroma, which could be partially responsible for the attenuated EPC mobilization and reduced EPC levels observed in diabetic patients.

INTRODUCTION

Premature atherosclerosis is a major complication in diabetes,[1] which is at least in part attributable to an impaired vascular regenerative potential of bone marrow-derived progenitor cells,[2] Diabetes is associated with endothelial cell dysfunction and impaired neovascularization after ischemia.[3-6] Healthy intact endothelium and maintenance of its integrity play a central role in protecting against the development of atherosclerotic disease.[7] Endothelial progenitor cells (EPC), a specialized subset of hematopoietic progenitor cells (HPC), circulate in peripheral blood and contribute to restoring damaged or lost endothelium and facilitate ischemic neovascularization.[8] EPC are capable of endothelial differentiation[9] and secretion of angiogenic growth factors and cytokines,[10,11] which is of paramount importance in neovascularization. Similar to HPC, EPC originate from the bone marrow, from which they are mobilized in response to mobilizing cytokines, such as vascular endothelial growth factor (VEGF),[12] granulocyte colony-stimulating factor (G-CSF),[13,14] and stromal-derived factor-1α (SDF-1α),[15] and neuronal impulses.[16,17] In the bone marrow HPC are localized in two distinguishable stem cell niches, i.e. the 'osteoblastic' and the 'vascular niche', which largely regulate progenitor cell proliferation and mobilization. Progenitor cell proliferation and quiescence is thought to be predominantly regulated by osteoblasts in the 'osteoblastic niche', which is further composed of a heterogeneous population of stromal cells that includes fibroblasts and endothelial cells. The sinusoidal endothelium is the essential component of the 'vascular niche' and plays a pivotal role in progenitor cell egress from the bone marrow to the circulation,[18-20] a process that requires nitric oxide (NO).[21]

Previous studies showed reduced HPC and EPC levels in type 1 and 2 diabetic patients.[22-24] Low levels of circulating EPC in peripheral blood may undermine the regenerative potential of the endothelium and thus contribute to accelerated cardiovascular disease development. Indeed, in prospective cohort studies, lower levels of EPC were associated with poor event-free survival.[25,26] Interestingly, endothelial dysfunction, measured by flow-mediated brachial artery reactivity, correlates with reduced EPC numbers in the peripheral circulation of patients at risk for cardiovascular disease.[27] This suggests that endothelial dysfunction and reduced EPC levels share a common pathogenic mechanism. Although circulating levels of EPC may also be reduced due to increased endothelial turnover or decreased cell survival, recent experimental evidence in diabetic murine models supports a role for impaired mobilization and a deranged bone marrow microenvironment[6,28-30] and bone marrow innervation.[31] We hypothesized that under diabetic conditions, endothelial progenitor cell levels are reduced due to impaired progenitor cell mobilization from the bone marrow in association with dysfunction of the bone marrow stroma.

METHODS

Animal model of diabetes

Diabetes was induced in 6-week-old C57Bl/6 mice (n=9; Harlan Laboratories Inc, Indianapolis, IN, USA) by a single intraperitoneal injection of 200 mg/kg streptozotocin (STZ, Serva Feinbiochemica GMBH, Heidelberg, Germany) in citrate buffer (pH 4.5), as published previously.[32] Buffer injected littermates served as controls (n=10). Diabetic mice received suboptimal insulin treatment by subcutaneous implantation of an insulin-releasing pellet (Linbit, Linshin Canada Inc, Toronto, Ontario, Canada) to prevent severe catabolism and lethal diabetes. Diabetic animals were required to have non-fasting blood glucose levels of >15 mmol/l after insulin pellet placement to be included in the study. Blood glucose levels were measured with a portable glucose meter (Medisense, Abbot Laboratories, Abbott Park, IL, USA). Further experiments were performed 4 weeks after confirmation of hyperglycemia. All experiments were performed at the same time of the day and in coupled-pairs of diabetic and non-diabetic animals to circumvent potential circadian influences on progenitor cell numbers.

Ethics statement

All protocols of animal experiments were approved by the animal ethical committee of the University Medical Center Utrecht, The Netherlands, or the Weill Cornell Medical Center, New York, USA. Protocols with respect to isolation of cells and cell lines were approved by the institutional review board of the University Medical Center Utrecht, The Netherlands, or the Weill Cornell Medical Center, New York, USA. All subjects provided written informed consent.

Quantification of HPC and EPC in blood and bone marrow

Flow cytometric quantification of both Sca-1$^+$ and c-Kit$^+$ HPC and EPC (Sca-1$^+$Flk-1$^+$) was performed in peripheral blood and bone marrow. On different time points EDTA anticoagulated blood was obtained via femoral vein cannulation and bone marrow cell suspension by flushing the femurs of the mice with RPMI medium (Invitrogen Ltd, Carlsbad, CA, USA) during the terminal experiment. Subsequently, 50µl of whole blood or a volume of bone marrow suspension containing 1x10^6 cells was stained with α-Sca1-FITC (BD Pharmingen, San Diego, CA, USA) and α-Flk1-PE (BD Pharmingen) or α-cKit-FITC (BD Pharmingen). Erythrocytes were lysed in an ammonium chloride buffer and remaining cells analyzed with flow cytometry (FC 500, Beckman Coulter, Fullerton, CA, USA). Cell numbers were quantified per ml of blood, estimated based on their relative proportion to the leucocytes in the flow cytometry sample and the total number of leucocytes in a complete blood cell count made on a hematocytometer (Cell-dyn 1800, Abbott laboratories), and for bone marrow cell numbers per femur were estimated based on their relative proportion in the sample and the total number of isolated cells. Additionally, progenitor cells were also quantified as percentage of the total number of WBC in either blood or bone marrow.

From bone marrow cell suspensions (diabetic, n=4; control, n=4; three experiments per mouse), an additional EPC quantification was performed by culturing bone marrow cells at a density of $2x10^6$ cells/ well on fibronectin-coated cover slips in 24-wells plates in EGM-medium supplemented with 20% FCS, 100ng/ml VEGF (R&D Systems Inc, Minneapolis, MN, USA) and penicillin/streptomycin (Invitrogen Ltd). After 4 days, non-adherent cells were washed away and adherent cells were incubated with DiI-labeled acetylated-LDL (ac-LDL, Invitrogen Ltd), fixed in paraformaldehyde, stained with FITC-labeled BS1-lectin (Bioconnect B.V., Huissen, The Netherlands), DAPI, and then mounted on slides using Vectashield (Vector Labs, Burlingame, CA, USA). EPC were identified as ac-LDL/ lectin double-positive cells using a fluorescence microscope and quantified using the average cell count in 3 random high-power fields.

Assessment of progenitor cell mobilization

HPC and EPC mobilization was assessed after subcutaneous injections of 250 µg/kg granulocyte-colony stimulating factor (G-CSF, Neupogen®, Amgen, Thousand Oaks, CA, USA) and 50 µg/kg stem cell factor (SCF, Amgen) in 0,9% NaCl for five consecutive days (experimental days 0 to 4), as previously published.[33] HPC and EPC in peripheral blood were quantified at baseline (prior to G-CSF and SCF injections) and at experimental days 2, 4, 7 and 10 after induction of progenitor cell mobilization.

In vivo model for vascular niche function: 5-Fluorouracil challenge

Control and diabetic mice were intravenously injected with 250 mg/kg body weight 5-fluorouracil (5-FU; American Pharmaceutical Partners, Schaumburg, IL, USA). White blood cell (WBC) and platelet counts were monitored in peripheral blood samples using a hematocytometer at baseline and after 4, 7, 10, 14 and 21 days.

Isolation of human $CD34^+$ hematopoietic progenitor cells

Human $CD34^+$ HPC were isolated from umbilical cord blood (CB) by magnetic activated cell sorting (MACS) using a commercially available $CD34^+$ isolation kit (Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's instructions. In brief, mononuclear cells were isolated using Ficoll-density gradient separation (Amersham Biosciences, Piscataway, NJ, USA) and incubated with magnetic microbead-conjugated α-CD34-antibodies and FcR-blocking solution. Cells were passed over a selection column (LS column, Miltenyi Biotech) placed in a magnetic field. After removal of the column from the magnetic field, positive cells were eluded and the procedure was repeated using a second column. Purity of selected $CD34^+$ cells was evaluated with flow cytometry using α-CD34-FITC (BD Pharmingen). Mean purity was 91% (range 71-96%) in the isolations performed for the experiments in this study. To prevent potential effects of differences in $CD34^+$ purity coupled diabetic and non-diabetic experiments using the same progenitor cell sample were performed throughout this study.

Ex vivo model for bone marrow stroma – progenitor cell interaction

Primary mouse bone marrow stromal cells (BMSC) were obtained by isolating the plastic-adherent fraction from crude bone marrow cell suspensions. Bone marrow cells were flushed from mouse femurs using RPMI medium and cultured in DMEM (Invitrogen Ltd) containing 20% FCS and penicillin/streptomycin (Invitrogen Ltd) at a density of $1x10^7$ cells per T25 culture flask. Medium was changed after one week and subsequently every 2-3 days until cells reached confluence. Subsequently, mouse BMSC were trypsinized and passed into a 12-wells plate and co-cultured with $1x10^5$ human cord blood $CD34^+$ HPC (CB-HPC) in X-VIVO-20 medium (Biowhittaker Inc, Chesterbrook, PA, USA) containing 2% FCS. After 10 days, non-adherent and trypsinized adherent cells were pooled and a fraction was plated in methylcellulose medium containing hematopoietic growth factors (Methocult complete, StemCell Technologies, Vancouver, BC, Canada). The number of colony forming units (CFU) was quantified after 14 days of culture (diabetic, n=8; control, n=10).

In vitro model for the bone marrow vascular niche

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described[34] and transfected with a lentiviral vector to express the E4Orf1 construct, providing endothelial cells with the capacity for long term support of hematopoietic cells in a confluent state as recently described.[35] E4Orf1-transfected HUVEC were grown to confluence in 12-wells plates, after which $1x10^5$ $CD34^+$ CB-HPC per well were added to the culture. Co-cultures were maintained in IMDM medium (Invitrogen Ltd) containing 0 or 30mM added D-Glucose (Sigma Aldrich, St. Louis, MO, USA). A small volume of fresh medium was added every 2-3 days and every two weeks excessive medium was carefully removed with minimal aspiration of non-adherent cells. Glucose concentrations were carefully monitored throughout the experiments to verify the normo- and hyperglycemic culture conditions. Glucose concentrations oscillated between 3-8mM for the normoglycemic experiments and were approximately 30mM in hyperglycemic cultures. After 2, 4 and 6 weeks, the number of non-adherent cells was counted and then again pooled with the adherent co-cultured cells, which were detached using trypsin-EDTA (Invitrogen Ltd). A fraction of these cells was plated in methylcellulose medium containing hematopoietic growth factors (Methocult complete, StemCell Technologies) and evaluated for generation of CFU after 14 days culture.

Assessment of direct effects of hyperglycemia on $CD34^+$ CB-HPC survival and migratory function

$CD34^+$ CB-HPC were incubated overnight in IMDM medium containing 10% FCS and 0 or 30mM added D-Glucose, or 30mM D-Mannitol (Sigma Aldrich) as osmotic control. Cell number was assessed using a Bürker-Türk counting chamber, counting only viable cells based on Trypan-Blue exclusion. Equal cell numbers were taken up in IMDM medium containing 1% FSC and 0 or 30mM added D-Glucose, or 30mM D-Mannitol and placed in transwell insert (5µm pore size, Corning Costar, Cambridge, MA, USA) in a 24-well

migration system with 100 ng/ml SDF-1α (R&D Systems) or vehicle added to the bottom wells. After 4 hours, migrated cells were counted using an automated cell counter (Cell-dyn 1800, Abbott Laboratories).

Statistical analysis

Data are expressed as means±SEM and were analyzed using SPSS version 20.0 (SPSS Inc, Chicago, IL, USA). After testing for normal distribution of data and equality of variances, differences between groups were analyzed using an unpaired-samples Student's *t*-test. In time elapsing experiments differences within groups compared to baseline were analyzed using repeated measures ANOVA with Bonferroni post-hoc testing. Between group differences in experiments with different time points were analyzed with a two-way ANOVA with Bonferroni post-hoc testing. A P-value of <0.05 was considered statistically significant.

RESULTS

Diabetic mice have normal bone marrow EPC levels, but EPC mobilization is impaired

The number of Sca-1$^+$Flk-1$^+$ EPC isolated per femur did not differ between diabetic and control mice (diabetic, 13.2±3.7; control, 10.5±3.4x10^3; P=0.591). A similar picture was observed for both Sca-1$^+$ (diabetic, 4.0±0.6; control, 3.4±0.6x10^6; P=0.449) and c-Kit$^+$ (diabetic, 1.8±0.2; control, 1.5±0.2x10^6; P=0.297) HPC bone marrow content (Table 1). Consistently, quantification of EPC by *ex vivo* culture of bone marrow cells also revealed no significant differences in cell number (diabetic, 24.1±2.2; control, 24.4±5.2 per high power field; P=0.965).

Despite the normal bone marrow progenitor cell levels observed in diabetic mice peripheral blood Sca-1$^+$Flk-1$^+$ EPC were lower in diabetic mice during steady state conditions (diabetic, 7.7±0.9; control, 13.3.±1.9x10^3/ml; P=0.020; Figure 1). Upon the mobilizing stimulus with G-CSF and SCF, a robust mobilization response was observed in control mice for all progenitor cell subtypes. Sca-1$^+$Flk-1$^+$ EPC levels reached 30.5±3.2x10^3 EPC per ml of blood after maximal stimulation at day 4, corresponding to a 129% increase of 17.2±3.0x10^3 EPC per ml (P=0.003; Figure 1). During the injection phase from day 0 to 4 EPC remained high and returned to baseline levels thereafter. In contrast, in diabetic mice EPC levels increased only moderately, although significant, with 4.3±0.8x10^3 EPC per ml in response to the G-CSF/SCF injections to reach values of 12.0±1.3x10^3 EPC per ml blood, corresponding to an increase of 56% compared to baseline levels (P=0.009). Hence EPC levels differed significantly between diabetic and non-diabetic animals after maximal stimulation at day 4 (P<0.001). Additionally, a similar pattern of lower steady state progenitor cell levels and a blunted mobilization in response to G-CSF and SCF was observed for both the Sca-1$^+$ and c-Kit$^+$ HPC in diabetic mice. If progenitor cell populations were expressed relative to the total number of WBC the differences were in general less pronounced (Table 1).

Table 1 Progenitor cell numbers in blood and bone marrow.

		Sca-1$^+$		Sca-1$^+$Flk-1$^+$ EPC		c-Kit$^+$	
Absolute numbers		**DM**	**Control**	**DM**	**Control**	**DM**	**Control**
PB (/ml)	Baseline	2.3±0.3x106, ##	4.1±0.3x106	7.7±0.9x103, #	13.3±1.9x103	0.12±0.03x106	0.15±0.02x106
	t=2	3.7±0.4x106, ##	7.5±0.9x106, *	13.0±1.4x103, ##	29.9±5.0x103, *	0.38±0.03x106, ##,**	0.61±0.07x106, **
	t=4	3.9±0.5x106, ##, *	9.4±0.6x106, **	12.0±1.3x103, ##, *	30.5±3.2x103, **	0.44±0.06x106, ##, *	0.74±0.06x106, **
	t=7	2.2±0.2x106, ##	4.7±0.3x106	12.7±5.7x103	15.5±2.2x103	0.20±0.05x106	0.28±0.06x106
	t=10	2.4±0.3x106, #	3.7±0.4x106	11.8±2.2x103	13.5±2.3x103	0.19±0.03x106	0.11±0.02x106
BM (/femur)		4.0±0.6x106	3.4±0.6x106	13.2±3.7x103	10.5±3.4x103	1.8±0.2x106	1.5±0.2x106
% Cells relative to WBC numbers							
PB	Baseline	5.1±0.2 ##	6.7±0.1	0.18±0.01	0.21±0.02	2.3±0.5	2.5±0.2
	t=2	4.2±0.2 **	4.6±0.3 **	0.15±0.01	0.17±0.02	4.5±0.1 *	3.7±0.2 *
	t=4	4.3±0.2 #	3.5±0.2 **	0.13±0.01 **	0.12±0.01 **	5.2±0.7 #	2.8±0.2
	t=7	5.3±0.2 ##	6.9±0.1	0.17±0.01	0.22±0.02	5.0±1.0	3.9±0.7
	t=10	4.1±0.3 ##, **	6.4±0.2	0.20±0.03	0.22±0.02	3.4±0.5 #	1.9±0.2
BM		7.8±0.7	7.5±0.5	0.03±0.01	0.02±0.01	3.6±0.3	3.8±0.4

DM = Diabetes Mellitus, PB = Peripheral Blood, BM = Bone Marrow, WBC = White Blood Cell. # P<0.05 compared to controls, ## P<0.01 compared to controls, * P<0.05 compared to baseline, ** P<0.01 compared to baseline.

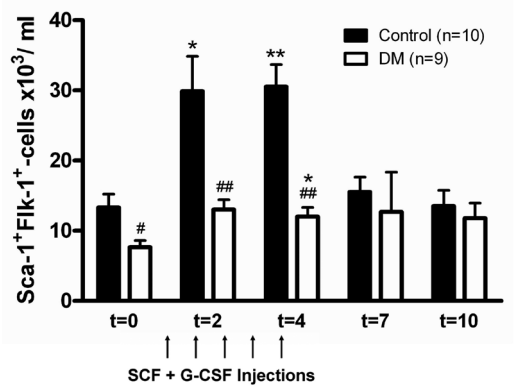

Figure 1 EPC levels and mobilization in diabetes. Diabetic animals have reduced levels of Sca1$^+$Flk-1$^+$ EPC in peripheral blood under steady-state (baseline) conditions compared to controls. After daily injection with mobilizing cytokines G-CSF and SCF from day 0 to 4, a significant mobilization of EPC was observed. EPC levels returned to baseline after cessation of cytokine injection. In contrast, diabetic animals showed a diminished mobilization response. P<0.001 for interaction of time and diabetes in 2-way ANOVA. #P<0.05 compared to controls, ## P<0.01 compared to controls, *P<0.05 compared to baseline, ** P<0.01 compared to baseline

Diabetic bone marrow fails to recover after 5-FU challenge

Diabetic and control animals were injected with 5-FU as an *in vivo* model for hemangiogenic and vascular niche recovery.[18] 5-FU injection causes destruction of proliferating bone marrow progenitor cells and sinusoidal endothelial cells, without affecting quiescent cells, and leads to a depression in peripheral blood WBC and platelet numbers. Recovery depends on the presence and function of quiescent multipotent stem cells, but particularly on functional vascular niche regeneration as a result of bone marrow neoangiogenesis.[18,36] After 5-FU injection we observed a reduction in both WBC and platelets that was most pronounced 7 days after injection (Figure 2A and B). As expected, control animals fully recovered with restoration of WBC and platelet levels, displaying a typical rebound thrombocytosis after recovery. In contrast, all diabetic animals died between day 10 and 14 due to a failure to repopulate with severe leukopenia and thrombopenia (Figure 2A and B).

Diabetes and hyperglycemia impair the HPC supportive function of bone marrow stroma

Plastic-adherent bone marrow stromal cells, a heterogeneous cell population including fibroblasts, endothelial cells, osteoblasts and potentially various other cell types, from diabetic and control primary cultures of mouse bone marrow cell suspensions were grown to confluence to serve as feeder layers for co-cultured human CD34$^+$ CB-HPC. Stromal cell morphology and growth pattern was similar between cultures from diabetic and control animals (data not shown). However, the number of CFU cells derived from 10-day co-cultures was significantly lower for diabetic stroma compared to control stroma (45±10 vs. 75±7 CFU/well; P=0.023, Figure 3A). The distribution over the various types of CFU

colonies was not affected (data not shown). Of note, cells were cultured using identical medium with a standard D-glucose concentration. As NO is essential for progenitor cell mobilization and maintenance we assessed the protein levels of the NO-producing enzyme eNOS by ELISA, which appeared to be significantly reduced in diabetic stroma (diabetic, 1.9±0.4; control, 4.1±0.4 AU; P=0.0055). Quantitative real-time PCR for eNOS mRNA showed a 3.9 fold reduced expression in diabetic stroma (P<0.01), suggesting that the reduced eNOS protein levels were due to decreased transcription of eNOS or enhanced posttranscriptional mRNA degradation.

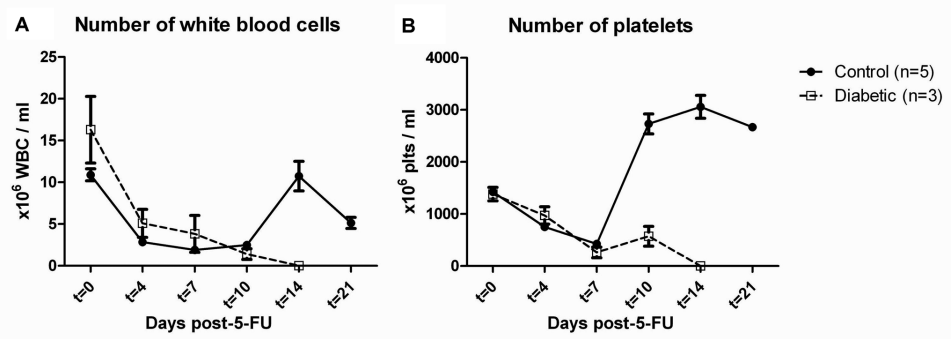

Figure 2 Diabetic mice do not recover after a 5-FU challenge. After 5-FU injection, a reduction in both WBC (A) and platelets (B) was observed in control and diabetic animals, which was maximal at 7 days after injection. Control animals fully recovered with restoration of WBC levels and platelets, displaying a typical rebound thrombocytosis after recovery. In contrast, diabetic animals died between day 10 and 14 with severe peripheral blood leukopenia and thrombopenia.

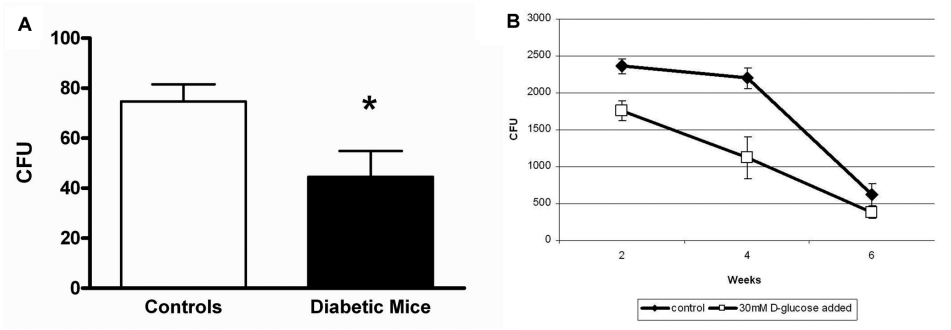

Figure 3 Progenitor cell support by bone marrow stroma ex vivo or endothelial cells in vitro. Plastic-adherent bone marrow stromal cells were grown to confluence from isolated bone marrow cell suspensions. Stromal layers from control (n=10) and diabetic (n=8) mice had a comparable morphology and growth pattern. Stromal layers were then used as feeder layer for human $CD34^+$ HPC. (A)The number of CFU derived from 10-day co-cultures was significantly lower for diabetic stroma than for control stroma. Human $CD34^+$ HPC were co-cultured with E4Orf1-transfected HUVEC for six weeks. At several time points, hematopoietic colonies (CFU) were counted. (B) Fewer CFU were obtained from $CD34^+$ HPC co-cultured on endothelium in the presence of hyperglycemia than under control conditions. *P<0.05

Because diabetes is associated with endothelial dysfunction and sinusoidal endothelium is important for progenitor cell support and egress, we studied whether the observed impairment of stromal cells to support progenitor cells under diabetic conditions might also be reflected in endothelial cells in an established *in vitro* model for the 'vascular niche'. For this purpose we co-cultured human $CD34^+$ CB-HPC with E4Orf1-transfected HUVEC in regular versus hyperglycemic conditions. Indeed, $CD34^+$ CB-HPC cultured on this vascular niche model in the presence of hyperglycemia generated less CFU-generating cells compared to normoglycemic conditions (Figure 3B). The distribution over the various types of CFU was not affected by hyperglycemia. We did not observe any direct effects of hyperglycemia on $CD34^+$ CB-HPC survival or migratory function when these cells were kept in suspension overnight in the absence of endothelium (data not shown).

DISCUSSION

Chronically reduced EPC levels that are relatively unresponsive to mobilizing cues may hamper maintenance and regeneration of the vascular endothelium and impair neovascularization in response to ischemia in diabetes. In this study we show impaired mobilization of endothelial and hematopoietic progenitor cells in diabetes, despite undisturbed bone marrow levels. Furthermore, diabetic bone marrow was unable to repopulate after 5-FU administration, a model for hemangiogenic bone marrow regeneration. Moreover, *in vitro* HPC supportive function of diabetic stroma and endothelium in a hyperglycemic environment was impaired, in conjunction with reduced stromal eNOS expression. Our *in vitro* and *in vivo* data suggest that even short-term diabetes impairs progenitor cell supportive and mobilizing capacity of the bone marrow, which might particularly result from 'vascular niche' dysfunction.

The reported amount of bone marrow stem cells in diabetes are discordant, some studies show normal primitive Lin- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) HPC levels,[6,37] whereas others report decreased[38] or even increased[30] quantities. These discordant results probably reflect temporal changes as an effect of diabetes duration. In our study bone marrow $Sca\text{-}1^+Flk\text{-}1^+$ EPC levels were undisturbed consistent with observations by others in early stage diabetes, [30,37,39] indicating that the attenuated mobilization response observed in the present study is not due to reduced pools of progenitor cells present in the bone marrow. In line with this, the number of *ex vivo* cultured bone marrow EPC was unaffected. Impaired progenitor cell mobilization may underlie the chronically reduced EPC levels under steady state conditions as well as the blunted EPC mobilization in response to progenitor cell mobilizing cues observed in diabetes. In the present study exogenous administration of G-CSF and SCF was used to mobilize progenitor cells from the bone marrow to the circulation. G-CSF and SCF are amongst the cytokines that are endogenously released in response to an ischemic event[40] and have been shown to synergistically induce progenitor cell mobilization.[33,41] In non-diabetic mice, a pronounced over 2-fold increase in circulating EPC levels was indeed observed, while this mobilization response after G-CSF/ SCF-

injections was significantly blunted in diabetic animals. This is in line with observations in animal and patient studies reporting less efficient progenitor cell mobilization in response to an acute ischemic event in diabetic compared to non-diabetic subjects.[6,29,42] Additionally, Ferraro et al. showed that diabetic patients that underwent G-CSF elicited HPC mobilization prior to autologous bone marrow transplantation for hematologic conditions were not able to mobilize $CD34^+$ HPC as effective as non-diabetic patients.[30] Furthermore, Fadini et al. reported that dysfunctional progenitor cell mobilization in response to exogenous G-CSF in both type 1 and type 2 diabetic patients extended to a broad range of progenitor cell populations, $CD34^+$ HPC and $CD34^+KDR^+$ EPC amongst others.[24]

Several pathologic processes that occur in the bone marrow under diabetic circumstances, such as dysfunction of the bone marrow niches,[30] altered gene expression[38,39] and cytokine signaling,[24,38] and bone marrow vasculo-[43] and neuropathy,[31] have been implicated to impair bone marrow function and progenitor cell differentiation and mobilization. Here we focused on the effects of diabetes on the progenitor cell supportive role of the stromal compartment of the bone marrow by studying the interplay between progenitor cells and supporting stromal cells in two *in vitro* models. First the progenitor cell supportive function of primary diabetic bone marrow stroma was assessed by co-culturing the plastic-adherent stromal bone marrow fractions from mice with human $CD34^+$ CB-HPC. This co-culture model supported HPC to survive and give rise to CFU after an *ex vivo* culture period of 10 days. However, when bone marrow stromal cells from diabetic mice were used fewer CFU were obtained compared to stromal cells from non-diabetic mice, indicating that diabetes induces impairment in the progenitor cell supportive function of the bone marrow stroma. Interestingly, these observations were made under normoglycemic culture conditions, suggesting that the diabetic stromal cell impairment is – at least to some extent – imprinted upon the cells. Furthermore, we showed that eNOS expression was reduced in diabetic stroma, which could partially explain the reduced supportive function, since NO is an important factor in progenitor cell mobilization, proliferation and maintenance.[44]

The second *in vitro* model particularly focused on the effects of hyperglycemia on a progenitor cell supporting endothelial environment resembling the 'vascular niche' of the bone marrow. Therefore isolated human endothelial cells expressing E4Orf1 were co-cultured with human $CD34^+$ CB-HPC. E4Orf1 expression in primary endothelial cell cultures results in a capacity of the endothelial cells to remain in a confluent state for a substantially prolonged period of time and the capacity to provide a functional vascular niche for progenitor cell expansion.[35] The extended endothelial survival is mediated by chronic Akt-phosphorylation and FGF-2/ FGF-R1 activation.[35] Using this model fewer CFU were obtained under hyperglycemic conditions compared to normoglycemic conditions. This observation suggests that the endothelial capacity to support progenitor cells is impaired under hyperglycemic conditions, which is possibly due to disturbed interaction between the progenitor and endothelial cells.

Diabetes is associated with systemic microangiopathy, which has been shown to extend to the bone marrow causing capillary rarefaction, increased microvascular permeability, and endothelial cell apoptosis and dysfunction.[43] To assess the function and regeneration

of the bone marrow vasculature *in vivo*, both diabetic and non-diabetic animals were injected with 5-FU. 5-FU causes a destruction of the cycling hematopoietic cells as well as the bone marrow sinusoids, while preserving quiescent stem and vascular progenitor cells. The recovery after 5-FU induced myelosuppression depends on a mutual restoration of hematopoiesis and angiogenesis and is therefore of particular value to study conditions that affect hemangiogenic restoration of the bone marrow.[18,36] Diabetic mice proved to be dramatically impaired in recovering from 5-FU challenge and died during the experiment from bone marrow failure while control animals all recovered. This failure of diabetic animals to recover from 5-FU challenge is consistent with dysfunctional hemangiogenic reconstitution due to impaired restoration of hematopoiesis, angiogenesis, or a combination hereof. We cannot rule out the fact that the hyperglycemic condition had an effect on the long-term repopulating stem cells in the bone marrow explaining reduced progenitor cell numbers and repopulating potential, however overnight exposure to hyperglycemia did not have any marked effects on $CD34^+$ HPC *in vitro* suggesting that impaired restoration of the 'vascular niche' is the key factor in the dysfunctional bone marrow recovery after 5-FU in diabetes. However the lack of effects after overnight exposure of $CD34^+$ HPC to hyperglycemia *in vitro*, cannot fully rule out effects after sustained *in vivo* exposure. Importantly, Orlandi et al. found no specific impairment of the 'vascular niche' in diabetic mice with 20 weeks duration of diabetes, however they focused on quantification of SLAM+-cells, a specific osteoblast type that is localized to the vascular niche, but did not study functionality of the 'vascular niche'. Furthermore, Orlandi et al. found that the bone marrow content of LSK HPC in diabetic mice is unaffected for up to 12 weeks duration of diabetes such as in our study, although a decline in number and engraftment efficiency was observed in mice after prolonged (20 weeks) duration of diabetes.[39] It is therefore possible that the bone marrow progenitor cell content remains unaffected in the initial phase of diabetes, but that long-term diabetes leads to reduced bone marrow EPC levels.[38,39] The present study aimed primarily at providing functional data on the interaction of bone marrow stromal and progenitor cells rather than complete mechanistic insight regarding the influence of diabetes on bone marrow function and progenitor cell mobilization. Further studies are required to identify the specific pathways involved in diabetes induced impairment of progenitor cell mobilization and the progenitor cell supportive role of bone marrow stroma and the bone marrow endothelium in particular. In the regulation of progenitor cell mobilization, various cytokines, proteases, and cell-cell contact proteins play a central role. A critical pathway involves MMP-9-mediated cleavage of membrane-bound kit-ligand, releasing soluble kit ligand or SCF, which triggers progenitor cells to transfer from a quiescent to a proliferative niche.[45,46] The activation of MMP-9 has been shown to be NO-dependent.[21] Based on bone marrow transplantation experiments between eNOS knockout mice and wild-type controls, it was shown that EPC mobilization in response to VEGF specifically depends on NO produced in bone marrow stromal cells; and not the progenitor cells themselves.[21] As diabetes and hyperglycemia have been shown to attenuate endothelial NO-production, this may be involved in the impaired function of the bone marrow endothelium. Indeed, eNOS in diabetic bone marrow displays an aberrant

enzyme function[47] and altered expression in bone marrow stroma, as shown in the current study, as well as in mononuclear cells[38] and increasing NO-availability by hyperoxia restored EPC levels in diabetic animals.[28] In addition, bone marrow oxidative stress levels are increased in diabetic animals[38,43] and increasing antioxidant levels prevented the development of bone marrow microangiopathy.[43] Also, in a study of bone marrow plasma cytokine levels in diabetic mice, various factors including VEGF were reduced.[39] In this study, SDF-1α levels were reduced without reaching statistical significance. However, Tepper et al. found the modulation of SDF-1α bone marrow levels in diabetic mice after peripheral skin injury to be significantly disturbed leading to impaired EPC mobilization. Restoration of SDF-1α induced mobilization by administration of plerixafor restored attenuated EPC mobilization supporting a pivotal role of the SDF-1α bone marrow 'mobilizing switch' in diabetes.[37] Abnormality of the SDF-1α signaling pathway, in particular disturbed upregulation of CD26/ DPP-4 in response to G-CSF, was shown to play a role in disturbed progenitor cell mobilization in humans with diabetes as well.[24,48]
In line with the observation in humans of Fadini and co-workers,[24] we found that the impaired mobilization of progenitor cells in diabetic animals was not restricted to the EPC subfraction of the HPC, but extends to a broader range of progenitor cells. HPC transplantation forms a widely accepted and well-established treatment strategy for both benign and malignant hematologic conditions,[49] and the novel applications of HPC transplantation or transplantation of its specific subpopulations in for instance autoimmune and cardiovascular disease have been appreciated more recently.[49,50] A common characteristic of these strategies is that their success – at least partly – depends on the number of $CD34^+$ progenitor cells that can be isolated from the blood or bone marrow.[51,52] It has been recently shown that diabetic patients undergoing HPC mobilization with G-CSF for hematologic conditions are 'poor mobilizers' of $CD34^+$ HPC,[30] which underlines that the results of the present study are also relevant beyond the cardiovascular field.
This study has some limitations. First, since this study particularly focused on the mobilization of EPC we primarily characterized these $Sca\text{-}1^+Flk\text{-}1^+$ cells. In order to study the influence of diabetes on a broader range of progenitor cells and to exclude an exclusive effect on EPC we additionally studied cells that were either $Sca\text{-}1^+$ or $c\text{-}Kit^+$. The veritable murine hematopoietic stem cells are Lin^- $Sca\text{-}1^+c\text{-}Kit^+$ cells, even if it would be expected that mobilization of these cells is disturbed in our animal model, we cannot draw conclusions on this specific progenitor cell population. Second, *in vitro* experiments were performed using $CD34^+$ CB-HPC, instead of bone marrow $CD34^+$ cells. The advantage of cord blood derived $CD34^+$cells is that they are relatively free of potential confounding 'environmental challenges', such as aging and disease, which could negatively affect cell function. Furthermore, we performed interspecies experiments, which could have potentially influenced the results of the individual experiments. However, such interspecies experiments have been performed previously, showing that mouse-derived stromal cells could effectively support human progenitor cells.[53,54] Additionally, cord blood cells have a relatively low immunogeneity compared to bone marrow cells,[55,56] which could have reduced the effects of interspecies differences. Moreover, these factors were all similar in

the diabetic as well as in the non-diabetic experiments and therefore unlikely to have resulted in bias.

In conclusion, early stage experimental diabetes results in reduced steady state EPC levels and a blunted mobilization response, despite undisturbed progenitor cell levels in the bone marrow. Furthermore, we show that hemangiogenic regenerative potential of diabetic bone marrow is impaired. Moreover, diabetic stroma, wherein eNOS expression is reduced, is less effective in its HPC supporting function *in vitro*, which could be a result of 'vascular niche' dysfunction in particular. Therapeutic interventions known to improve endothelial function, such as enhanced NO bioavailability, may also have beneficial effects on the endothelium residing in the vascular niche, hence partly restoring the dysfunctional EPC mobilization in diabetes.

ACKNOWLEDGEMENTS

The technical advice from Dr. Geurt Stokman and Dr. Jaklien Leemans from the Amsterdam Medical Center, the Netherlands, is gratefully acknowledged.

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9

PART THREE

Role of endothelial progenitor cells and platelet function in cardiovascular disease

CHAPTER 10

Bone Marrow Alterations and Lower Endothelial Progenitor Cell Numbers in Critical Limb Ischemia Patients

Plos One. 2013; 8(1): e55592

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ABSTRACT

Background

Critical limb ischemia (CLI) is characterized by lower extremity artery obstruction and a largely unexplained impaired ischemic neovascularization response. Bone marrow (BM) derived endothelial progenitor cells (EPC) contribute to neovascularization. We hypothesize that reduced levels and function of circulating progenitor cells and alterations in the BM contribute to impaired neovascularization in CLI.

Methods

Levels of primitive ($CD34^+$ and $CD133^+$) progenitors and $CD34^+KDR^+$ EPC were analyzed using flow cytometry in blood and BM from 101 CLI patients in the JUVENTAS-trial (NCT00371371) and healthy controls. Blood levels of markers for endothelial injury (sE-selectin, sICAM-1, sVCAM-1, and thrombomodulin), and progenitor cell mobilizing and inflammatory factors were assessed by conventional and multiplex ELISA. BM levels and activity of the EPC mobilizing protease MMP-9 were assessed by ELISA and zymography. Circulating angiogenic cells (CAC) were cultured and their paracrine function was assessed.

Results

Endothelial injury markers were higher in CLI ($P<0.01$). CLI patients had higher levels of VEGF, SDF-1α, SCF, G-CSF ($P<0.05$) and of IL-6, IL-8 and IP-10 ($P<0.05$). Circulating EPC and BM $CD34^+$ cells ($P<0.05$), lymphocytic expression of CXCR4 and CD26 in BM ($P<0.05$), and BM levels and activity of MMP-9 ($P<0.01$) were lower in CLI. Multivariate regression analysis showed an inverse association between IL-6 and BM $CD34^+$ cell levels ($P=0.007$). CAC from CLI patients had reduced paracrine function ($P<0.0001$).

Conclusion

CLI patients have reduced levels of circulating EPC, despite profound endothelial injury and an EPC mobilizing response. Moreover, CLI patients have lower BM $CD34^+$-cell levels, which were inversely associated with the inflammatory marker IL-6, and lower BM MMP-9 levels and activity. The results of this study suggest that inflammation-induced BM exhaustion and a disturbed progenitor cell mobilization response due to reduced levels and activity of MMP-9 in the BM and alterations in the SDF-1α/ CXCR4 interaction contribute to the attenuated neovascularization in CLI patients.

INTRODUCTION

Critical limb ischemia (CLI) is a major health care problem, associated with a high risk of limb loss [1] as well as a high short-term cardiovascular ischemic event rate and increased mortality.[2-4] CLI is caused by obstruction of lower extremity arteries – most often due to atherosclerosis – in combination with a yet largely unexplained impaired ischemic neovascularization response. Postnatal neovascularization in response to tissue ischemia occurs not only by migration and proliferation of resident mature endothelial cells but also involves bone marrow (BM) derived endothelial progenitor cells (EPC).[5] In response to hypoxia, the local production of chemokines and growth factors such as stromal cell-derived factor-1α (SDF-1α) and vascular endothelial growth factor (VEGF) is upregulated, leading to elevated blood levels. In the BM microenvironment this induces release and activation of matrix metalloproteinases (MMPs) causing EPC, which are positive for the SDF-1α receptor CXCR4 and VEGF receptor 2 (VEGFR-2, KDR) to mobilize to the circulation.[6] EPC subsequently contribute to neovascularization, either by physical incorporation into the endothelial layer or by excretion of paracrine factors that stimulate proliferation of resident endothelial cells,[5] the latter being likely the paramount mechanism,[7, 8] occurring in delicate concert with other circulating cells, such as monocytes.[9] Patients with CLI have a large burden of cardiovascular risk factors and endothelial dysfunction, characterized by reduced nitric oxide (NO) bioavailability. The presence of cardiovascular risk factors and overt cardiovascular disease have been associated with reduced numbers and impaired function of circulating EPC.[10-14] Although it has been clearly demonstrated that circulating EPC increase in response to acute tissue injury or ischemia,[15-17] studies that have reported on EPC number and function in patients with chronic continuous ischemia as a result of ongoing cardiovascular disease, as is the case in chronic CLI, are scarce. In patients with chronic ischemic heart disease, the number of circulating EPC was reduced.[18, 19] Thus far, only few small studies have reported reduced numbers of circulating EPC in chronic CLI.[12, 13, 20, 21] Only Fadini et al. reported on circulating angiogenic cells (CAC), which like circulating EPC exert their angiogenic effects mainly via a paracrine mechanism,[22] and found reduced clonogenic and adhesive function of these cells in 15 patients with PAD, however the proportion of CLI patients was not defined, as compared to control subjects.[13] Levels of progenitor cells in the BM of patients with cardiovascular disease have rarely been studied relative to the healthy situation. Heeschen et al. observed no differences in the percentage of BM-MNC expressing CD34 in 18 patients with ischemic cardiomyopathy compared to healthy controls, but significant impairment of BM progenitor cell function.[19] This observation was later confirmed by Kissel and colleagues in a population of 94 ischemic cardiomyopathy patients.[18] Oda et al. recently reported no significant differences in the fraction of BM-MNC expressing CD34, but hypocellularity of the BM and hence an absolute reduction of BM $CD34^+$-cell content in a small heterogeneous group of 16 CLI patients (atherosclerosis obliterans and other causes of CLI) compared to healthy controls.[23]

We hypothesized that in patients with CLI circulating EPC are dysfunctional and numerically reduced due to a direct negative impact of this systemic disease on the BM, causing BM exhaustion, impaired progenitor cell mobilization, and cellular dysfunction. In 101 CLI patients participating in an ongoing trial [24] on the effects of BM mononuclear cell (MNC) administration, we investigated the numbers of hematopoietic and endothelial progenitor cells in BM and peripheral blood (PB), as well as CAC outgrowth from PB-MNC and their paracrine function, in comparison to healthy controls. EPC mobilizing (eg SDF-1α and VEGF) and inflammatory (eg interleukin-6 and interleukin-8) factors were assessed in PB and MMPs (MMP-2 and 9) were assessed in BM as factors mediating and CXCR4 and CD26 expression as factors modulating [25, 26] EPC mobilization from the BM.

METHODS

Study subjects

We collected PB and BM samples from 101 patients with CLI participating in the JUVENTAS study; a trial evaluating the clinical effects of intra-arterial infusion of BM-MNC in CLI (clinicaltrials.gov NCT00371371).[24] Patients with chronic CLI, an ankle-brachial index (ABI) of 0.6 or less, or an unreliable index (non-compressible or not in proportion to the Fontaine classification), and who were not candidate for conventional revascularization are included in this trial. 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 impossibility to complete follow-up. Control PB samples were collected from 37 gender- and age-matched healthy subjects. Control BM samples were obtained from patients without a history of peripheral arterial disease undergoing orthopaedic surgery (n=12 and n=8 for flow cytometry and ELISA, respectively).

In patients with CLI a total volume of 100 ml BM was aspirated from the iliac crest under local anaesthesia and conscious sedation for use in the clinical trial. Seven ml of BM was reserved for (functional) characterization of BM cells. In the BM control group approximately 15 ml of BM was harvested from the surgical site under general anaesthesia. CLI patients and PB controls were asked to fill out health-related questionnaires, and underwent a brief physical examination and laboratory testing (complete blood count, liver enzymes, kidney function, lipid spectrum, and glucose and homocysteine level). BM control subjects filled out a similar questionnaire and underwent physical examination and laboratory testing as part of the hospital's pre-operative screening program.

Ethics statement

The study has been approved by the Medical Ethics Committee of the University Medical Center Utrecht, was performed conform the Declaration of Helsinki, and all patients gave written informed consent.

Plasma and serum measurements

Biochemical parameters (liver enzymes, kidney function, lipid spectrum, and glucose and homocysteine level) were measured using standard clinical laboratory procedures.

All PB samples were collected in potassium-EDTA or serum tubes. All BM samples were collected in a sodium heparin solution. Markers for vascular endothelial activation or injury, soluble E-Selectin (sE-selectin), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1) (R&D Systems, Minneapolis, MN, USA) and thrombomodulin (Diaclone, Stamford, CT, USA), were determined in PB samples using commercially available enzyme immunoassay (ELISA) kits according to manufacturer's instructions. Additionally, PB SDF-1α levels, total (active and inactive) BM plasma MMP-2 (gelatinase A) and 9 (gelatinase B) levels were measured using commercially available ELISA kits (R&D Systems) according to manufacturer's instructions.

Customized group I and II bio-plex multiplex cytokine assays (Bio-rad Labarotories, Hercules, CA, USA) were used to measure levels of cytokines and growth factors (Basic fibroblastic growth factor [FGF-b], 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-β], Tumor necrosis factor-alpha [TNF-α], Tumor necrosis factor related apoptosis inducing ligand [TRAIL], and Vascular endothelial growth factor [VEGF]) in PB serum, according to manufacturer's instructions.

Asymmetric dimethylarginine (ADMA) competitively inhibits production of NO from the substrate arginine by NO synthase, whereas symmetric dimethylarginine (SDMA) may limit NO production by competing with arginine for cellular uptake. Arginine, ADMA, and SDMA were determined in PB plasma using high-performance liquid chromatography (HPLC) with fluorescence detection as previously described,[27] using modified chromatographic separation conditions.[28]

Zymographic analysis

Gelatinolytic activities of BM plasma samples were assessed by zymography as described previously (Figure S1).[29] In short, BM plasma samples were diluted 10 times with phosphate buffered saline and supplemented with loading buffer (0.25 mol/L Tris-HCL, 8% SDS, 40% glycerol, and 0.004% Bromophenol Blue). PageRuler pre-stained protein ladder #SM0671 (Fermentas Life Sciences, Burlington, Ontario, Canada) was used to determine the weights of the gelatinases. Loading buffer was used as negative control and 5 µl of 1:25000 83 kDa active MMP-9 (Calbiochem, La Jolla, CA, USA) was used as positive control. Proteins were separated on a 4% polyacramide stacking gel onto a 10% polyacramide running gel, copolymerized with 2mg/mL gelatine (Sigma-Aldrich, St. Louis, MO, USA). Subsequently gels were incubated overnight at 37ºC in Brij solution (10 mmol/L $CaCl_2$, 0.05% Brij 35 solution [Sigma-Aldrich], 50 mmol/L Tris-HCL pH 7.4)

and stained (25% MeOH, 15% AcOH, and 1% Coomassie Blue). Gels were photographed and picture analysis was performed using NIH ImageJ 1.42q software. The lytic zones of the BM samples were normalized to the positive control and expressed as arbitrary units (AU).

Flow cytometry analysis of circulating and bone marrow progenitor cells

A volume of 100 µl of PB or a BM volume containing $1x10^6$ white blood cells was incubated with anti-CD34-FITC (BD Pharmingen, San Diego, CA, USA), anti-KDR-PE (R&D Systems) and anti-CD45-PE-Cy7 (BD Pharmingen) antibodies for 45 minutes. Erythrocytes were lysed in an ammonium chloride buffer and remaining cells were washed and analyzed by flow cytometry (FC 500, Beckman Coulter, Fullerton, CA, USA). Circulating EPC were identified as $CD34^+KDR^+$ cells in the lymphocytic region of the forward/sideward scatter plot (Figure S2).[30] To assess primitive hematopoietic progenitors, 100 µl of PB or a BM volume containing $1x10^6$ white blood cells was incubated with anti-CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). Primitive hematopoietic progenitors were identified as $CD133^+$ cells in the lymphocytic region of the forward/sideward scatter plot. Cell numbers were quantified per millilitre of blood and relative to $1x10^6$ granulocytic events in the BM. Granulocytic events were identified based on their typical distribution on the forward/sideward scatter plot. Measurements were performed in duplo and results were averaged. Isotype-stained samples served as negative controls. Expression of CXCR4 and CD26 was assessed by incubating 100 µl of EDTA blood or a BM volume containing $1x10^6$ white blood cells with anti-CXCR4-PE (BD Pharmingen) and anti-CD26-FITC (AbD Serotec, Oxford, United Kingdom). Expression of CXCR4 and CD26 was assessed in the complete MNC population as well as in the lymphocyte and monocyte subpopulations. Lymphocytes were identified according to their distribution on the forward / sideward scatter plot. Cells were also incubated with anti-CD14-ECD (Immunotech, Coulter, France) to allow for the separation of monocytes.

Circulating angiogenic cell quantification and functional characterization

CAC numbers obtained from PB-MNC were assessed as described previously.[31] In brief, MNC were isolated from PB samples using density gradient centrifugation (Histopaque 1077, Sigma-Aldrich). To evaluate CAC numbers in culture, MNC were seeded on a human fibronectin (Sigma-Aldrich) coated 6-well plate ($10x10^6$ per well), using EGM-2 medium (Cambrex, Walkersville, MD, USA), supplemented with accompanying aliquots, 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 ng/ml recombinant VEGF-165 (R&D Systems) and antibiotics. Medium was changed after 4 days to remove non-adherent cells. After 7 days, medium was removed and the CAC were placed on serum free medium (EBM-2 with hEGF, hydrocortisone, GA-1000, R3-IGF-1, ascorbic acid, heparin and antibiotics) overnight. Finally, this conditioned medium (CM) was collected and stored for functional experiments. In the applied protocol essentially every adherent cell after the 7-day culture period stained double-positive for acetylated-LDL (ac-LDL) and Ulex europaeus lectin (UEA-lectin; Figure S3). For the experiments in this study CAC obtained

using this protocol were detached using trypsin and cell scraping, and automatically counted on a hemocytometer (Cell-dyn 1800, Abbott laboratories, Abbott Park, IL, USA). To examine the capacity of CAC to excrete paracrine factors that stimulate endothelial cell migration, an *in vitro* scratch wound assay was performed as described previously.[32] Human microvascular endothelial cells (HMEC, Centers for Disease Control and Prevention, Atlanta, GA, USA) were grown to confluence on fibronectin coated 48-well plates, using MCDB-131 medium, supplemented with 10% fetal bovine serum, 5% L-glutamine, 0.1% hEGF, 0.1% hydrocortisone, and antibiotics. Lines were drawn on the bottom of each well to serve as a reference during image acquisition. A straight mechanical scratch was created using a p200 pipette tip and the HMEC monolayer was carefully washed with phosphate buffered saline. CM obtained from the CAC cultures was subsequently placed on the cells. The scratched area was photographed using a light microscope at start and after 6 hours of incubation at 37 ºC. CAC culture medium and CAC serum free culture medium served as positive and negative controls, respectively. After 6 hours the extent of scratch closure was determined relative to the scratch width before incubation, using Image-Pro Plus software version 3.0 (Media Cybernetics, Bethesda, MD, USA). Each sample was measured in two separate wells and each scratch was examined at two separate reference points per well. Cell closure measurements were averaged for data analysis.

Statistical analysis

Continuous data are expressed as mean ± standard deviations (SD) or median and 25th and 75th pecentiles (P25-P75), depending on the normality of the data. Continuous variables were tested whether they fulfilled the assumptions for parametric tests. A Levene's test was used to test for equality of variances between the groups. Independent samples *t*-tests were used to test for differences of parametric variables between two groups. The Mann-Whitney *U*-test was used to test for differences of non-parametric variables between groups. Categorical variables were compared using a chi-square test. False discovery rate (FDR) control of the comparisons of chemokines, growth factors and progenitor cell numbers between CLI patients and healthy controls was performed using the Benjamini and Hochberg method.

Univariate analyses were performed by calculating Spearman's rho, since the majority of variables did not meet the assumptions for the Pearson correlation. Multivariate analyses were performed by performing step forward multivariate linear regression analyses. Variables that did not meet the assumption of normality and constant variance of the residuals were Log^{e}-transformed prior to multivariate linear regression. P-values <0.05 were considered statistically significant. All analyses were performed using SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. Age and sex did not differ between CLI patients and healthy controls. Besides total and LDL-cholesterol levels, which were higher in the healthy controls, classical cardiovascular risk factors, such as presence of hyperhomocysteinemia, hypercholesterolemia and diabetes were all significantly more prominent in CLI patients.

Serum and plasma markers for endothelial activation

Levels of markers for vascular endothelial activation or injury, sICAM-1, sVCAM-1, sE-selectin and thrombomodulin, were significantly higher in CLI patients compared to healthy controls (Table 2). Levels of sVCAM-1 were significantly higher in CLI patients with ulcers or gangrene (Fontaine grade IV) than in CLI patients with ischemic rest pain only (Fontaine grade III) (607±185 ng/ml vs 481±123 ng/ml; P=0.005). Diabetic CLI patients had significantly higher sVCAM-1 levels in comparison to non-diabetic CLI patients (647±200 ng/ml vs 491±127 ng/ml; P=0.006). In addition sVCAM-1 levels correlated with ABI, homocysteine and creatinine levels (P=0.049, P=0.003 and P=0.001, respectively; Table S1).

Widespread inflammatory and progenitor cell mobilizing response in CLI

CLI patients show markedly increased levels of factors associated with progenitor cell recruitment and mobilization, such as SDF-1α, VEGF, SCF, G-CSF, and HGF (Table 2). Inflammatory cytokines, such as IL-6, Il-8, and IP-10 were significantly higher in CLI patients compared to healthy controls, whereas tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which has been associated with anti-inflammatory and anti-atherosclerotic effects was lower in CLI.[33] Levels of IL-8 were higher in CLI patients with diabetes compared to non-diabetic patients (18.7 [13.4-25.2] pg/ml vs 14.3 [9.9-22.5] pg/ml, P=0.048) and IL-6 levels showed a similar trend (10.5 [3.9-15.3] pg/ml vs 4.5 [1.4-11.2] pg/ml, P=0.050). Additionally, both cytokines showed a trend towards an association with disease severity (rho=0.310, P=0.002; rho=0.188, P=0.059, for IL-6 and IL-8, respectively). The progenitor cell mobilizing factors SCF and GRO-α were significantly higher in Fontaine grade IV patients compared to patients with Fontaine grade III (131 [70-173] pg/ml vs 169 [116-212] pg/ml, P=0.025; 93±105 pg/ml vs 131±103 pg/ml, P=0.043, respectively; Table S2).

Reduced L-arginine blood levels in CLI

Arginine was lower in PB plasma of CLI patients than in healthy controls (Table 3). No differences were found in PB for the arginine analogues ADMA and SDMA. Arginine levels were significantly lower in patients with ulcers or gangrene compared to patients with rest pain only (58.1 [46.1-68.6] µmol/l vs 68.5 [61.3-80.6] µmol/l, P=0.005), while SDMA levels were higher in the Fontaine grade IV patients (0.54 [0.46-0.65] µmol/l vs 0.62 [0.54-0.87] µmol/l, P=0.026; Table S1).

Table 1 Subject characteristics.

	CLI patients (n=101)	Healthy controls (n=37)	BM controls (n=12)
Age (years)	65.3±11.8	62.4±14.4	58.6±13.8
Male gender	71 (70%)	23 (62%)	7 (58%)
History of cardiovascular disease			
Previous revascularisation of affected leg	85 (85%)	0 (0%)*	0 (0%)*
CABG	16 (16%)	0 (0%)*	1 (8%)
Congestive heart failure	6 (6%)	0 (0%)	0 (0%)
Myocardial infarction or angina pectoris	35 (35%)	0 (0%)*	3 (25%)
TIA	6 (6%)	0 (0%)	0 (0%)
Stroke	8 (8%)	0 (0%)	0 (0%)
End stage renal disease	3 (3%)	0 (0%)	0 (0%)
Body mass index (kg/m²)	26.6±4.7	24.0±2.9*	25.4±2.4
Currently smoking	26 (26%)	0 (0%)*	3 (25%)
Diabetes	40 (40%)	0 (0%)*	2 (17%)
Hypertension	89 (88%)	2 (5%)*	7 (58%)*
Systolic blood pressure (mmHg)	138±22	129±22*	137±14
Hypercholesterolemia	95 (94%)	2 (5%)*	†
Total cholesterol (mmol/l)	4.3±1.1	5.0±1.0*	†
HDL-cholesterol (mmol/l)	1.1 (0.9-1.5)	1.4 (1.1-1.6)*	†
LDL-cholesterol (mmol/l)	2.3±0.9	3.3±0.8*	†
Triglycerides (mmol/l)	1.3 (0.9-1.9)	0.6 (0.6-0.9)*	†
Statins	90 (89%)	0 (0%)*	3 (25%)*
ACE inhibitors/Angiotensin receptor blocker	55 (55%)	0 (0%)*	4 (33%)
Beta-blockers	46 (46%)	0 (0%)*	4 (33%)
Diuretics	49 (49%)	0 (0%)*	1 (8%)*
Anticoagulants	36 (36%)	0 (0%)*	1 (8%)
Antiplatelet aggregation therapy	71 (70%)	0 (0%)*	2 (17%)*
Homocysteine (µmol/l)	14.9 (12.1-19.9)	12.1 (9.6-13.9)*	†
Currently hyperhomocysteinemic	33 (33%)	0 (0%)*	†
Creatinine (µmol/l)	90.5 (76.0-117.0)	81 (76.0-91.5)*	88 (74.0-95.0)
Hemoglobin (mmol/l)	8.1±1.1	8.9±0.8*	7.5±1.1
Fontaine classification (grade III/IV)	38/63	0/0*	0/0*
Rutherford classification (grade 4/5/6)	38/60/3	0/0/0*	0/0/0*
Ankle-brachial pressure index	0.47±0.26	†	†

Values are presented as absolute numbers and percentage (n [%]) for categorical variables and mean ± SD or medians and P25-P75, unless otherwise specified.
Presence of hypertension, hypercholesterolemia, and hyperhomocysteinemia were determined at the time of inclusion. Hypertension was defined as having a systolic blood pressure >140 mmHg or taking antihypertensive medication. Hypercholesterolemia was defined as having a total cholesterol level >6.5 mmol/l or taking cholesterol reducing medication. Hyperhomocysteinemia was defined as having a homocysteine level >19 µmol/l for men or >17 µmol/l for women. * P<0.05 compared to JUVENTAS patients, † data not available

Decreased EPC levels in CLI and reduced hematopoietic stem cell levels in BM

Circulating levels of $CD34^+$ hematopoietic stem cells (HSC) were not different between CLI patients and healthy controls (Table 4; Figure 1). However, the proportion co-expressing the endothelial lineage marker KDR was significantly lower in CLI patients, resulting in lower circulating EPC ($CD34^+KDR^+$-double-positive EPC; 313 [124-574]

Table 2 Endothelial dysfunction markers, chemokines and MMPs.

	CLI patients	Controls	P-value
Endothelial markers			
sE-selectin (ng/ml)	37.3 (27.6-45.4)**	29.2 (21.7-33.7)	0.002
sICAM-1 (ng/ml)	266 (180-295)**	136 (113-163)	<0.0001
sVCAM-1 (ng/ml)	546±169**	395±108	<0.0001
thrombomodulin (ng/ml)	0.42 (0.18-0.58)**	0.15 (0.04-0.36)	0.001
Chemokines and Growth Factors			
FGF-b (pg/ml)	26.9 (11.7-37.8)	22.1 (10.8-40.9)	0.736
G-CSF (pg/ml)	21.0±14.7*	15.6±24.0	0.017
GRO-a (pg/ml)	120±84**	67±62	0.001
HGF (pg/ml)	541 (442-812)**	297 (208-392)	<0.0001
IL-6 (pg/ml)	6.6 (1.8-14.5)*	4.1 (1.0-7.0)	0.023
IL-8 (pg/ml)	17.0 (11.2-24.5)**	10.6 (7.9-13.8)	<0.0001
IP-10 (pg/ml)	951 (625-1604)**	688 (480-840)	<0.0001
MCP-1 (pg/ml)	55.2±27.9	48.0±27.4	0.191
PDGF-bb (ng/ml)	11.2 (8.7-15.3)	11.5 (8.9-15.7)	0.845
RANTES (ng/ml)	7.7 (6.1-9.6)	8.1 (6.2-11.3)	0.438
SCF (pg/ml)	150 (115-192)*	124 (91-150)	0.013
SCGF-β (ng/ml)	14.5 (10.4-20.0)	15.2 (11.0-19.3)	0.689
SDF-1α (ng/ml)	2.6 (2.2-3.0)*	2.4 (2.2-2.7)	0.012
TNF-α (pg/ml)	9.9 (1.8-22.0)	12.2 (3.1-22.0)	0.867
TRAIL (pg/ml)	92 (68-126)**	124 (98-150)	0.005
VEGF-A (pg/ml)	150±145*	124±89	0.017
MMPs			
MMP-2 (BM) (ng/ml)	129 (117-161)**	95 (78-114)	0.001
MMP-9 (BM) (ng/ml)	381 (270-520)**	660 (570-799)	<0.0001
MMP activity			
MMP-9 (BM) (AU)	414±205**	717±194	0.002
Pro-MMP-9 (BM) (AU)	57±43	110±95	0.167
MMP-2 (BM) (AU)	193±74	133±82	0.064

Data represent means ± SD or medians and P25-P75. * P<0.05 and ** P<0.01 compared to control subjects. Endothelial markers were assessed in a subgroup of 54 CLI-patients and 22 controls.

cells/ml vs 566 [198-1099] cells/ml, P=0.010). In addition the amount of circulating $CD133^+$ primitive hematopoietic cells (ie hemangioblasts) was significantly lower in CLI patients. In a step forward multivariate linear regression model including all patient characteristics (Table 1) age, BMI, and use of antiplatelet drugs were negatively correlated with the amount of $CD34^+$-cells, no correlations of patient characteristics were observed with circulating EPC levels, and $CD133^+$-cells were negatively associated with disease severity (Table S3 shows results of univariate correlations).

The BM of CLI patients contained significantly lower numbers of $CD34^+$-cells compared to that of healthy controls (738 [459-997] cells/$1x10^6$ granulocytes vs 990 [688-1561] cells/$1x10^6$ granulocytes, P=0.018). No differences for $CD34^+KDR^+$-cells and $CD133^+$-cells were observed (Table 4; Figure 2). Of the patient characteristics there was only a negative association of $CD34^+$-cells with ABI and Log^e converted creatinine levels. The other cell types in BM were not correlated with any of the demographic data (Table S3).

Relationship between cell populations and humoral factors

Since MMP-9 and MMP-2 have been suggested to be involved in EPC mobilization [6, 34] levels and activity were studied in the BM. MMP-9 was found to be significantly lower in CLI patients compared to healthy controls, while MMP-2 was significantly higher (Table 2; Table S4 shows univariate correlations of MMP-levels with patient characteristics). Both levels and activity of these MMPs were not related to circulating EPC levels, however a weak positive correlation of MMP-9 with $CD34^+$-cells in circulation was observed (rho=0.255, P=0.011).

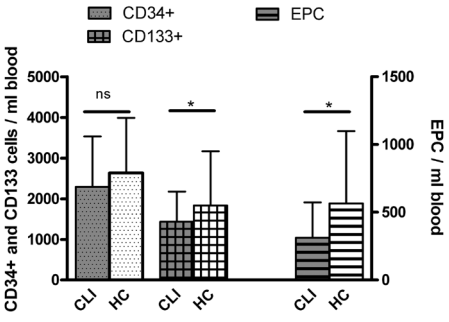

Figure 1 Lower circulating progenitor cell levels in CLI patients. Data represent median and P75. Circulating progenitor cell numbers in CLI patients (n=101) and healthy controls (HC; n=37). The number of circulating $CD133^+$ and $CD34^+KDR^+$ EPC was significantly reduced in CLI patients (* P<0.05). No significant (ns) differences in circulating numbers were observed for $CD34^+$ cells.

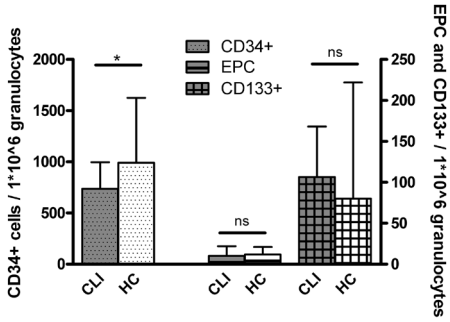

Figure 2 BM $CD34^+$ progenitor cell are reduced in CLI patients. Data represent median and P75. BM progenitor cell numbers in CLI patients (n=101) and healthy controls (HC; n=12). $CD34^+$ cells were significantly reduced (* P<0.05) in CLI patients compared to health controls. No significant (ns) differences in $CD133^+$-cells and $CD34^+KDR^+$ EPC in the BM.

Table 3 Arginine analogues.

	CLI patients	Controls	P-value
Arginine (µmol/l)	62.1 (51.1-74.4)**	78.7 (68.7-86.6)	<0.0001
ADMA (µmol/l)	0.44 (0.41-0.49)	0.46 (0.42-0.51)	0.327
SDMA (µmol/l)	0.59 (0.49-0.76)	0.59 (0.53-0.63)	0.564

Data represent medians and P25-P75. ** P<0.01 compared to control subjects. Arginine analogues were measured in the blood of 74 CLI patients and in 23 healthy controls.

Table 4 Progenitor cells.

	CLI patients	Controls	P-value
Peripheral Blood			
CD34 (cells/ml)	2294 (1630-3530)	2632 (1619-3989)	0.645
%KDR of CD34	12.4 (6.3-27.7)*	22.5 (11.5-36.8)	0.016
CD34KDR (cells/ml)	313 (124-574)*	566 (198-1099)	0.010
CD133 (cells/ml)	1437 (946-2176)*	1836 (1112-3169)	0.040
Bone Marrow			
CD34 (cells/1*10^6gran)	738 (459-997)*	990 (688-1561)	0.018
%KDR of CD34	1.5 (0.7-3.1)	1.1 (0.9-2.8)	0.932
CD34KDR (cells/1*10^6gran)	10.0 (4.9-21.9)	12.0 (6.5-36.0)	0.349
CD133 (cells/1*10^6gran)	107 (67-168)	80 (33-210)	0.292

Data represent medians and P25-P75. * P<0.05 compared to control subjects. Progenitor cells were assessed in 101 CLI patients and in 37 and 12 healthy controls for PB and BM, respectively.

In order to find explanatory factors for the reduction of circulating $CD34^+KDR^+$-cells and BM $CD34^+$-cells in CLI patients, correlations with cytokines and growth factors were studied. In univariate analysis there was a significant positive correlation of circulating $CD34^+KDR^+$-cells with SCGF-β, which remained the only significant predictor in a step forward multivariate linear regression model, including all cytokines and growth factors. In CLI patients the levels of BM $CD34^+$-cells, which were reduced compared to healthy controls, were negatively correlated with circulating levels of HGF and IL-6. After multivariate regression Log^e converted IL-6 was the only factor that was negatively associated with BM $CD34^+$-cells (P=0.007).

Altered expression of CXCR4 and CD26

Expression of CXCR4 and CD26 on blood and BM cells is involved in the mobilization of progenitor cells from the BM, via the modulation of the response to SDF-1α,[25, 26] and was therefore studied in both blood and BM. The percentage of CXCR4 expressing cells in the blood was not different in CLI patients compared to healthy controls for the complete mononuclear cell population (7.5% [4.7-13.4] vs 7.0% [4.6-9.6], P=0.215), as was the case

for mean CXCR4 expression, percentage of CD26 expressing MNC and mean CD26 expression by MNC. The percentage of lymphocytes expressing CXCR4 was significantly higher in CLI patients compared to healthy controls (7.9% [5.2-15.0] vs 5.5% [3.9-8.4], P=0.004), whereas the percentage of CD26 expressing lymphocytes did not significantly differ (1.8% [1.0-2.9] vs 1.7% [1.2-3.1], P=0.582) (Figure 3). The mean CXCR4 expression per monocyte was significantly higher in healthy controls (1.2 [0.9-1.8] vs 1.9 [1.5-2.2], P<0.001), while the percentage of CXCR4 expressing monocytes did not differ significantly. The percentage of CXCR4 and CD26 expressing MNC and lymphocytes in the circulation was not influenced by severity of CLI or presence of diabetes.

In the BM of CLI patients the percentage of MNC expressing CXCR4 (11.1% [7.4-16.8] vs 41.1% [18.5-48.6], P<0.001), and MNC expressing CD26 (1.5% [1.0-2.0] vs 2.3% [1.2-2.7], P=0.024) and the mean expression of CXCR4 (4.6 [4.4-5.0] vs 8.6 [6.4-12.2], P<0.001) were all lower as compared to controls. The percentage of CXCR4 expressing lymphocytes was significantly lower compared to healthy controls (10.3% [7.3-16.2] vs 40.0% [22.0-46.1], P<0.001), which was also the case for the percentage of CD26 expressing lymphocytes (1.4% [1.0-2.0] vs 2.4% [1.2-2.9], P=0.026) (Figure 3). In addition mean CXCR4 expression per lymphocyte in the BM was lower in CLI patients (4.6 [4.3-5.1] vs 8.5 [5.8-12.5], P<0.001). No differences were observed for monocytic expression of both CXCR4 and CD26 in the BM. The percentage of CXCR4 expressing MNC and lymphocytes in the BM were both negatively associated with disease severity (rho=-0.255, P=0.015 and rho=-0.245, P=0.020), but not with diabetes. $CD34^{+}KDR^{+}$ EPC levels both in the circulation as well as in the BM were positively correlated with mean MNC expression of CXCR4 in the BM (rho=0.323, P=0.002; rho=0.370, P<0.001, respectively). This pattern with respect to $CD34^{+}KDR^{+}$ EPC levels was also observed for the mean lymphocytic CXCR4 expression in the BM (rho=0.315, P=0.003; rho=0.374, P<0.001, respectively).

The circulating levels of SDF-1α were neither related to levels of CD26 expressing cells in the circulation nor in the BM compartment. Moreover, SDF-1α levels were not related to the percentage of cells expressing CXCR4 in the BM or the circulation.

Reduced paracrine function despite equal CAC numbers

CAC numbers obtained from cultured PB-MNC were not different between CLI patients and healthy controls (6±8 CAC/1000 MNC vs 10±9 CAC/1000 MNC; P=0.137). In CLI patients the number of CAC was not affected by the presence of diabetes (5±8 CAC/1000 MNC vs 8±9 CAC/1000 MNC; P=0.088) and showed no change with disease severity (9±9 CAC/1000 MNC vs 6±6 CAC/1000 MNC; P=0.116). CAC number was not related to any of the circulating progenitor cell populations.

An important function of CAC during the neovascularization process is the secretion of pro-angiogenic factors.[35] CM obtained from CAC cultures of CLI patients had a significantly reduced capacity to stimulate endothelial cell migration in a scratch wound assay compared to CM obtained from healthy controls (39.7% [28.0-49.8] vs 53.0% [50.0-58.5]; P<0.0001) (Figure 4). In CLI patients the capability of diabetic CM to stimulate scratch wound closure

showed a clear trend to be lower than that of non-diabetic CM (34.3% [11.7-41.3] vs 46.1% [35.1-51.9]; P=0.052) no differences were observed between patients with Fontaine III or IV (P=0.882). After correction for CAC number present in the primary culture the presence of CLI remained a significant negative predictor for scratch wound closure (P<0.0001).

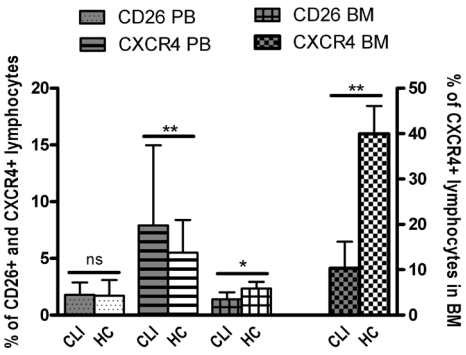

Figure 3 Altered expression of CD26 and CXCR4 in blood and BM of CLI patients. Data represent median and P75. The percentage of CXCR4 expressing lymphocytes was higher in the blood of CLI patients (n=101) compared to healthy controls (HC; n=37), while a significant reduction was observed in the BM (n=101 and n=12 for CLI patients and healthy controls, respectively). The percentage of CD26 expressing lymphocytes was not different in the PB, while a lower percentage of lymphocytes in the BM expressed CD26 in CLI patients. * P<0.05, ** P<0.01

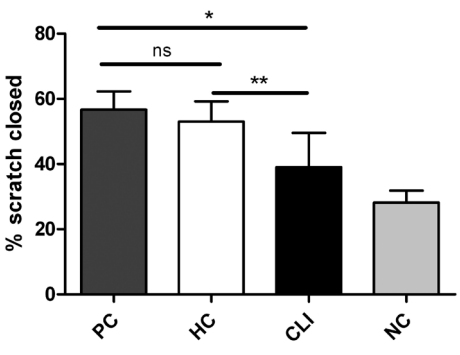

Figure 4 Conditioned medium obtained from CAC cultured from CLI patients has impaired paracrine effects. Data represent median and P75. Percentage of scratch wound closure stimulated with conditioned medium (CM) obtained from CLI derived CAC (n=33) was significantly reduced (P<0.0001) compared to CM obtained from healthy control (HC; n=25) derived CAC. Wound closure after stimulation with HC derived CM was equal to stimulation with a positive control (PC; n=4). CAC serum free culture medium served as a negative control (NC; n=4). * P<0.05, ** P<0.01

DISCUSSION

Our study shows that circulating EPC levels are significantly reduced in patients with CLI on regular medication, despite significant endothelial injury and upregulation of progenitor cell mobilizing factors in the circulation such as SDF-1α and VEGF. The observed reduction of $CD34^+$-cells in the BM of CLI patients suggests that the low circulating EPC levels are at least in part due to BM exhaustion, which appears related to increased systemic inflammation. In addition, BM levels and activity of the progenitor cell mobilizing factor MMP-9 are reduced, indicating that an impairment in the progenitor cell mobilizing response is also involved. Moreover, paracrine function of CAC was impaired. The results of this study suggest that reduced levels and function of circulating progenitor cells and concomitant BM exhaustion and dysfunction contribute to the attenuated neovascularization response in CLI patients.

Our observations of significantly elevated levels of progenitor cell mobilizing chemokines, such as VEGF, SDF-1α, and G-CSF suggest that the ischemic signalling response is not critically disturbed in CLI patients, as has been observed in diabetic animal models.[36] Although our study does not allow definitive mechanistic conclusions, the reduction of BM $CD34^+$-cells in CLI patients observed in the present study suggests BM exhaustion as a potential mechanism that contributes to reduced progenitor cell availability in the circulation. It has been suggested that prolonged exposure to pro-inflammatory stimuli may lead to exhaustion or suppression of the progenitor cell pool in the BM,[37-40] leading to attenuated release of EPC into the circulation and a shift towards the release of more immature and dysfunctional EPC. Indeed, the pro-inflammatory state in CLI with high levels of inflammatory cytokines, such as IL-6, IL-8, and IP-10 [41, 42] and the significant negative correlation between IL-6 levels and $CD34^+$ progenitor cell numbers in the BM of CLI patients supports a role for inflammatory suppression or exhaustion of the BM in CLI.

In addition to inflammation-induced suppression or exhaustion of the BM progenitor pool, an impaired mobilization response of progenitor cells from the BM may contribute to the reduction in circulating EPC. We explored how mobilization cues in the BM environment are affected in CLI. The process of EPC mobilization from the BM is critically dependent on MMP-9.[6, 43] In MMP-9 knockout mice VEGF administration or hindlimb ischemia failed to induce progenitor cell mobilization.[6, 44] The NO-pathway is also essential for the mobilization of BM progenitor cells as eNOS knockout mice show defective VEGF-induced EPC mobilization and profoundly reduced MMP-9 activity, thus phenotypically resembling MMP-9 knockout mice.[45] MMP-9 has been identified as a major target for NO, activating MMP-9 by S-nitrosylation.[46] Patients with cardiovascular diseases, such as CLI have decreased NO bioavailability.[47] Indeed we observed reduced levels of L-arginine, the most important natural occurring substrate for the generation of NO, in CLI, which may negatively affect NO-bioavailability.[45, 47] Furthermore, we show that MMP-9 levels and activity are reduced in the BM of CLI patients. Reduced NO availability in CLI may well extend to the BM compartment and thus — in part – explain the lower MMP-9 levels and activity in CLI patients and hence a reduced mobilization response to tissue ischemia.

The SDF-1α / CXCR4 interaction is another important pathway in the mobilization of progenitor cells from the BM. SDF-1α is a critical mediator for ischemia-specific recruitment of progenitor cells. Gradients of SDF-1α are sensed by cells expressing the SDF-1α receptor CXCR4. We observed altered expression patterns of CXCR4 and CD26 – which is known to inhibit the SDF-1α / CXCR4 interaction[25] – in CLI patients. Since the physical interaction of CXCR4 and SDF-1α retains progenitor cells in their BM niche, disruption of this interaction by CD26 seems an important step in the mobilization of these cells.[26] Therefore the reduced CD26 expression in the BM of CLI patients could hamper the physical release of these cells from the BM to the circulation and thus contribute to a disturbed BM response to tissue ischemia.

In our study, absolute number of $CD34^+KDR^+$ EPC in blood and BM are relatively low, which raises the question about whether these cells could play an important role in neovascularization. However, numbers of $CD34^+KDR^+$ EPC in our control population are

similar to reports by others. In our population – if converted to EPC numbers per 10^6 flow cytometric events – an average of 80 $CD34^+KDR^+$ EPC/ 10^6 events was observed, whereas others report 35 to 70 $CD34^+KDR^+$ EPC/ 10^6 events;[12] 40.9 to 87.4 $CD34^+KDR^+$ EPC/ 10^6 events;[13] 39 to 74 $CD34^+KDR^+$ EPC/ 10^6 events.[48] Furthermore, several studies have shown that the number of $CD34^+KDR^+$ EPC correlates with cardiovascular prognosis and disease severity,[10-13] suggesting a role for these cells in cardiovascular health and vascular regeneration despite the low numbers present in the circulation. Whether this vasculoprotective function is mediated through actions as actual building blocks in neovascularization, or via paracrine pathways[7, 8] cannot be determined by our study.

Other circulating cell types, such as monocytes and lymphocytes, may also be involved in neovascularization.[9, 49, 50] Here we focused on CAC, also known as early EPC, or monocytic EPC. CAC lack characteristics that are required for cells to be considered as true 'progenitors', such as a capacity for clonal expansion, and their capability to form sustainable endothelium in vivo has been challenged. However, CAC can be obtained from PB in relatively high numbers and are potent secretors of proangiogenic factors. In contrast to reduced numbers of circulating $CD34^+KDR^+$ EPC, the number of CAC, was not different between CLI patients and healthy controls. $CD34^+KDR^+$ EPC represent a defined subset of true BM-derived progenitor cells with the ability for clonal expansion from single cells into endothelial-like cell colonies,[51, 52] whereas CAC are mostly derived from monocytes/ macrophages.[35] The pro-inflammatory state in CLI may explain a relatively undisturbed quantity of EPC with inflammatory cell origin. Importantly, we found a marked reduction in their paracrine actions. Such functional impairment of CAC may contribute to the impaired neovascularization response in CLI. Moreover, this may have negative impact on the therapeutic potential of autologous progenitor cell therapy.

In our CLI patients we found a general lack of association of progenitor cell levels with patient characteristics and cardiovascular risk factors, for instance diabetes, which is in contrast to previous reports. This may be explained by the end-stage phase of vascular disease in CLI, where the conditions and risk factors that originally caused and enhanced the development of the disease become overwhelmed by the systemic influence of CLI per se, hence the influence of patient characteristics and cardiovascular risk factors on EPC levels becomes less evident.

This study has limitations, which are in large part related to the limited availability of human BM. We were not able to obtain blood samples from the same control population as where we obtained the BM, ie patients undergoing orthopedic surgery. Therefore our data do not allow conclusions on influence of circulating factors on the BM compartment and vice versa in healthy controls. Furthermore, differences in BM puncture sites between patients and controls, the iliac crest and femoral head respectively, could have influenced composition of the BM. However, we normalized the BM progenitor cell numbers to granulocyte numbers to correct for potential differences in BM composition. No differences were observed in granulocyte numbers between patients and controls.

In conclusion we show that CLI patients, despite profound endothelial injury and an upregulation of progenitor cell mobilizing growth factors and cytokines, have significantly reduced circulating $CD34^{+}KDR^{+}$ EPC. Our data suggest that inflammation-induced suppression or exhaustion of the BM progenitor cell pool, as well as a defective progenitor cell mobilization response due to reduced levels and activity of MMP-9 in the BM and alterations in the SDF-1α / CXCR4 interaction contribute to the defective neovascularization response in CLI.

ACKNOWLEDGEMENTS

We kindly acknowledge Petra de Bree for technical laboratory support, Joost Sluijter for his advice regarding zymogram analysis, and Arjen Schoneveld for his advice and support regarding the multiplex cytokine assays.

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SUPPLEMENTAL INFORMATION

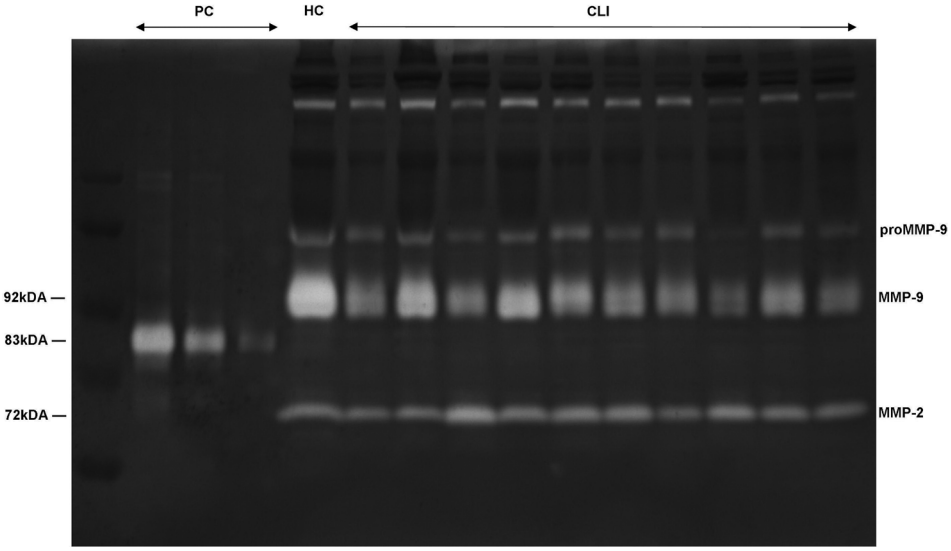

Figure S1 Representative picture of gelatine zymography of BM plasma. Representative picture of a gelatine zymogram showing increased MMP-9 activity in healthy control (HC) BM plasma compared to BM plasma obtained from CLI patients even on gross inspection. For analysis the lytic zones were normalized to the highest concentration of 83kDA active MMP-9 used as a positive control and expressed as arbitrary units (AU).

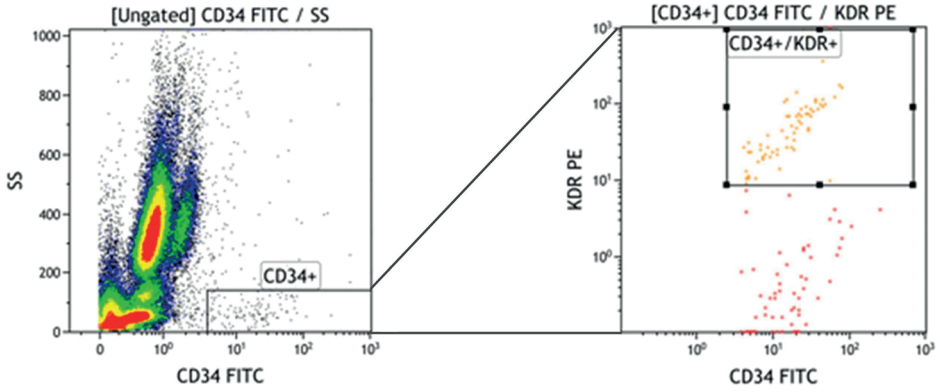

Figure S2 Gating Strategy for detection of CD34+ progenitor cells and CD34+KDR+ EPC. CD34+ progenitor cells were identified in the lymphocytic range of the sideward scatter plot and the KDR+-cells in the CD34+ gate were defined as CD34+KDR+ EPC.

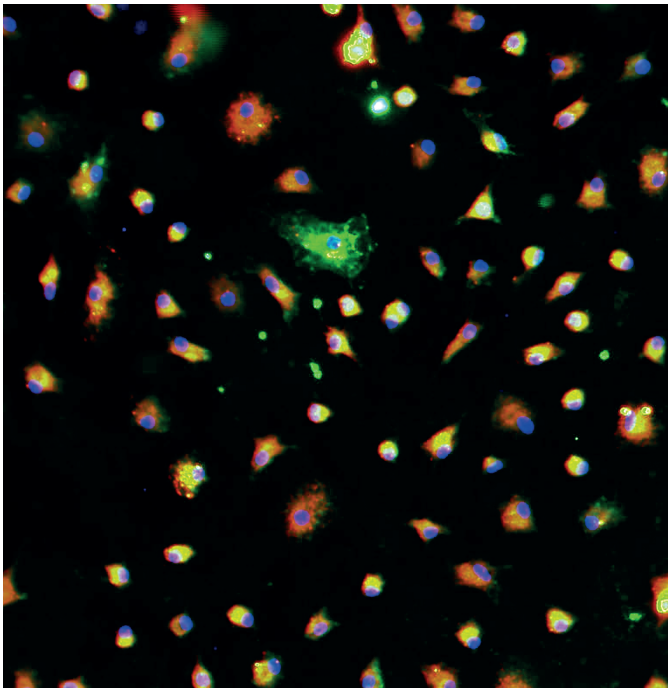

Figure S3 Representative image showing ac-LDL/ UEA-lectin double-staining CAC obtained after defined 7-day culture protocol. PB-MNC after 7-day culture on fibronectin-coated plates in a defined endothelium specific medium acquire endothelial like characteristics and double-staining for ac-LDL/ UEA-lectin, specific for CAC. Red = DiI-labeled ac-LDL, Green = FITC-labeled UEA-lectin, Blue = DAPI, Yellow/Orange = double-stain ac-LDL/ UEA-lectin.

Table S1 Univariate correlation of cardiovascular risk factors and endothelial markers and arginine analogues in CLI patients.

	sE-selectin	sICAM-1	sVCAM-1	thrombomodulin	Arginine	ADMA	SDMA
Age	-.21	-.31*	.27	.09	-.09	.17	-58**
Male gender	-.03	.17	-.18	-.10	-.08	-.16	.04
Body mass index	.07	.06	.01	.09	-.13	.14	-.10
Currently smoking	.09	.28**	-.20	-.16	.03	-.09	-.30**
Diabetes	.06	-.16	.37**	.09	-.31**	-.01	.41**
Hypertension	.14	.07	.08	.00	.02	.03	.12
Systolic blood pressure	-.06	-.13	.07	.04	.02	-.06	.11
Hypercholesterolemia	-.01	-.02	.05	-.06	-.04	.06	-.03
Total cholesterol	.14	.16	-.09	.06	.09	-.03	-.10
HDL-cholesterol	.14	.18	.09	.03	.05	-.21	.00
LDL-cholesterol	.01	.10	-.06	.14	.19	.09	-.13
Triglycerides	-.11	-.03	-.16	-.12	-.16	.03	-.02
Homocysteine	-.02	-.16	.41**	.33*	-.12	.39**	.66**
Creatinine	-.07	-.14	.44**	.37**	-.06	.11	.66**
Fontaine classification (grade III/IV)	.02	-.02	.39**	.23	-.33**	.13	.26*
Medication use							
Statins	.16	.14	-.25	.14	.16	-.24*	-.14
ACEI/ARB	.20	-.17	.17	.21	.07	.20	.35**
Beta-blockers	-.16	-.02	-.06	-.08	-.13	.07	.09
Diuretics	.15	-.03	.16	.36*	-.11	.11	.33**
Anticoagulants	-.09	-.25	.07	-.29*	-.30**	.02	-.12
APT	-.01	.15	-.19	.10	.27*	-.01	.01

Data represent Spearman's rho or point-biserial correlation coefficients (rpb) in case one of the variables is nominal. Presence of hypertension, hypercholesterolemia, and hyperhomocysteinemia were determined at the time of inclusion. Hypertension was defined as having a systolic blood pressure >140 mmHg or taking antihypertensive medication. Hypercholesterolemia was defined as having a total cholesterol level >6.5 mmol/l or taking cholesterol reducing medication. ACEI/ARB=ACE inhibitor or angiotensin receptor blocker. APT=Antiplatelet therapy. Light grey cells indicate significant positive correlations and dark grey cells significant negative correlations. * P<0.05, ** P<0.01

Table S2 Univariate correlation of cardiovascular risk factors and chemokines and growth factors in CLI patients.

	FGF-b	G-CSF	GRO-a	HGF	IL-6	IL-8	IP-10	MCP-1	PDGF-bb	RANTES	SCF	SCGF-β	SDF-1α	TNF-α	TRAIL	VEGF-A
Age	-.21*	-.08	-.01	-.01	.02	-.01	.17	-.17	-.16	-.24*	.20*	-.14	.21*	-.09	-.03	-.10
Male gender	.07	-.12	.06	.00	-.08	.02	.12	-.10	-.06	.00	-.15	-.12	-.06	-.05	.08	-.18
Body mass index	-.06	.04	.08	.14	-.04	-.02	.02	.00	.02	.15	.03	-.14	.08	-.09	.13	.12
Currently smoking	.04	.05	-.05	.03	-.06	-.16	-.37**	.05	.15	.21*	-.19	.11	-.10	-.12	-.06	.08
Diabetes	-.06	-.08	.19	.19	.21*	.20*	.10	-.06	-.24*	-.08	.34**	-.11	.16	.08	-.02	.08
Hypertension	-.06	-.09	.01	.06	.02	-.01	.11	-.02	-.11	-.16	.12	.10	.01	-.12	.03	-.02
Systolic blood pressure	-.13	-.14	-.20*	-.12	-.26**	-.17	.02	.04	-.11	.24*	-.05	.18	-.03	-.20*	.13	-.17
Hypercholesterolemia	-.11	.00	-.09	.04	-.10	-.09	-.12	-.07	.04	.08	.12	-.06	.00	-.17	.20*	-.09
Total cholesterol	-.20*	-.30**	-.29**	-.23*	-.32**	-.27**	-.03	-.17	-.08	.06	-.18	-.08	-.34**	-.21*	.21*	-.33**
HDL-cholesterol	-.07	-.11	-.24*	-.26*	-.24*	-.23*	.01	-.08	.08	.01	-.31**	-.10	-.22*	-.13	-.01	-.24*
LDL-cholesterol	-.11	-.21*	-.27**	-.27**	-.17	-.25*	-.13	-.09	.01	.04	-.19	.01	-.32**	-.10	.19	-.24*
Triglycerides	-.13	-.13	.05	.14	-.11	.02	.10	-.06	-.17	-.02	.15	.02	.01	-.10	.23*	.05
Homocysteine	-.11	-.12	.14	.21*	.12	.21*	.16	-.10	-.06	-.19	.36**	.03	.32**	.04	-.04	-.02
Creatinine	-.19	-.03	.01	.05	.04	.02	.10	-.01	-.11	-.15	.67**	.12	.46**	-.07	-.12	-.01
Fontaine classification	.02	-.06	.20*	.11	.30**	.19	-.10	-.03	-.16	-.34**	.22*	-.14	.12	.11	-.08	.10

Table S2 continued

Medication use																
Statins	-.02	.06	.06	-.10	-.03	-.02	-.03	.13	-.11	-.13	.04	-.11	-.02	-.09	.00	-.07
ACEI/ARB	-.21*	-.16	.00	-.11	-.06	-.16	-.04	-.02	-.33**	-.11	.19	-.01	.13	-.12	-.09	-.11
Beta-blockers	-.19	-.09	.06	-.04	-.07	-.04	.09	-.01	-.27**	-.14	.17	-.14	.15	-.20*	-.08	-.03
Diuretics	.02	-.07	-.07	.13	-.01	.03	.10	-.19	-.10	-.01	.31**	.09	.18	.02	-.04	-.07
Anticoagulants	.16	.01	.07	.14	.12	.21*	.18	-.10	-.17	.14	.10	.00	.06	-.01	-.02	-.05
APT	-.04	.13	-.05	-.12	-.02	-.05	-.11	.23*	.16	-.03	-.15	-.06	-.07	.11	-.10	.09

Data represent Spearman's rho or point-biserial correlation coefficients (rpb) in case one of the variables is nominal. Presence of hypertension, hypercholesterolemia, and hyperhomocysteinemia were determined at the time of inclusion. Hypertension was defined as having a systolic blood pressure >140 mmHg or taking antihypertensive medication. Hypercholesterolemia was defined as having a total cholesterol level >6.5 mmol/l or taking cholesterol reducing medication. ACEI/ARB=ACE inhibitor or angiotensin receptor blocker. APT=Antiplatelet therapy. Light grey cells indicate significant positive correlations and dark grey cells significant negative correlations. * P<0.05, ** P<0.01

Table S3 Univariate correlation of cardiovascular risk factors and progenitor cell numbers in CLI patients.

	Peripheral Blood				Bone Marrow				CAC	
	CD34	%KDR of CD34	CD34KDR	CD133	CD34	%KDR of CD34	CD34KDR	CD133	CAC	Paracrine effect (n=32)
Age	-.29**	.10	-.07	-.26**	-.13	.03	.00	-.11	-.12	-.04
Male gender	-.08	.07	-.01	.15	.01	.12	.13	.03	-.06	-.02
Body mass index	-.07	-.03	-.08	-.06	-.01	-.03	-.06	-.10	.13	-.21
Currently smoking	.00	.11	.16	.14	-.02	.08	.08	-.13	.06	.02
Diabetes	-.19	-.03	-.14	-.34**	-.15	.18	.10	-.07	-.18	-.36*
Hypertension	.11	.02	.14	.05	-.05	-.04	-.07	-.05	.00	-.07
Systolic blood pressure	.11	.08	.20*	.07	-.04	-.03	-.07	-.09	.07	.03
Hypercholesterolemia	-.11	.21*	.15	.07	-.14	.15	.10	-.14	-.14	N/A
Total cholesterol	.04	.00	.04	.06	.20	-.16	-.06	.07	.07	-.31
HDL-cholesterol	.01	-.03	-.01	.01	.10	.02	.10	.17	.05	.27
LDL-cholesterol	.05	-.03	.04	.18	.11	-.21*	-.15	.07	.02	-.33
Triglycerides	.13	.03	.05	-.04	.20*	-.11	-.03	-.05	.09	-.31
Homocysteine	-.13	.10	.04	-.05	-16	.00	-.08	-.12	-.12	-.20
Creatinine	-.05	.03	.02	-.15	-.18	.02	-.03	-.26*	-.14	-.05
Fontaine classification (grade III/IV)	-.08	-.11	-.10	-.26**	-.20*	-.04	-.13	-.07	-.16	.03
Medication use										
Statins	.04	-.13	-.10	-.17	-.08	-.05	-.14	-.02	.14	.39*
ACEI/ARB	.07	-.06	-.04	-.03	.00	.02	.03	.03	-.10	-.16
Beta-blockers	.13	-.03	.04	.05	.09	-.19	-.16	-.02	.08	.06
Diuretics	-.07	.01	-.07	-.11	-.14	.03	-.01	.01	-.18	-.47**
Anticoagulants	.10	.13	.21*	.14	-.09	.20*	.14	.01	.08	.09
APT	-.12	-.05	-.17	-.13	.04	-.14	-.13	-.10	.03	-.05

Data represent Spearman's rho or point-biserial correlation coefficients (rpb) in case one of the variables is nominal. Presence of hypertension, hypercholesterolemia, and hyperhomocysteinemia were determined at the time of inclusion. Hypertension was defined as having a systolic blood pressure >140 mmHg or taking antihypertensive medication. Hypercholesterolemia was defined as having a total cholesterol level >6.5 mmol/l or taking cholesterol reducing medication. ACEI/ARB=ACE inhibitor or angiotensin receptor blocker. APT=Antiplatelet therapy. Light grey cells indicate significant positive correlations and dark grey cells significant negative correlations. * P<0.05, ** P<0.01

Table S4 Univariate correlation of cardiovascular risk factors and MMP-2 and 9 levels and activity in bone marrow of CLI patients.

	Levels		Activity		
	MMP-2	**MMP-9**	**MMP-2**	**Pro-MMP-9**	**MMP-9**
Age	.33**	-.09	-.02	.02	-.07
Male gender	-.09	.12	-.14	-.07	-.10
Body mass index	-.03	.03	-.22	-.14	-.07
Currently smoking	-.34**	.22**	-.02	.08	.02
Diabetes	.45**	-.08	.21	-.02	-.15
Hypertension	-.10	-.11	.02	-.18	-.14
Systolic blood pressure	-.08	-.04	.13	-.04	-.03
Hypercholesterolemia	-.11	.18	.04	.05	.02
Total cholesterol	-.26**	-.08	-.19	-.10	.00
HDL-cholesterol	.05	-.12	-.03	-.18	-.17
LDL-cholesterol	-.30**	-.08	-.11	-.08	.03
Triglycerides	-.13	.06	-.12	.01	.07
Homocysteine	.31**	.07	.14	.20	.15
Creatinine	.44**	-.02	.28*	-.15	-.11
Fontaine classification (grade III/IV)	.33**	-.04	.22	.02	.08
Medication use					
Statins	-.13	-.13	-.19	-.15	-.17
ACEI/ARB	.26**	-.13	.31*	.08	.08
Beta-blockers	.06	.00	.19	.04	.13
Diuretics	.15	.00	.13	.03	.05
Anticoagulants	-.08	.07	-.05	.03	.19
APT	.06	-.10	.03	.03	-.06

Data represent Spearman's rho or point-biserial correlation coefficients (rpb) in case one of the variables is nominal. Presence of hypertension, hypercholesterolemia, and hyperhomocysteinemia were determined at the time of inclusion. Hypertension was defined as having a systolic blood pressure >140 mmHg or taking antihypertensive medication. Hypercholesterolemia was defined as having a total cholesterol level >6.5 mmol/l or taking cholesterol reducing medication. ACEI/ARB=ACE inhibitor or angiotensin receptor blocker. APT=Antiplatelet therapy. Light grey cells indicate significant positive correlations and dark grey cells significant negative correlations. * P<0.05, ** P<0.01

APPENDIX I

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10

PART THREE

Role of endothelial progenitor cells and platelet function in cardiovascular disease

CHAPTER 11

Bone marrow microvascular and neuropathic alterations in critical limb ischemia patients

In preparation

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ABSTRACT

Rationale

The impact of severe cardiovascular disease and critical limb ischemia (CLI) on the bone marrow (BM) is largely unknown.

Objective

Investigate microvascular and neuropathic changes in BM of CLI patients.

Methods and Results

BM biopsies were obtained from CLI patients (n=12) included in the Juventas-trial (NCT00371371) and controls (n=12). We performed histomorphometry and immunohistochemistry of the BM to assess microvascular density (MVD), and to evaluate pan-neuronal and sympathetic innervation, which is involved in progenitor cell (PC) mobilization. MVD was significantly reduced in CLI compared to controls (P=0.03), as was sympathetic (P=0.01) and pan-neuronal innervation (P=0.001). No differences between CLI patients with and without diabetes were observed.

Conclusions

CLI is associated with both BM microvascular and neuropathic changes, which may impede PC mobilization and hence neovascularization.

INTRODUCTION

Critical limb ischemia (CLI), the most advanced stage of peripheral artery disease (PAD), is associated with high risk for major amputation and death. It is characterized by obstruction of the lower limb arteries and an impaired neovascularization response. Patients with cardiovascular disease, including CLI, have low levels of circulating progenitor cells (PCs), which may contribute to their impaired neovascularization response.[1,2] The low PC levels have been suggested to be secondary to alterations at the bone marrow (BM) level, however the effects of CLI on BM structure in humans have not been reported.

Animal studies have shown that diabetes, which is a major risk factor for cardiovascular disease and associated with impaired mobilization of PCs from the BM,[1,3-5] induces pathological processes in the BM, including niche dysfunction,[5] altered cytokine signaling,[3] and BM vasculopathy[6] and neuropathy,[7] which relate to impaired PC function and mobilization. In a recent issue of this journal, Spinetti and colleagues for the first time provided evidence for a detrimental effect of type-2 diabetes on *human* BM, causing a reduction of hematopoietic tissue, fat deposition, and microvascular rarefaction, particularly when associated with CLI.[8] Although their findings suggests that severity of systemic vascular disease impacts BM remodeling, their study does not allow conclusions on the impact of CLI in absence of diabetes, as they did not include CLI patients without DM. In this Research Commentary, we confirm and extend the observations of Spinetti et al. by studying BM microvascular and neuropathic changes in CLI with and without diabetes.

MATERIAL AND METHODS

For details see Supplemental Methods section.

Study population and protocol

Iliac crest biopsies were obtained from 12 CLI patients, included in the Juventas-trial (NCT00371371),[9] and 12 age and sex-matched controls. The study complied with the Declaration of Helsinki and was approved by the local institutional review board. Informed consent was obtained.

Tissue samples were formalin-fixed, decalcified, and paraffin-embedded prior to immunohistochemical staining. Histomorphometry was performed on H&E-stained sections. Microvascular, arteriolar, sympathetic nerve, and pan-neuronal innervation were quantified using CD34, alpha smooth muscle actin (α-SMA), tyrosine hydroxylase (TH) and protein gene product 9.5 (PGP9.5) based protocols, respectively. Scoring researchers were unaware of biopsy origin.

RESULTS

Subject characteristics

See Online Table 1. Groups did not differ with respect to age or gender. One-third of CLI patients had a history of diabetes. Patients with CLI had a substantial burden of cardiovascular risk factors and history of cardiovascular disease.

CLI induces alterations in BM vasculature and innervation

General cellularity, i.e. BM area comprised of hematopoietic tissue, was lower in CLI patients with 32±5% compared to 48±5% in controls, respectively. Less microvessels, i.e. capillaries and sinusoids, were present in BM of CLI patients compared to healthy controls (41.1±3.8 vs. 51.8±2.4 microvessels per mm^2; P=0.03) (Table 1; Figure 1). Arterioles tended to be less in CLI, however not significant (P=0.10). Total PGP9.5-positive arterioles were less in CLI (P=0.001). Number of sympathetic nerve terminals in CLI was approximately 50% of that observed in controls (P=0.01).

Table 1 Bone marrow histomorphometry and immunohistochemistry.

Characteristic	CLI	Control	P-value
Microvessels, n/mm^2	41.1±3.8	51.8±2.4	0.03
Arterioles, n/mm^2	7.8±1.2	11.7±2.0	0.10
PGP 9.5+ arterioles, n/mm^2	0.5±0.2	2.1±0.3	0.001
Tyrosine hydroxylase+ fibers, n/mm^2	5.8±0.8	11.6±1.9	0.01
Cellularity, %	32±5	48±5	0.04

Numbers are means±SEM. Both groups n=12, except for TH for CLI (n=10).

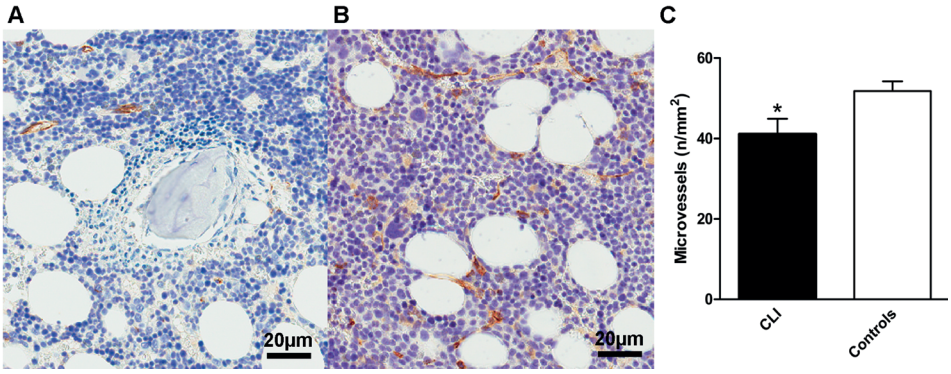

Figure 1 CLI is associated with decreased microvascular density. Microscopic photographs of human CLI (A) and control (B) BM stained with microvascular marker CD34 showing reduced microvascular density in CLI. C) Bar graph showing mean±SEM for microvascular density. *P<0.05. Both CLI and controls n=12.

CLI induces alterations in BM vasculature and innervation independent of DM presence
Between CLI patients with (n=4) and without diabetes (n=8) no differences in BM cellularity (P=0.87), microvascular (P=0.97) and arteriolar density (P=0.89), and nerve innervation (P=0.23 and P=0.56 for TH and PGP9.5, respectively) were observed. We found no significant correlations between presence of diabetes and BM characteristics. In non-diabetic CLI patients numbers of microvessels (40.9±3.7 vs. 51.8±2.4 per mm2; P=0.02), sympathetic nerve terminals (5.0±2.1 vs. 11.6±1.9 per mm^2; P=0.03), and PGP9.5-positive arterioles (0.4±0.2 vs. 2.1±0.3 per mm^2; P=0.001) were lower than in controls.

DISCUSSION

In this Research Commentary we show for the first time that CLI, also in the absence of diabetes, induces structural changes in the BM, characterized by lower microvascular density, and reduced general as well as sympathetic nervous system (SNS) innervation. Our findings confirm and extend the observations of Spinetti et al. recently published in this journal, who provided first human evidence for a damaging effect of type-2 diabetes on BM.[8]

Spinetti et al. reported microvascular changes, alterations in BM composition and PC content in patients with diabetes and showed most striking microvascular remodeling in diabetic CLI patients. However, they performed their analyses in CLI patients on the proximal part of the amputated femoral bone which may have influenced their results. We obtained BM from the iliac crest in both CLI patients and controls, excluding effects from variances in BM structure in different anatomic areas, and confirm the microvascular changes observed by Spinetti et al. Moreover, our study included CLI patients with and without diabetes, showing similar changes in microvasculature, thus providing proof that CLI in itself, independent of diabetes, induces microvascular changes in the human BM.

In addition to the difference in microvasculature between CLI patients and controls we found that CLI is associated with a marked reduction in general and sympathetic innervation of the BM. It has been shown that neuronal impulses, especially of the SNS, regulate PC proliferation in and egress from the BM[10,11] and that diabetic neuropathy in animals extends to the BM and results in impaired endothelial progenitor cell (EPC) release.[7] However no previous studies have addressed the relation between vascular disease in humans and altered innervation of the BM. Our data suggest that impaired general and sympathetic innervation of the BM may contribute to disturbed PC homeostasis and impaired mobilization of PCs from the BM in CLI.

Altogether, our study shows that CLI, irrespective of diabetes, induces microvascular rarefaction and impairment of the SNS in the human BM. These alterations in BM structure may contribute to the reduced circulating PC counts in CLI and reflect impaired vasculoregenerative potential.

ACKNOWLEDGEMENTS

The authors acknowledge Drs. Liane C. te Boome for performing and Dr. Sofie L.J. Verbeke for histomorphometric analyses of the BM biopsies.

SOURCES OF FUNDING

The reported work was supported by the "Stichting Vrienden UMC Utrecht" on behalf of the Dirkzwager-Assink foundation (The Netherlands, grant CS 06.007), the Dutch Heart Foundation (grant 2008B094), The Netherlands Organisation for Scientific Research (ZonMw-TAS grant 116001026) and foundation "De Drie Lichten" (The Netherlands, grant 10/06). M.C. Verhaar is supported by The Netherlands Organization for Scientific Research (Vidi grant 016.096.359).

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SUPPLEMENTAL MATERIAL

Study population

Patients with documented CLI (n=12), participating in the Juventas-trial; a clinical trial evaluating the clinical effects of intra-arterial infusion of BM mononuclear cells in CLI (clinicaltrials.gov NCT00371371), were included for the present study.[1] In short, the Juventas-trial included patients with chronic CLI, an ankle-brachial index (ABI) of 0.6 or less, or an unreliable index (non-compressible or not in proportion to the Fontaine classification), who were not candidate for conventional revascularization. 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 impossibility to complete follow-up. All patients underwent a BM aspiration procedure, during which approximately 100mL of BM was harvested according to the trial protocol. In patients included in the current study an additional BM biopsy was taken from the iliac crest distant to the site of the BM aspiration. The control population consisted of normal routine iliac crest biopsies (n=12) selected to match age and sex of the patient population.
The study was performed according to the Declaration of Helsinki, the study protocol was approved by the institutional review board of the University Medical Center Utrecht, The Netherlands, and patients provided written informed consent.

Histomorphometry and immunohistochemistry

Biopsy samples were fixed in buffered formalin for 24h and subsequently decalcified in saturated Na_2EDTA (125 g/L) solution in distilled water for 2 days under constant agitation. Tissues were embedded in paraffin. 3 µm sections were mounted on 3-aminopropyl-3-triethoxysilane/bovine serum albumin coated slides and deparaffinized in xylene, ethanol, to distilled water.
For histomorphometry slides were stained with conventional hematoxylin-eosin (H&E). Cellularity (percentage area comprised of hematopoietic tissue) was estimated by two experienced independent pathologists, and results were averaged (Pearson's correlation of both pathologists 0.97, $P<0.001$). To identify vascular structures in the BM CD34-antibody was used,[2] alpha smooth muscle actin (α-SMA) antibody was used to identify arterioles, tyrosine hydroxylase (TH) and protein gene product 9.5 (PGP9.5) antibodies were used for sympathetic and pan-nerve characterization, respectively.
Immunohistochemical stainings for CD34 (mouse anti-human antibody, 1:800 dilution; Immunotech, Beckman Coulter Inc, Marseille, France; 0786) and alpha-SMA (mouse anti-human antibody, 1:32,000 dilution; Sigma-Aldrich Corp, St. Louis, MO, USA; A2457) were performed using the Bond automated staining machine (Leica Microsystems GmbH, Wetzlar, Germany) with the Bond Polymer Refine Detection kit (Leica Microsystems GmbH). Antigen retrieval was performed with Bond Epitope Retrieval Solution 2 (Leica Microsystems GmbH) for 20 min at 99°C (only for CD34), then slides were incubated with the primary antibody for 15 min, the Bond Polymer Detection kit for 8 min, and 10

min with 3,3'-diaminobenzidine (DAB) all at room temperature (RT).
For TH and PGP9.5 manual staining protocols were applied. Sections were treated with peroxidase block for 15 min and incubated in citrate-HCl buffer at 100°C for 20 min. For TH sections were stained with rabbit anti-human antibody to TH (1:100 dilution; Abcam plc, Cambridge, UK; ab59276) for 1 h at RT. For PGP9.5 sections were stained with rabbit anti-human antibody to PGP9.5 (1:400 dilution; Abcam plc; ab15503) for 1 h at RT. Sections were incubated with Bright Vision Poly horseradish peroxidase (HRP)-anti rabbit IgG (Immunologic BV, Duiven, The Netherlands; DPVR55HRP) for 1h at RT. Finally, incubation with the NovaRED Peroxidase substrate kit (Vector Laboratories Inc, Burlingame, CA, USA; SK-4800) for 10 min was applied and sections were counterstained with hematoxylin.
All sections were scanned and converted to digital images. Analyses and scoring of all sections was performed using Aperio ImageScope software (Aperio Technologies Inc, Vista, CA, USA) by one investigator (RR) blinded for the origin of the biopsies and cross-checked by a second investigator (MT).
Vascularity of the BM was assessed using the "hot spot" method as previously described.[3,4] Briefly, three microvascular hot spots were identified at 100x magnification in each CD34-stained section[2] and counted at 400x magnification. Presence of a clearly discernible lumen was not defined as a requirement to identify a structure as a microvessel. Results were expressed as the average of the three hot spots. Arterioles were identified in the α-SMA-stained sections. The total area of hematopoietic tissue was measured. In the complete section the number of arterioles was scored at 70x magnification and divided by the total area of hematopoietic tissue.
Sympathetic nerve fibers were scored in three random high-power fields identified at 100x magnification and nerve fibers were subsequently counted at 400x magnification. Results were expressed as the average of the three fields. The pan-nerve staining with PGP9.5 was scored by counting the positively staining arterioles, since aspecific staining of especially megakaryocytes made it necessary to count structures that clearly classify as PGP9.5 positive nerve terminals, such as in the walls of arterioles.

Statistical Analyses

Continuous data are expressed as means±SEM or medians with 25th and 75th percentiles (P25-P75), depending on the normality of the data. Normality of variables was tested using the Kolmorov-Smirnov test. A Levene's test was used to test for equality of variances between the groups. Independent samples *t*-tests were used to test for differences of parametric variables between groups. The Mann-Whitney *U*-test was used to test for differences of non-parametric variables between groups. Categorical variables were compared using the Fisher's exact test.
Univariate analyses for correlations were performed by calculating the Pearson's *R*. Two-sided P-values <0.05 were considered statistically significant. All analyses were performed using IBM SPSS Statistics for Windows version 20.0 (IBM Corp, Armonk, NY, USA).

SUPPLEMENTAL FIGURES

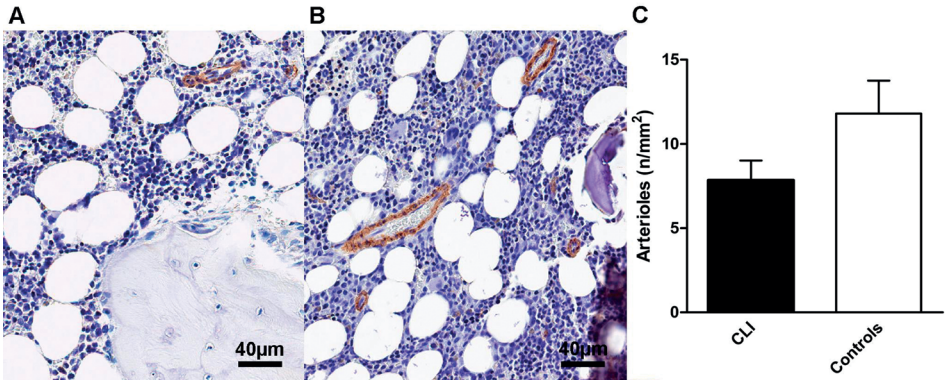

Figure S1 Trend towards lower arteriolar density in CLI. Microscopic photographs of human CLI (A) and control (B) BM stained with smooth muscle marker α-SMA showing trend towards lower arteriolar density in CLI. C) Bar graph showing mean±SEM for arteriolar density. Both CLI and controls n=12.

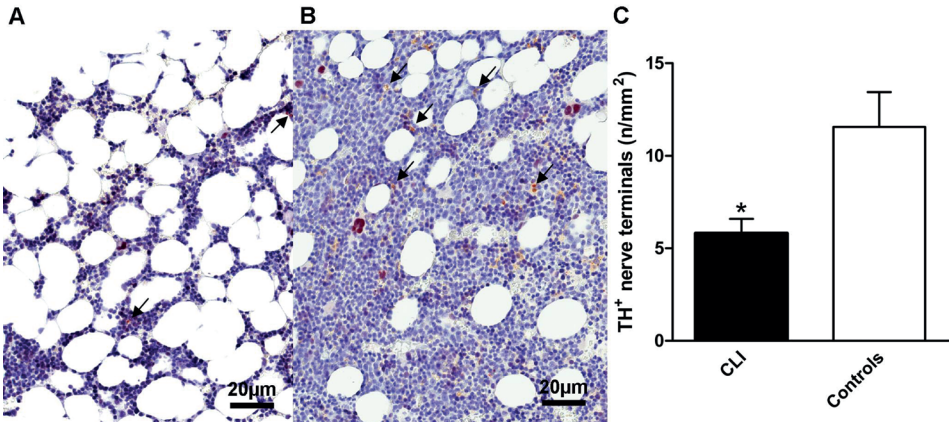

Figure S2 Lower number of sympathetic nerve terminals in CLI. Microscopic photographs of human CLI (A) and control (B) BM stained with sympathetic nerve marker TH showing reduced sympathetic nerve terminals (arrows) in CLI. C) Bar graph showing mean±SEM for number of sympathetic nerve terminals per mm2. *P<0.05. CLI, n=10 and controls, n=12.

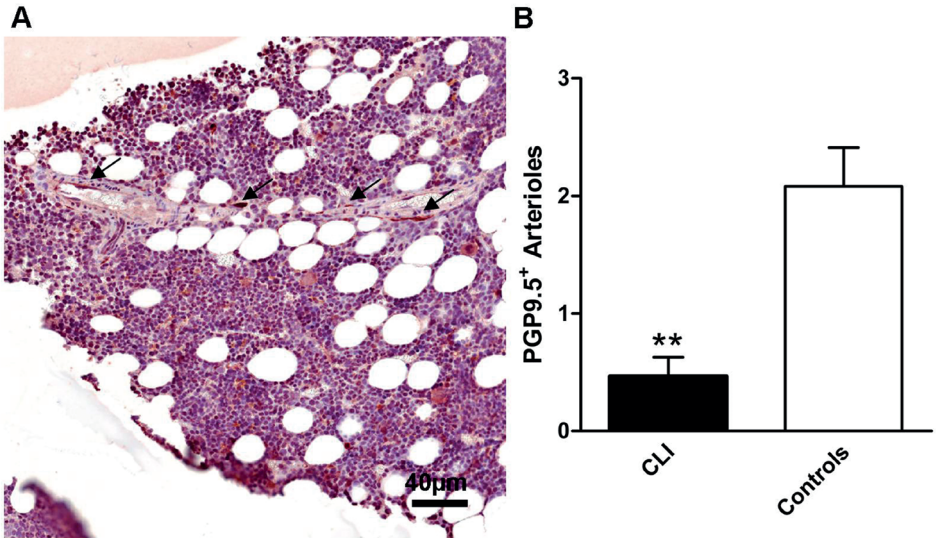

Figure S3 Lower number of pan-nerve PGP9.5+ arterioles in CLI. A) Microscopic photograph of human BM stained with pan-nerve marker PGP9.5 showing positive nerve staining in a wall of an arteriole. B) Bar graph showing mean±SEM for number of PGP9.5+ arterioles per mm2. **P<0.01. Both CLI and controls n=12.

Table S1 Demographic and Clinical Characteristics*

Characteristic	CLI (N = 12)	Controls (N = 12)
Age, years	61±5	55±5
Male sex, no. (%)	9 (75)	8 (67)
History of cardiovascular disease, no. (%)		
Myocardial infarction or chest pain	3 (25)	NA
TIA or Stroke	1 (8)	NA
Dialysis dependent renal disease	2 (17)	NA
Previous angioplasty	9 (75)	NA
Previous bypass	7 (58)	NA
Major amputation	0 (0)	NA
Body-mass index, kg/m2	25.5±1.3	–
Currently smoking, no. (%)	1 (8)	–
Diabetes, no. (%)	4 (33)	NA
Non-fasting glucose, mmol/L	6.7±0.6	–
Hypertension, no. (%)†	12 (100)	–
Hyperlipidemia, no. (%)‡	10 (83)	–
Total cholesterol, mmol/L	3.9±0.3	–
HDL-cholesterol, mmol/L	1.13±0.13	–
Triglycerides, mmol/L	2.0±0.4	–
Hyperhomocysteinemia, no. (%)§	4 (33)	–
Homocysteine, µmol/L	18.2±3.0	–
Renal insufficiency, no. (%)‖	4 (33)	–
Creatinine, µmol/L	92 (63 - 203)	–
Cardioprotective drug use, no. (%)		
Antiplatelet therapy	10 (83)	–
Coumarines	5 (42)	–
Statins	9 (75)	–
ACE-inhibitors	3 (25)	–
Disease characteristics		
Rutherford stage, no. (%)		
4	5 (42)	NA
5	6 (50)	NA
6	1 (8)	NA
Ankle-brachial index	0.53±0.07	–
tcO_2, mmHg	38±6	–

* P>0.05 for all characteristics. Values are means±SEM or medians and interquartile ranges. CLI Critical Limb Ischemia, NA Not applicable. † Hypertension was defined as having systolic blood pressure ≥140, or diastolic blood pressure ≥90, or antihypertensive drug use. ‡ Hyperlipidemia was defined as having blood levels of total cholesterol ≥6.50 mmol/l, triglycerides ≥2.3 mmol/l, or HDL-cholesterol ≤1.0 mmol/l, or lipid lowering drug use. § Hyperhomocysteinemia was defined as having homocysteine blood levels of >19 µmol/l in males and >17 µmol/l in females. ‖ Renal insufficiency was defined as having creatinine blood levels >120 µmol/l.

SUPPLEMENTAL REFERENCES

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PART THREE

Role of endothelial progenitor cells and platelet function in cardiovascular disease

CHAPTER 12

Reduced platelet reactivity in critical limb ischemia patients

Submitted

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ABSTRACT

Background

Patients with critical limb ischemia (CLI) have a high risk to develop cardiovascular events (CVE), despite aspirin therapy. We hypothesized that platelets from CLI patients display increased baseline platelet activation and platelet reactivity.

Objectives

We investigated baseline platelet activation, platelet reactivity, and the effect of aspirin on platelet function in patients with CLI.

Patients/Methods

In this case-control study baseline platelet activation and platelet reactivity in response to five major platelet agonists were determined, in 23 CLI patients (11 on aspirin, 9 on oral anticoagulant and 3 on aspirin + clopidogrel) included in the Juventas-trial (clinicaltrials.gov NCT00371371) and in 17 healthy controls. Platelet activation was quantified with flow cytometric measurement of platelet P-selectin expression and fibrinogen binding. Aspirin effectiveness was measured by the additional stimulatory effect of arachidonic acid (AA) to convulxin (CVX) stimulation.

Results

CLI patients not using aspirin showed higher baseline platelet activation compared to healthy controls. Platelet reactivity was not different between cases and controls, except for $\alpha_{IIB}\beta_3$ activation in response to CVX, which was lower in cases than in controls ($p=0.009$). Platelet reactivity of CLI patients was inversely associated with presence of cardiovascular risk factors. Aspirin treatment decreased platelet reactivity to AA, while other pathways remained unaffected.

Conclusions

Our results suggest increased systemic baseline activation of platelets in CLI, whereas platelet reactivity was not different or even decreased. Additionally, reactivity of platelets was inversely correlated with cardiovascular risk factors. Our novel platelet reactivity assay offers a new quantitative measurement for aspirin effect on platelet function.

INTRODUCTION

Critical Limb Ischemia (CLI), the most advanced stage of peripheral arterial disease (PAD), is characterized by ischemic rest pain or tissue loss as well as a profound risk for cardiovascular complications and mortality.[1,2] Platelets play a central role in the development of arterial thrombosis and subsequent cardiovascular events (CVE).[3] Abnormal platelet function with an increased tendency to aggregate is implicated in the pathogenesis of atherosclerosis[4] and development of superimposed acute ischemic events.[5-7] Conflicting results have been reported regarding platelet reactivity in PAD, possibly related to different patterns of platelet reactivity in different stages of PAD.[8-10]

Antiplatelet therapy reduces the risk for future CVE in patients with previous cardiovascular disease and is therefore the cornerstone of medical therapy in PAD.[11] Inhibition of platelets' cyclooxygenase-1 (COX-1) by aspirin has been proven to reduce CVE in a wide range of populations.[12] However, many patients will experience CVE despite aspirin therapy.[13] Recent studies have shown significant variability in platelet reactivity between patients taking either aspirin[14,15] or clopidogrel[16] and high on-treatment platelet reactivity (HPR) is associated with an increased risk of CVE.[16,17]

We hypothesized that CLI patients display increased baseline platelet activation and platelet hyperreactivity, which may contribute to their increased cardiovascular risk. Platelet reactivity was assessed as P-selectin expression and fibrinogen binding, which reflects $\alpha_{IIB}\beta_3$ activation, using a flow cytometry based method, in CLI patients at baseline and in response to concentration series of the thrombin receptor agonist SFLLRN (TRAP), adenosine diphosphate (ADP), the thromboxane analog U-46619 (Tx) and convulxin (CVX), thus covering all major platelet activation pathways. In addition we investigated whether assessing the added stimulatory effect of arachidonic acid (AA) to CVX stimulation could be used as a measure for aspirin effectiveness in CLI patients and thus provide a novel thromboxane dependent functional platelet reactivity assay, which could have future value to identify HAPR.

METHODS

Study subjects

23 patients with documented CLI, participating in the Juventas-trial; a clinical trial evaluating the clinical effects of intra-arterial infusion of bone marrow mononuclear cells in CLI (clinicaltrials.gov NCT00371371), were included for the present study.[18] In short, the Juventas-trial included patients with chronic CLI, an ankle-brachial index (ABI) of 0.6 or less, or an unreliable index (non-compressible or not in proportion to the Fontaine classification), who were not candidate for conventional revascularization. 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 bone marrow aspirate, known infection with human immunodeficiency virus, hepatitis B or C virus, and

an impossibility to complete follow-up. In all 23 patients, 4.5 mL citrate-anticoagulated venous blood samples were obtained.
The antiplatelet therapy regimen was left to the discretion of the vascular surgeon and was recorded at inclusion and verified based on pharmaceutical supply records. For the remainder of the manuscript CLI patients not on antiplatelet therapy are referred to as CLI A- patients, patients using aspirin, but not clopidogrel as CLI A+ patients, and patients using both aspirin and clopidogrel as CLI AC+ patients.
Healthy controls were recruited from the mini donor service of the University Medical Center (UMC) Utrecht, consisting of healthy employees of the UMC Utrecht. Healthy controls were primarily compared with the CLI A- patients and we aimed for at least two healthy controls per CLI A- patient. Ultimately, blood was obtained from 17 healthy controls, who did not use antiplatelet drugs for at least 7 days prior to blood withdrawal. This study was conducted in accordance to the Declaration of Helsinki and procedures were approved by the institutional review board of the UMC Utrecht. All patients gave written informed consent.

Study procedures

General platelet reactivity and aspirin resistance were assessed within 90 minutes from blood withdrawal. Reactivity of platelets was determined with concentration series of: thrombin receptor agonist SFLLRN (TRAP) ranging from 0.038 to 625 μM, adenosine diphosphate (ADP) ranging from 0.008 to 125 μM, the thromboxane analog U-46619 ranging from 0.8 to 12500 μg/mL (Tx), convulxin (CVX) ranging from 0.13 to 80 μg/mL with and without arachidonic acid (AA) 125 μg/mL (modified procedure of previously published method[19]). Serial dilutions were prepared in 50 μL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO4, 5 mM KCl, pH 7.4 4, 5 mM KCl, pH 7.4) with 2 μL phycoerythrin-labeled mouse α-human P-selectin antibodies and 1 μL fluorescein isothiocyanate-labeled mouse α-human fibrinogen antibodies. The platelet activation test was initiated by addition of 5 μL whole blood to each sample of the serial dilutions. After 20 min of incubation, the samples were fixed with 500 μL 0.2% formyl saline (0.2% formaldehyde in 0.9% NaCl) and kept at 4°C. All samples were analyzed on a FACS Calibur flow cytometer from BD Biosciences (Franklin Lakes, NJ, USA) within 1 day after processing. Single platelets were gated on the basis of forward-scatter and side-scatter properties, and their median fluorescence intensity (MFI) was measured. All assays were performed by a single observer blinded to subject characteristics.

Study parameters

Platelet reactivity

For each individual subject, dose–response graphs and areas under the curves (AUC) expressed in arbitrary units were produced with PRISM software version 5.01 (Graphpad Software, La Jolla, CA, USA) for each agonist separately. Baseline platelet activation was determined by averaging MFI's from the lowest concentrations of the TRAP, ADP, CVX, and U-46619 concentration series.

Aspirin effectiveness
The added stimulatory effect of AA stimulation to CVX stimulation was used as a measure of aspirin effectiveness and was calculated by the following formula: Arachidonic Acid effect (AA-effect) = (AUC(CVX+AA) - AUC(CVX)) / AUC(CVX) * 100%.

Statistics
Dichotomous variables are presented as frequencies and percentages and continuous variables are presented as means with standard deviation (SD). CLI A- patients were compared to healthy controls and to CLI A+ patients. To assess clopidogrel effect CLI A+ patients were compared to CLI AC+ patients. Differences between groups were tested with a Student's t-test. Associations between patient's baseline characteristics and platelet reactivity parameters were tested using the Spearman's rank correlation tests. Statistical significant difference was considered at a two-sided p-value below 0.05. All analyses were performed using SPSS software version 20.0 (IBM, Chicago, IL, USA).

RESULTS

Baseline Characteristics
Twenty-three CLI patients and 17 healthy controls were included for this study. Mean age was 64.3±15.2 years for CLI patients and the majority of the CLI patients were male (74%; Table 1). The healthy controls were younger (45.1±9.6) and the majority was female (65%). The different groups of CLI patients (CLI A-, CLI A+, and CLI AC+) were not different with respect to age, sex, cardiovascular history and medication use.

Baseline platelet activation is increased in CLI patients
Baseline platelet activation was determined with the P-selectin expression or the fibrinogen binding capacity of the patients platelets without stimulation with an agonist. Baseline platelet $\alpha_{IIB}\beta_3$ activation did not differ between CLI A- patients and healthy controls (73.7±12.9 vs. 80.3±9.7, p=0.682). However, baseline P-selectin expression was significantly higher in CLI patients as compared to healthy controls (59.4±4.8 vs. 40.3±8.6, p<0.001; Figure 1).

In-vitro platelet reactivity is reduced in CLI patients
Platelet reactivity was determined with P-selectin expression or the fibrinogen binding capacity after stimulation to five major platelet agonists. Overall, *in-vitro* platelet reactivity in CLI A- patients was not elevated for any of the agonists, when compared to healthy controls. Instead, $\alpha_{IIB}\beta_3$ activation was decreased for CVX activation both with (p=0.009) and without (p=0.037) AA (Figure 2). P-selectin expression on TRAP activation showed a trend towards a reduced response in CLI patients (AUC 50866±8725 vs. 44420±7498, p=0.076, respectively).

Table 1 Baseline Characteristics.

	CLI patients (n=23)
Age (years)	64.3±15.2
Male gender	17 (74%)
History of cardiovascular disease	
Previous revascularisation of affected leg	19 (83%)
CABG	5 (22%)
Myocardial infarction or angina pectoris	8 (35%)
TIA or stroke	5 (22%)
Cardiovascular risk factors	
Currently smoking	7 (33%)
Diabetes	9 (39%)
Hypertension	15 (65%)
Medication use	
Aspirin	11 (48%)
Aspirin + Clopidogrel	3 (13%)
Anticoagulants	9 (39%)
Statins	20 (87%)
ACE inhibitors	8 (35%)
Angiotensin receptor blocker	2 (9%)
Beta-blockers	6 (26%)
Diuretics	5 (22%)
Laboratory parameters	
Total cholesterol (mmol/l)	4.0±1.3
HDL-cholesterol (mmol/l)	1.2±0.4
HbA1c (mmol/mol)	50.4±17.1
Homocysteine (μmol/l)	18.3±12.7
Creatinin (μmol/l)	106.0±38.4
Ureum (mmol/l)	7.5±4.1
Fontaine classification (grade IIB/III/IV)	1/5/17

Values are presented as absolute numbers and percentage (n [%]) for categorical variables and mean ± standard deviation (SD) for continuous variables. History of hypertension was defined as reported history of hypertension or being on antihypertensive medication.

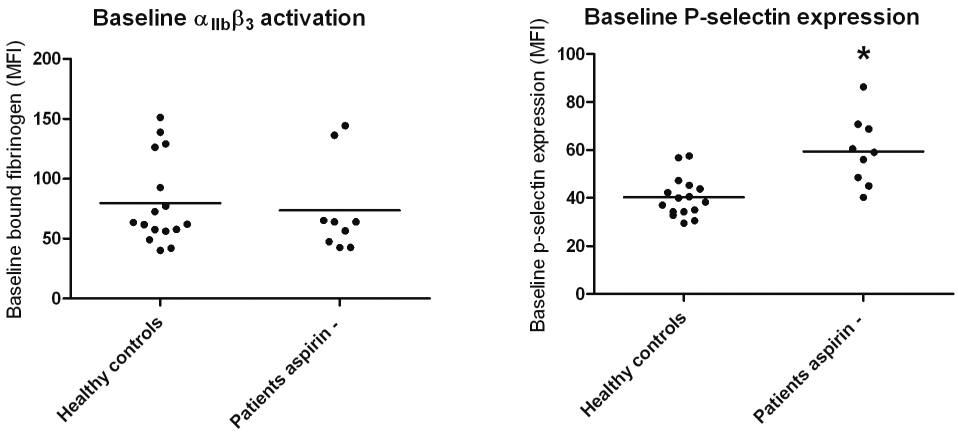

Figure 1 Baseline platelet activation of CLI A- patients versus healthy controls. Mean MFI for bound fibrinogen or P-selectin expression, stratified for CLI A- patients versus healthy controls. Baseline P-selecting expression was higher in CLI patients, MFI median fluorescence intensity, * p<0.05.

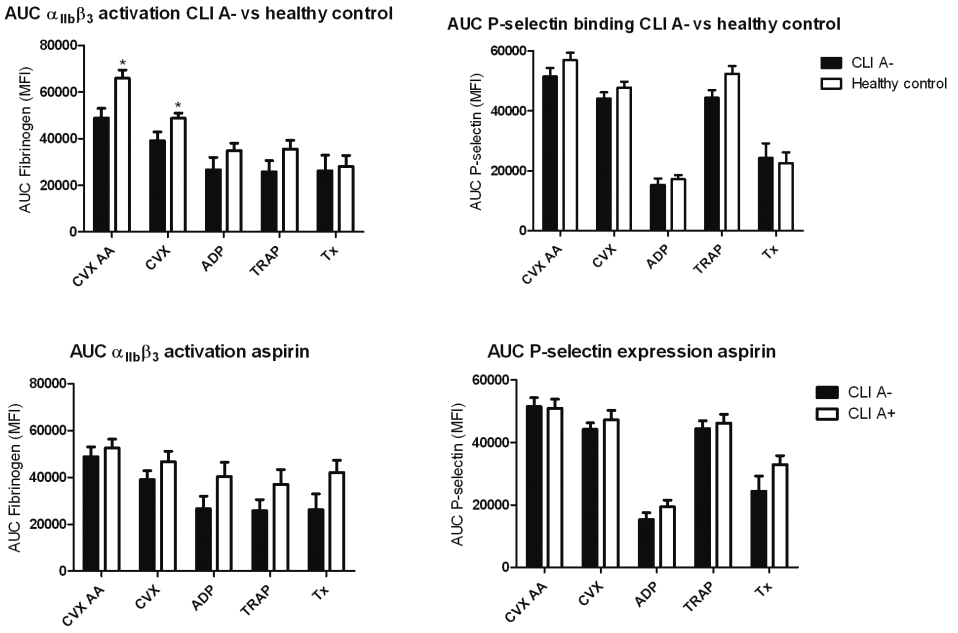

Figure 2 Platelet reactivity of CLI A- patients versus healthy controls and CLI A- patients versus CLI A+ patients. AUC of MFI for fibrinogen binding or P-selectin expression per agonist, stratified for CLI A- patients versus healthy controls and CLI A- patients versus CLI A+ patients. *CLI* A- CLI patients not treated with aspirin, *CLI* A+ CLI patients treated with aspirin, *AUC* area under the curve, *MFI* median fluorescence intensity, *CVX*+AA Convulxin and arachidonic acid, *CVX* Convulxin, *ADP* Adenosine Diphosphate, *TRAP* Thrombin receptor agonist SFLLRN, *Tx* Thromboxane receptor agonist, * *p*<0.05.

Increased cardiovascular disease burden is associated with reduced *in-vitro* platelet activation

Associations between patient's baseline characteristics and platelet reactivity parameters were tested in the CLI patients not using clopidogrel (CLI A- and CLI A+ patients). In CLI patients not using clopidogrel, platelet reactivity was in general negatively correlated with a history of angina pectoris or myocardial infarction. An inverse trend was also observed for the correlation between platelet reactivity and markers for renal function, creatinine and urea. Age, presence of diabetes, HbA1c and homocysteine levels showed a tendency towards an inverse association with platelet response to several of the platelet activators (Table 2).

Aspirin specifically reduces arachidonic acid effect in CLI patients

Aspirin effectiveness was measured by the additional stimulatory effect of arachidonic acid (AA) to convulxin (CVX) stimulation. Overall, platelet reactivity of CLI A- and CLI A+ patients did not differ significantly for any of the agonists (Figure 2). However, the additional effect on platelet reactivity of AA superimposed on CVX in platelets from aspirin treated patients was substantially lower for P-selectin (AA-effect 8.7±7.6 vs. 16.2±6.2%, p=0.029) and a similar trend was observed for $\alpha_{IIB}\beta_3$ activation (AA-effect 15.6±15.8 vs. 27.4±13.8%, p=0.095; Figure 3).

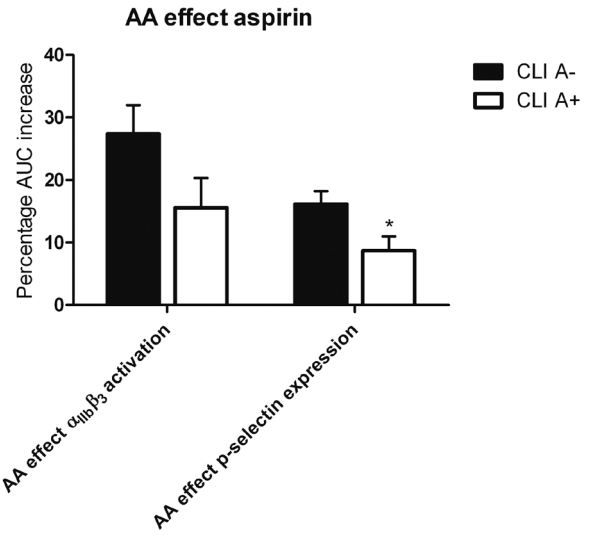

Figure 3 Additional effect of arachidonic acid for CLI A- patients versus CLI A+ patients. Percentage increase of AUC of MFI for fibrinogen binding or P-selectin expression as an effect of addition of AA, stratified for CLI A- patients versus CLI A+ patients. *CLI* A- CLI patients not treated with aspirin, *CLI* A+ CLI patients treated with aspirin, *AUC* area under the curve, *MFI* median fluorescence intensity, *CVX*+AA Convulxin and arachidonic acid, *CVX* Convulxin, ADP Adenosine Diphosphate, *TRAP* Thrombin receptor agonist SFLLRN, *Tx* Thromboxane receptor agonist, *AA effect* Additional effect arachidonic acid, * $p<0.05$.

Table 2 Correlations of patient factors with platelet reactivity.

	CVX + AA		CVX		ADP		TRAP		Tx		AA effect	
	Fibr	**P-sel**	**Fibr**	**P-sel**	**Fibr**	**P-sel**	**Fibr**	**P-sel**	**Fibr**	**P-sel**	**Fibr**	**P-Sel**
Age	-0.31	-0.31	-0.24	-0.26	-0.29	-0.12	-0.26	-0.47*	-0.26	-0.43†	-0.10	-0.05
Sex	0.13	0.21	-0.08	0.17	-0.19	-0.10	0.38	0.19	0.08	0.21	0.19	0.02
Hypertension	-0.28	0.26	-0.25	0.19	-0.25	0.16	-0.06	0.28	-0.28	0.28	-0.25	0.06
Diabetes mellitus	-0.41†	-0.28	-0.27	-0.27	-0.23	-0.21	-0.28	-0.41†	-0.35	-0.39†	-0.14	0.00
HbA1c	-0.42	-0.05	-0.35	-0.07	-0.42	-0.26	-0.52†	-0.53†	-0.40	-0.29	0.32	0.02
Smoking	0.30	0.06	0.19	0.06	0.34	0.28	0.33	0.22	0.39†	0.31	0.15	0.01
History of AP or MI	-0.62**	-0.25	-0.45*	-0.23	-0.42	0.02	-0.25	0.02	-0.28	0.17	0.04	-0.02
History of CABG	-0.65**	-0.26	-0.65**	-0.39†	-0.65**	-0.24	-0.48*	-0.15	-0.48*	-0.13	0.30	0.30
History of TIA or CVA	-0.33	-0.04	-0.35	-0.04	-0.41†	-0.15	-0.41†	-0.26	-0.28	-0.30	0.28	0.11
Previous revascularization	-0.11	0.02	-0.13	0.11	-0.33	-0.15	-0.24	0.11	-0.22	-0.04	0.04	0.09
Fontaine classification	-0.07	-0.20	0.04	-0.11	0.10	0.24	0.22	0.10	0.34	0.43	-0.13	-0.28
Cholesterol	0.28	0.26	0.24	0.32	0.21	-0.09	0.08	0.09	0.03	-0.20	-0.04	-0.23
HDL	0.26	0.15	0.16	0.17	0.14	-0.10	0.22	0.35	0.23	0.23	-0.09	-0.24
Creatinin	-0.30	-0.34	-0.16	-0.28	-0.13	-0.18	-0.23	-0.66**	-0.31	-0.35	0.02	-0.19
Ureum	-0.31	-0.34	-0.18	-0.31	-0.15	-0.17	-0.18	-0.60**	-0.20	-0.38†	-0.07	-0.12
Homocysteine	-0.43†	-0.28	-0.38†	-0.31	-0.36	-0.34	-0.16	-0.01	-0.16	0.03	0.07	0.04
Number of platelets	-0.13	0.00	-0.20	-0.07	-0.15	0.21	0.11	0.38	0.25	0.52*	0.18	0.08
Mean platelet volume	0.43†	0.09	0.43†	0.15	0.44*	-0.07	0.15	-0.21	0.09	-0.25	-0.23	-0.12
Aspirin use	0.11	-0.03	0.25	0.11	0.39	0.10	0.34	0.10	0.43†	0.36	-0.46*	-0.53*
Statin use	-0.28	-0.35	-0.16	-0.28	-0.16	-0.01	-0.09	-0.11	0.04	0.01	-0.16	-0.01
Use of Diuretics	-0.31	-0.07	-0.25	-0.13	-0.25	0.09	-0.15	0.09	-0.35	-0.03	0.05	0.01
Use of ACE-inhibitor	-0.41†	0.05	-0.46*	0.01	-0.63**	-0.12	-0.41†	-0.06	-0.46*	-0.17	0.28	0.17
Beta-blocker use	-0.39†	-0.02	-0.28	-0.07	-0.28	0.13	-0.15	0.22	-0.17	0.17	0.15	0.13

Values represent Spearman's rho or point-biserial correlation coefficients (rpb) in case one of the variables is categorical. *CVX+*AA Convulxin and arachidonic acid, *CVX* Convulxin, *ADP* Adenosine Diphosphate, *TRAP* Thrombin receptor agonist SFLLRN, *Tx* Thromboxane receptor agonist, *AA effect* Additional effect arachidonic acid, *Fibr* Fibrinogen binding, *P-sel* P-selectin expression, † $p<0.10$, * $p<0.05$, ** $p<0.01$

A significant association of the AA-effect for $\alpha_{IIB}\beta_3$ activation and P-selectin expression was observed, for all patients and CLI A+ patients separately (p=0.003 and p=0.011, respectively). As expected, the three patients on aspirin with the highest AA-effect for $\alpha_{IIB}\beta_3$ activation had the highest AA-effect for P-selectin expression as well (Figure 4).

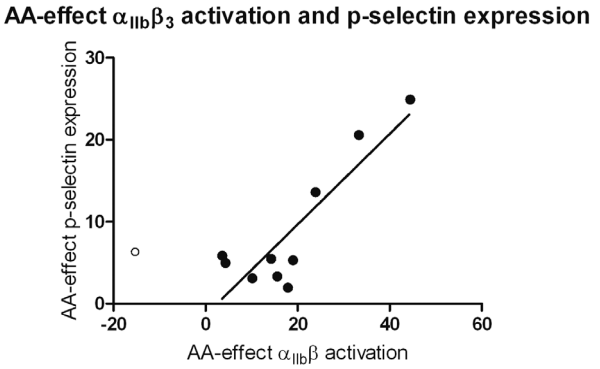

Figure 4 Association of additional effect of arachidonic acid for fibrinogen binding and P-selectin expression for CLI A+ patients. Percentage increase of area under the curve of median fluorescence intensity as an effect of the addition of AA for fibrinogen binding plotted against that for P-selectin expression in patients treated with aspirin. oExcluded value, *CLI* A+ CLI patients treated with aspirin, *AA-effect p-selection expression* percentage increase of area under the curve of median fluorescence intensity as an effect of the addition of AA for P-selectin expression, *AA-effect fibrinogen binding* percentage increase of area under the curve of median fluorescence intensity as an effect of the addition of AA for fibrinogen binding.

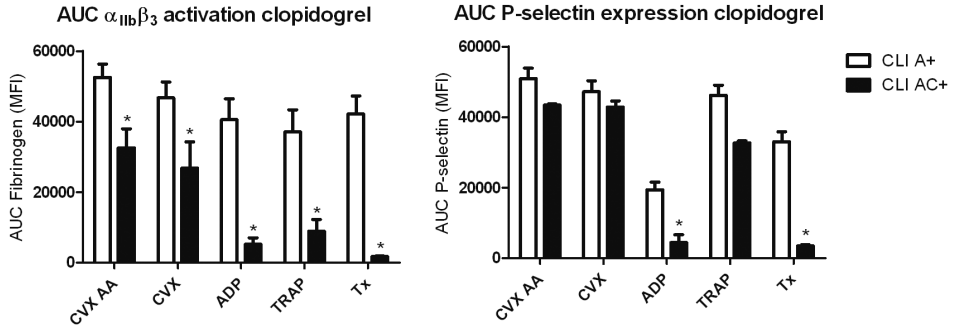

Figure 5 Platelet reactivity of CLI A+ patients versus CLI AC+ patients. AUC of MFI for fibrinogen binding or P-selectin expression per agonist, stratified for CLI A+ patients versus CLI AC+ patients. *CLI* A+ CLI patients treated with aspirin, *CLI* AC+ CLI patients treated with aspirin and clopidogrel, *AUC* area under the curve, *MFI* median fluorescence intensity, *CVX*+AA Convulxin and arachidonic acid, *CVX Convulxin*, *ADP* Adenosine Diphosphate, *TRAP* Thrombin receptor agonist SFLLRN, *Tx* Thromboxane receptor agonist, * p<0.05.

Additional clopidogrel for dual antiplatelet therapy strongly reduces platelet reactivity
In CLI AC+ patients the platelet reactivity was profoundly reduced compared to CLI A+ patients. Clopidogrel significantly reduced the platelet reactivity to all of the agonists when fibrinogen binding was considered (Figure 5). For P-selectin expression this difference was generally less pronounced except for ADP and U-46619.

DISCUSSION

Our study shows increased baseline activation of circulating platelets in CLI patients who are not on aspirin therapy. The reactivity of circulating platelets to ADP, TRAP, and Tx in CLI patients was not different from healthy controls, while reactivity to CVX was attenuated in CLI patients compared to healthy controls. In line, attenuated platelet reactivity to multiple agonists was associated with several traditional risk factors for cardiovascular disease. A third major finding of our study is that with our novel thromboxane dependent platelet activation assay, that measures platelet reactivity to arachidonic acid, we showed that aspirin treatment decreased platelet reactivity to AA, while other platelet activation pathways remained unaffected. This novel test may be used to identify HAPR.
There is growing evidence for an pathogenic role of platelets in atherogenesis. Platelet adhesion to activated endothelial cells or the denuded vessel wall is an early event in the atherosclerotic process.[20,21] However, the exact role of platelets and platelet function in atherosclerotic progression in established and extensive atherosclerotic states, such as CLI, is still under debate.[9,10,22,23] Our findings of an increased baseline platelet P-selectin expression and no difference in basal $\alpha_{IIB}\beta_3$ activation in patients with CLI compared to healthy controls confirm the previously published findings of Cassar et al.[9] It is unknown what causes the observed divergence between baseline activation and reactivity. $\alpha_{IIB}\beta_3$ is involved in firm adhesion to activated endothelial cells, the subendothelial matrix, and platelet aggregation by fibrinogen and von Willebrand factor binding, while P-selectin is involved in initial rolling of platelets on the endothelial surface upon activation.[24] One possible explanation could be that platelets with moderate $\alpha_{IIB}\beta_3$ activation are captured from the circulation, while those with moderate P-selectin expression circulate for a prolonged period of time. Hence P-selectin expression of circulating platelets might provide a more realistic representation of the *in-vivo* platelet activity.
Our observations of a tendency to a decreased platelet reactivity to most agonists in patients with end stage CLI compared to healthy controls seems in conflict with several other studies suggesting increased platelet reactivity in claudicants compared to healthy controls.[9,22] However, a similar decreased platelet reactivity to ADP in CLI patients has been reported.[9] The divergent results between claudicants and CLI patients indicate a different platelet reactivity in different PAD stages. Furthermore, the present study shows that *in-vitro* platelet reactivity is also inversely associated with the burden of cardiovascular disease and risk, i.e. history of myocardial infarction or angina pectoris, decreased renal function, and elevated homocysteine and HbA1c levels, which is an additional indication for

attenuated *in-vitro* reactivity of circulating platelets in patients with extensive atherosclerotic disease. A potential explanation for the reduced reactivity of the circulating platelet subfraction in CLI patients is that a subpopulation of the most reactive platelets are captured by the diseased endothelium and existing atherosclerotic plaques, resulting in a residual circulating pool of relatively activation resistant platelets, as has been suggested previously.[9] Another potential explanation is that increased proteolytic shedding of surface receptors involved in platelet activation potentially down-regulates the platelet reactivity to its agonists,[25-27] since proteases responsible for this surface receptor shedding are elevated in cardiovascular diseases.[28,29] Furthermore, subclinical intra-plaque hemorrhage is associated with progression of atherosclerotic lesions and may occur more frequently is patients with low platelet reactivity.[2] Subsequently, low platelet reactivity might contribute to progression of atherosclerotic lesions which could lead to CLI. Further research is warranted to elucidate the particular mechanisms underlying this observation and to assess the predictive value of platelet reactivity with respect to future CVE in patients with CLI.

Antiplatelet therapy reduces CVE and mortality in patients with a history of cardiovascular disease.[30] Aspirin is the most often prescribed antiplatelet drug in this patient population. We observed that aspirin treatment decreased platelet reactivity to AA, while other platelet activation pathways remained unaffected. Aspirin inhibits platelet activation via irreversible inactivation of COX-1, thereby blocking the conversion of AA to thromboxane A2. The other platelet activation pathways are independent of thromboxane A2, which was confirmed by our findings that these pathways were not negatively affected by aspirin use. Unexpectedly, we observed a trend towards increased $\alpha_{IIB}\beta_3$ activation in response to thromboxane receptor stimulation in patients using aspirin, and a similar pattern for the other activators. This suggests a compensatory hyperreactivity of platelets via thromboxane A2 independent pathways, which might have a relation with the increased CVE risk observed during perioperative aspirin cessation.[31] The general tendency of platelets to be hyperreactive in response to different *in-vitro* stimuli during treatment with aspirin is not easily unraveled and should be confirmed in future studies.

In this study we introduced a novel thromboxane dependent platelet reactivity assay to quantify aspirin mediated platelet inhibition. With this test, we showed that patients on aspirin therapy displayed a lower AA-effect for both P-selectin expression and $\alpha_{IIB}\beta_3$ activation and that there was a significant association between both. In line, we identified three patients that displayed the highest AA-effect for both $\alpha_{IIB}\beta_3$ activation and P-selectin expression indicating that this new test seems to reflect the effect of aspirin on platelet inhibition. According to the available evidence on HAPR incidence from different HAPR-tests the proportion of patients with HAPR lies around 24%,[32] which is similar to our observation in the current population of CLI patients. Reliable determination of HAPR is of clinical importance since it is a risk factor for subsequent CVE[17] and improving aspirin compliance or additional antiplatelet treatment might prevent CVE.[33,34]

Aspirin therapy reduced platelet activation after stimulation by arachidonic acid, but did not influence platelet reactivity for other agonists. In contrast, we showed that clopidogrel in is an extremely potent inhibitor of platelet reactivity by modulating multiple activation

pathways, an effect that substantially exceeds that of treatment with aspirin alone.
Our study has some limitations. First, our study was not randomized and therefore sensitive to confounding factors. We cannot exclude that differences in age and sex between CLI patients and healthy donors might have influenced our results. It is known however that platelet reactivity in a healthy population is not dependent on age,[35] while platelet reactivity in females is generally higher,[36] but no relation of age or sex with any of the platelet reactivity parameters was observed in our control population. There were no differences in baseline characteristics among the different subgroups of CLI patients. The cross-sectional design of our study does not allow drawing conclusions on the relation of platelet reactivity to different agonists and future CVE risk, this should be investigated in future longitudinal studies. Additionally, our study has a relatively small sample size and hence low power. However, studying the platelet reactivity in such detail as in this small study provides a basis for future focused studies in larger patient populations. Noteworthy, platelet reactivity was only assessed using flow cytometric analysis, which does not assess the speed of platelet activation. Addition of platelet aggregometry is likely to show similar results as both methods objectify $\alpha_{IIB}\beta_3$ activation. Moreover, flow cytometry is more sensitive and has a broader quantitation range than aggregometry.

CONCLUSION

Our study shows that CLI patients have increased baseline activation of circulating platelets compared to healthy controls, whereas the reactivity of circulating platelets to stimulatory agents is not different or even decreased. Additionally, *in-vitro* reactivity of circulating platelets is inversely correlated to established risk factors of cardiovascular disease. Prospective studies are required to investigate whether platelet reactivity to different agonists predicts future CVE in CLI patients and other populations at high risk for CVE. The novel thromboxane dependent flow cytometric assay introduced in this study provides a quantitative method to assess response to aspirin and future studies should validate its value to reliably identify HAPR in clinical practice.

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PART FOUR

Discussion and summary

CHAPTER 13

General discussion and future directions

GENERAL ISSUES AND CURRENT MANAGEMENT OF CRITICAL LIMB ISCHEMIA

Critical limb ischemia (CLI), the most advanced stage of peripheral artery disease (PAD), is characterized by severely impaired perfusion of the lower limbs, which results in rest pain and/ or tissue loss. CLI, especially in Western society, is usually caused by atherosclerotic disease. It is associated with high risk for major amputation (as high as 40% at 6 months)[1,2] and cardiovascular events resulting in 6-month mortality rates up to 20%.[3,4] These figures exceed numbers for every other form of occlusive cardiovascular disease, including symptomatic coronary artery disease,[5,6] and reflect the high systemic atherosclerotic burden in CLI. Furthermore, CLI is associated with high treatment costs.[7] With an estimated yearly incidence of 500-1,000 new cases per million individuals per year,[1,2] CLI poses a substantial burden on patients, health care providers and resources.

In **Chapter 2** we describe how treatment of CLI, and PAD in general, has evolved over the past decade, with the introduction of novel surgical and endovascular technologies, such as nitinol and drug-eluting stents. These developments are paralleled by a gradual improved prognosis of CLI, for instance adjusted odds ratios of 0.95 (95% CI: 0.95-0.95, $p<0.001$) for major amputation per year between 2000 and 2008 were reported,[8] but no causal relationship between prognosis and altered management has been established. **Chapter 3**, which was embedded in the "Guidelines for critical limb ischaemia and diabetic foot" of the European Society of Vascular Surgery, provides recommendations on revascularizing treatment in CLI, based on currently available evidence.[9] While an endovascular-first strategy is becoming more widely adopted this is not based on evidence derived from clinical trials that proves its superiority in CLI.[3] Evidence-based treatment of CLI would benefit from a robust, generally accepted and widely adopted definition of CLI and more well-designed clinical trials. Current recommendations in guidelines for the treatment of CLI are generally based on expert opinion and extrapolation of results from studies in milder PAD populations.[1,2] Joining forces in transatlantic or continental initiatives may be necessary to be able to complete large randomized controlled trials in this specific population, ultimately leading to improved evidence-based treatment in CLI (**Chapter 2 and 3**).

CLI patients are at high risk for atherosclerotic cardiovascular complications. Therefore cardiovascular risk factors should be aggressively treated. Over the years, treatment of cardiovascular risk factors in CLI and PAD patients has improved, however recent reports suggest that there is still room for improvement in prescription and use of platelet inhibitory drugs, statins, and antihypertensive drugs, ACE-inhibitors in particular (**Chapter 2**).[3,10,11] It has been estimated that more widespread use of cardiovascular preventive drugs in PAD could prevent approximately 200,000 cardiovascular events (myocardial infarction, stroke and cardiovascular death) each year, in North America and Western Europe alone.[12] Efforts

to increase awareness of the high cardiovascular risk and need for adequate secondary prevention in CLI should be taken to optimize medical treatment in these patients.

Apart from a poor prognosis with respect to both life and limb, CLI patients experience substantial functional impairment and pain, which leads to poor quality of life as we report in **Chapter 4**. This, together with the large number (25-40%) of CLI patients that,[1,2] despite the evolving revascularizing therapies, is not eligible for conventional revascularization and the lack of effective pharmacological therapies urges the need for novel therapeutic options aimed at improved limb perfusion.

BONE MARROW DERIVED CELL THERAPY IN CRITICAL LIMB ISCHEMIA

Available evidence

The pioneering research by Asahara and colleagues, which in 1997 resulted in the discovery of a putative bone marrow (BM)-derived endothelial progenitor cell (EPC), resulted in a concept that BM-derived cells contribute to postnatal neovascularization.[13] This shifting paradigm, led to the notion that BM cells might be used as a therapeutic agent in ischemic disease. This soon resulted in first-in-man clinical trials to investigate whether BM-derived cells, mainly mononuclear cells (MNC), could restore tissue perfusion in ischemic cardiovascular disease, such as myocardial infarction[14] and PAD.[15,16] In 2002, a small first-in-man clinical trial reported safety and promising effects of BM-derived cell therapy in CLI patients.[17] Since then, multiple studies have suggested benefit of BM-derived cell therapy for CLI.[15,16] However, studies were small, lacked appropriate controls, often did not consider clinically relevant endpoints and thus did not provide definite proof on clinical effectiveness. In **Chapter 5**, we report our recently performed meta-analysis on twelve randomized clinical trials (RCTs) that studied BM-derived cell therapy in CLI. This meta-analysis underlines the promising potential of this therapy, but also shows divergent results between placebo-controlled and non-placebo-controlled RCTs, stressing the need for a large, well-designed, placebo-controlled RCT with clinically relevant endpoints.[18]

Juventas-trial

Our randomized, double-blinded, placebo-controlled Juventas-trial, initiated in 2006, was designed to investigate whether repetitive intra-arterial infusion of BM-MNC reduces amputation rates in a large cohort of no-option CLI patients.[19] With inclusion of 160 patients the Juventas-trial is currently the largest RCT in the field. The trial results in **Chapter 6** show no benefit of BM-MNC compared to placebo infusion on all endpoints, e.g. amputation rates, amputation-free survival (AFS), ankle-brachial index and transcutaneous oxygen measurements, and quality of life. Moreover, it shows that even in the placebo group general improvement was observed for both objective as well as

subjective endpoints. Our results contradict the reports of mainly small, non-placebo-controlled and non-randomized studies[16] but seem in line with the results of our meta-analysis in **Chapter 5**.[18]

One could argue that the opted approach might have resulted in a lack of effect of the cell therapy in our study. The majority of published trials in CLI and PAD have used either mobilized blood derived MNC or BM-MNC, similar to our trial, and positive results have been reported for both cell types, while there is no evidence for superiority of either cell type.[20,21] The administration route to infuse the cells, i.e. intra-muscular vs. intra-arterial, is another factor that has differed among published studies. While superiority of the intra-muscular route has been suggested,[16] studies directly comparing the two showed no differential effects.[22-24] The infused amount of $CD34^+$ progenitor cells and total amount of BM-MNC have been proposed as predictors of treatment effect,[21,25,26] but this could also mirror a more beneficial prognosis in those patients where a larger amount of these cells can be isolated, and hence be a reflection of systemic disease severity rather than a predictor of therapeutic efficacy. The amount of cells administered in our study was at the low end of the spectrum published in literature, however, beneficial effects have been reported for even lower amounts in PAD[25,26] as well as in the heart.[27]
Cell manufacturing procedures have been reported to influence BM-MNC potency. Use of heparin during the cell isolation procedure, in a similar amount as used in our protocol, has been shown to negatively affect homing capacity of cells by disturbing the interaction of SDF-1α and its receptor CXCR4, which is important for recruitment and homing of cells,[28] being a prerequisite for contribution to neovascularization. We cannot exclude such an effect in our trial, however previously published studies using similar protocols, have reported potential clinical effectiveness.[17,25,27] In line, it has been shown that red cell contamination of the BM-MNC product might impede effectiveness.[29] Assmus et al. showed that there was substantial impairment of the BM-MNC product when $>0.2x10^9$ erythrocytes/mL are present. The amount of erythrocytes in our cell product was far below this threshold ($0.08x10^9$/mL±0.06 $x10^9$/mL) and is therefore unlikely to have influenced our product.

The Juventas-trial included mainly elderly (median age of 67 years) Caucasian patients with atherosclerotic PAD, with a high atherosclerotic burden and prevalence of cardiovascular disease, characteristic of a western CLI population. In contrast, several initial positive studies were conducted in Asian populations, often including relatively young patients with thromboangiitis obliterans.[16] Aging and comorbidities have been shown to induce functional impairment of BM-MNC which may limit the therapeutic potential of autologous BM-derived cell therapy. In part the lack of benefit in our patients could be due to BM cell dysfunction in patients with high atherosclerotic burden.[30] Furthermore, it could be argued that patients with severe disease and extensive tissue loss may have reached a stage where they cannot benefit from any therapy.

Altogether, the results from the Juventas-study are consistent with a lack of benefit of BM-MNC administration in CLI. The general improvement in both the placebo as well as the verum group during follow-up and the observations in our meta-analysis that placebo-controlled RCTs show modest or even a lack of difference between the intervention and control group,[18] suggest that placebo-effects are present and may have influenced previously published study results. Interestingly, discrepant results have also been observed for the more recent and larger cell-based trials in ischemic heart disease as compared to smaller pioneering studies.[31] Based on our trial's results, we cannot exclude a potential beneficial effect of different BM cell subpopulations, different administration routes or higher cell doses in CLI. Further study is warranted to investigate whether cell therapy strategies with selected cell populations, enhanced BM cell function or different modes of administration can provide therapeutic benefit in patients with CLI. The observations that in our clinical trial (**Chapter 5**) there is a general improvement in clinical parameters in both groups during the course of follow-up stresses the need for future RCTs to implement a rigorous double-blinded, placebo-controlled design.

Factors influencing cellular yield

BM aspirations have been performed for decades to obtain stem and progenitor cells for BM transplantations to treat (non)malignant hematologic disorders. Successful allogeneic hematopoietic BM transplantation depends on the total number of nucleated donor cells transplanted.[32,33] The number of administered BM cells has also been suggested to be a determinant of clinical effectiveness in regenerative medicine (RM) interventions.[25,34,35] In **Chapter 7** we report that differences in diameter of the BM aspiration needle affect the composition of the harvested BM. In our study population the use of a large 8 Gauge needle resulted in an over 30% increase in white blood cell content of the harvested BM, while an over 60% increase was observed for $CD34^+$ progenitor cells, compared to a smaller 15 Gauge needle. Although the results of Juventas will not lead to implementing BM-MNC therapy for treatment of CLI our findings may have relevance for other clinical BM cell applications.

BONE MARROW, PROGENITOR CELL, AND PLATELET RELATED DISTURBANCE IN CARDIOVASCULAR DISEASE

Impaired bone marrow and progenitor cell function

The presence of cardiovascular risk factors and overt cardiovascular disease are known to impair quantity and quality of BM-derived progenitor cells involved in vascular regeneration and neovascularization.[36,37] In **Chapter 8** we discuss the available literature that reports the influence of diabetes, as a paramount risk factor for cardiovascular disease, on EPC numbers and the underlying mechanisms leading to reduced circulating EPC numbers. Several processes in the BM, the circulation and at tissue level, are involved in

reducing circulating EPC numbers which may contribute to the impaired cardiovascular regenerative capacity in diabetes. In **Chapter 9** we confirm previous reports,[38-40] that mobilization of progenitor cells in a type-1 diabetes model is impaired. Moreover, our results suggest that diabetes induces an altered interaction of BM stroma and progenitor cells and that the hemangiogenic regenerative potential of diabetic BM is impaired. These studies underline the role of a disturbed BM environment in the impaired vasculoregenerative and neovascularization capacity in diabetes.

Cardiovascular risk factors and overt cardiovascular disease reduce EPC numbers, whereas acute ischemic events increase EPC levels in circulation. Studies on EPC number and function in patients with chronic continuous ischemia as a result of ongoing cardiovascular disease, as is the case in chronic CLI, are scarce. We studied the quantity of several progenitor cell populations in both the BM as well as the blood by flow cytometry in CLI patients in **Chapter 10**. We showed that CLI is associated with decreased circulating EPC numbers and a reduced number of primitive $CD34^+$ progenitor cells in the BM. Furthermore, paracrine (neovascularization stimulating) function of circulating angiogenic cells (CAC) is profoundly impaired in CLI. The amount and activity of MMP-9, required for progenitor cell egress from the BM,[42,43] was significantly reduced. Activity of MMP-9 is associated with nitric oxide (NO) availability,[44] which is reduced in atherosclerotic disease, and we indeed showed that arginine , the most important natural occurring substrate for NO production, was reduced in CLI patients. Additionally, levels of the inflammatory cytokine interleukin-6 were inversely associated with the number of $CD34^+$ progenitor cells in the BM. It has been previously shown that chronic inflammation can result in BM exhaustion[45,46] and our results suggests that a parallel process is involved in BM progenitor cell exhaustion in CLI. Together these results show that CLI is a systemic disease leading to systemic disturbance, including the BM, of vascular homeostasis and progenitor cell biology.

In **Chapter 11** we report that CLI is associated with BM microvascular alterations. It has previously been shown that diabetes induces microscopic changes in the BM of diabetic animal models, i.e. microvascular rarefaction,[47] BM niche alterations,[48] and neuropathic changes.[49] Spinetti et al. recently showed that diabetes is also associated with microvascular changes in human BM, particularly in patients with vascular complications, i.e. CLI.[50] However, this study did not allow conclusions on whether CLI in absence of diabetes has similar effects. In **Chapter 11** we show reduced numbers of microvessels in BM of patients with CLI compared to healthy controls. Since neuronal impulses, from sympathetic nerves in particular,[51,52] play a role in progenitor cell mobilization from BM we studied neuropathic changes and showed that both general as well as sympathetic innervation of the BM were affected in CLI. These observations were independent of the presence of diabetes. BM alterations could impair progenitor cell mobilization from the BM and hence provide an additional explanation for the reduction of circulating EPC in CLI patients.

Altered platelet responsiveness in critical limb ischemia

Platelets play a central role in the development of arterial thrombosis and subsequent cardiovascular events.[53] Abnormal platelet function with an increased tendency to aggregate is implicated in the pathogenesis of atherosclerosis[54] and development of superimposed acute ischemic events.[55,56] However, conflicting results have been reported for platelet reactivity in PAD, which might be related to the severity of the disease.[57-59] In **Chapter 12** we show increased baseline platelet activity in CLI, but reduced platelet reactivity to stimulation of all major platelet activation pathways in a flow cytometry based assay. Platelet reactivity was inversely associated with the presence of cardiovascular risk factors. Future prospective studies should confirm whether platelet reactivity using our detailed flow cytometric assay predicts future cardiovascular events in CLI and other populations at risk for cardiovascular events.

Conclusions and future perspectives

The poor quality of life in CLI (**Chapter 4**) and the substantial number of patients not eligible for conventional therapeutic strategies (**Chapter 2 and 3**), as discussed in the first part of this thesis, underlines that there is a need for novel therapeutic approaches in CLI. The core of this thesis (**Part II**), comprises of studies that focus on cell-based interventions in CLI. These approaches have been shown to be a promising revascularizing strategy for no-option CLI (**Chapter 5**). In the large randomized, double-blinded, placebo-controlled Juventas-trial, discussed in **Chapter 6**, we showed no beneficial effects of repetitive intra-arterial administration of BM-MNC into the common femoral artery compared to placebo in no-option CLI on a broad range of clinical parameters. While these results seem to contradict the results of previously published mainly small, non-blinded and non-placebo-controlled studies they are in line with the recently published larger well-designed, blinded and placebo-controlled studies that report more modest effects as discussed in the meta-analysis in **Chapter 5**. The lack of effect of BM-MNC in the Juventas-trial could be related to our observations of reduced progenitor cell number and function in atherosclerotic CLI and related cardiovascular risk factors (**Chapter 8, 9, and 10**), which could hamper effect of autologous cell therapy in this patient population. Future studies are warranted to investigate whether modified cell-based approaches, such as via administration of specific cell subpobulations or interventions to restore progenitor cell function via pre-treatment strategies are effective in CLI. We are currently studying these options in a translational research project funded by the Netherlands Organization for Scientific Research (NWO; ZonMw-TAS grant 116001026). Importantly, the observation in the Juventas-trial that the placebo and BM-MNC group improved over a broad range of endpoints during the course of follow-up, stresses the need for the implementation of a rigorous placebo-controlled and double-blind design in future RCTs in no-option CLI patients.

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PART FOUR

Discussion and summary

CHAPTER 14

Nederlandse Samenvatting

ACHTERGRONDEN EN HUIDIGE BEHANDELING VAN KRITIEKE ISCHEMIE

Kritieke ischemie, de ernstigste vorm van perifeer arterieel vaatlijden, wordt veroorzaakt door een kritische beperking van de doorbloeding van het been en wordt gekenmerkt door rustpijn en/ of weefselverval. Atherosclerose (slagaderverkalking) is, met name in de Westerse samenleving, de belangrijkste oorzaak van dit ziektebeeld. Kritieke ischemie gaat gepaard met een hoog risico op amputatie (tot 40% binnen 6 maanden) en cardiovasculaire complicaties met als gevolg sterftecijfers tot 20% binnen 6 maanden na de diagnose. Deze aantallen overstijgen die voor elke andere vorm van cardiovasculair lijden, waaronder symptomatisch vaatlijden van de kransslagaders. Dit exceptioneel hoge risico op cardiovasculaire complicaties is een weerspiegeling van de aantasting van de vaten in het gehele lichaam. De behandeling van kritieke ischemie gaat gepaard met hoge kosten. Met een geschatte incidentie van 500-1.000 nieuwe gevallen per miljoen personen per jaar vormt kritieke ischemie een belangrijke belasting voor zowel patiënten, zorgverleners als de gezondheidszorg.

Patiënten die lijden aan kritieke ischemie hebben een verhoogd risico op het ontwikkelen van cardiovasculaire complicaties, zoals bijvoorbeeld een hart- of herseninfarct. Het is daarom van belang dat cardiovasculaire risicofactoren, zoals hypertensie (hoge bloeddruk), hypercholesterolemie (hoog cholesterol), en diabetes (suikerziekte), adequaat worden behandeld. In de afgelopen jaren is de behandeling van deze risicofactoren duidelijk verbeterd. Recente literatuur laat echter zien dat er nog altijd ruimte is voor verdere verbetering (**Hoofdstuk 2**). Er wordt geschat dat alleen al in Noord-Amerika en West-Europa jaarlijks 200.000 cardiovasculaire complicaties (hartinfarct, herseninfarct en sterfte als direct gevolg van cardiovasculaire complicaties) kunnen worden voorkomen bij patiënten met perifeer arterieel vaatlijden door een toename van het gebruik van medicijnen die het cardiovasculaire risico verlagen. Daarom is het van belang om het besef ten aanzien van het hoge cardiovasculaire risico en de noodzaak tot behandeling van risicofactoren bij patiënten met kritieke ischemie te vergroten, om zo de behandeling van deze patiënten verder te verbeteren.

In **Hoofdstuk 2** van dit proefschrift hebben we laten zien dat in het afgelopen decennium de behandeling van kritieke ischemie, en perifeer arterieel vaatlijden in zijn algemeenheid, is veranderd. Deze veranderingen in de behandeling gaan gepaard met een verbeterende prognose van kritieke ischemie, waarbij het risico op amputatie elk jaar lijkt af te nemen. Er is echter onvoldoende bewijs dat een verbetering van de revascularisatiemogelijkheden (behandelingen om de doorbloeding van het been te herstellen) de directe oorzaak is van deze positieve trend ten aanzien de prognose van kritieke ischemie. **Hoofdstuk 3**, onderdeel van de Europese richtlijn voor de behandeling van kritieke ischemie en de diabetische voet van de "European Society of Vascular Surgery", verschaft adviezen ten aanzien van de

behandeling van kritieke ischemie, gebaseerd op de meest recente literatuur. Goede literatuur op het gebied van kritieke ischemie is echter beperkt, als gevolg van onder andere het ontbreken van een goede en eenduidige definitie van het ziektebeeld en het gebrek aan kwalitatief goed opgezette en uitgevoerde studies. Als gevolg hiervan zijn behandeladviezen ten aanzien van kritieke ischemie veelal gebaseerd op de mening van experts of op resultaten uit studies bij patiënten met mildere vormen van perifeer arterieel vaatlijden. Het is daarom van belang om internationaal de handen in één te slaan om grote en kwalitatief goede studies op te zetten en uit te voeren en zo uiteindelijk de behandeling van kritieke ischemie in de toekomst verder te verbeteren (**Hoofdstuk 2 en 3**).

Patiënten met kritieke ischemie worden niet alleen bedreigd door een hoog risico op amputatie en sterfte, zij ervaren tevens aanzienlijke functionele beperkingen en pijnklachten, hetgeen leidt tot een slechte kwaliteit van leven (**Hoofdstuk 4**). De ernstig beperkte kwaliteit van leven en het feit dat een aanzienlijk deel van de patiënten (25-40%) niet in aanmerking komt voor de bestaande behandelingsmogelijkheden onderstreept de grote noodzaak voor nieuwe behandelingsopties voor kritieke ischemie.

BEENMERGCELTHERAPIE ALS BEHANDELING VOOR KRITIEKE ISCHEMIE

Het beschikbare bewijs

In 1997 werd door de onderzoeksgroep van Asahara een cel in het beenmerg ontdekt die als voorloper zou fungeren van de endotheelcel, de binnenbekleding van de bloedvaten, en betrokken zou zijn bij vaatnieuwvorming en herstel van vaatschade. Deze cel wordt de endotheliale progenitorcel (EPC) genoemd. Tot op dat moment werd gedacht dat daadwerkelijke vaatnieuwvorming (vasculogenese), dus niet als gevolg van vertakking vanuit pre-existente vaatjes (angiogenese), uitsluitend voor de geboorte plaats zou vinden. Als gevolg van dit verschuivend paradigma is men de mogelijkheid om beenmergcellen als therapie toe te passen gaan onderzoeken bij ziekten waarbij vaatschade en doorbloedingsstoornissen een rol spelen. Spoedig na de ontdekking van de EPC werden de eerste studies verricht naar het effect van beenmergceltoediening op het herstel van weefseldoorbloeding bij patiënten met hart- en vaatziekten, onder andere na een hartinfarct of bij perifeer arterieel vaatlijden. Sindsdien zijn verscheidene studies uitgevoerd naar het effect van beenmergceltherapie bij patiënten met kritieke ischemie. Deze studies waren over het algemeen klein, van matige kwaliteit en beschreven vaak klinisch weinig relevante uitkomsten, waardoor zij geen definitief bewijs voor de effectiviteit van beenmergceltoediening bij kritieke ischemie hebben geleverd. In **hoofdstuk 5** beschrijven we de resultaten van een recente meta-analyse die we hebben uitgevoerd, welke de resultaten van twaalf gerandomiseerde studies naar het effect van beenmergcellen bij

kritieke ischemie samenvat. Deze meta-analyse onderstreept het veelbelovende karakter van de therapie, maar toont vooral ook effectverschillen tussen studies die placebo- en niet-placebogecontroleerd zijn uitgevoerd, hetgeen de noodzaak van een grote, goed ontworpen en placebogecontroleerde studie met klinisch relevante uitkomsten benadrukt.

Juventas-trial

Onze gerandomiseerde, dubbelblinde, placebogecontroleerde Juventas-studie, welke van start ging in 2006, is ontworpen met als doel te onderzoeken of herhaaldelijke intra-arteriële (in de slagader) toediening van beenmergcellen het aantal amputaties bij patiënten met uitbehandelde kritieke ischemie kan verminderen. De Juventas-trial is met 160 patiënten op dit moment de grootste gerandomiseerde studie op dit gebied. De studieresultaten, beschreven in **hoofdstuk 6**, laten op geen enkele uitkomst een effect zien van intra-arteriële toediening van beenmergcellen ten opzichte van placebo. Verder laat de studie zien dat de placebogroep gedurende de studie een algehele verbetering vertoont op zowel objectieve als subjectieve uitkomsten. Daarmee spreken onze resultaten die van de hoofdzakelijk kleine, niet-placebogecontroleerde en niet-gerandomiseerde studies tegen. De resultaten liggen echter in het verlengde van de resultaten van de meta-analyse in **hoofdstuk 5**, waar de placebogecontroleerde studies aanzienlijk gematigder resultaten laten zien dan de niet-placebogecontroleerde studies.

Voor het gebrek aan effect van beenmergceltherapie, zoals dat gezien wordt in onze trial, bestaan naast een daadwerkelijke ineffectiviteit van beenmergcellen als therapie bij patiënten met kritieke ischemie, nog verscheidene andere potentiële verklaringen. Andere mogelijke verklaringen zouden kunnen liggen in (1) de keus voor het toegediende celtype, (2) de wijze van toediening (intra-arterieel versus intra-musculair), (3) de toegediende celdosis, of (4) de wijze van bewerking van de cellen voorafgaand aan toediening. Voor elk van deze factoren zoals die in onze studie golden zijn eerder positieve resultaten gerapporteerd. We kunnen echter op basis van onze studieresultaten niet uitsluiten dat bepaalde subpopulaties van beenmergcellen, andere toedieningswegen of hogere celdoseringen effectief kunnen zijn bij kritieke ischemie.

In de Juventas-studie zijn voornamelijk oudere patiënten met atherosclerotisch vaatlijden geïncludeerd, waarbij uitgebreide atherosclerose en hart- en vaatziekten aanwezig zijn, karakteristiek voor de westerse populatie met kritieke ischemie. Daarentegen werden de eerste positieve resultaten beschreven bij Aziatische populaties met relatief jonge patiënten, waarbij kritieke ischemie veelal het gevolg was van thromboangiitis obliterans (een ontstekingsachtig beeld van de bloedvaten). Hogere leeftijd en co-morbiditeit hebben een bewezen negatief effect op het functioneren van de beenmergcellen, waardoor het therapeutisch effect van deze cellen beperkt kan worden. Het is dus mogelijk dat het gebrek aan effect in onze studie deels het gevolg is van de beenmergceldysfunctie bij onze patiënten met uitgebreid atherosclerotisch vaatlijden.
Al met al tonen de resultaten van de Juventas-studie een gebrek aan effectiviteit van

beenmergceltoediening bij patiënten met kritieke ischemie, althans wanneer het wordt toegepast zoals in ons studieprotocol. De algehele verbetering in zowel de placebo- als in de beenmergcelgroep in onze studie, tezamen met de uitkomsten van onze meta-analyse waarbij de placebogecontroleerde studies geen of een geringer effect van de celtherapie laten zien, is suggestief voor het feit dat er sprake is van belangrijke placebo-effecten waardoor mogelijk eerder gepubliceerde studieresultaten kunnen zijn beïnvloed. Opvallend genoeg is eenzelfde beeld te zien bij studies in ischemische hartziekten (hartziekten als gevolg van doorbloedingsprobleem), waarbij er discrepante resultaten worden gerapporteerd voor de meer recente grotere studies ten opzichte van de eerste kleine studies. Toekomstige studies zijn vereist om te onderzoeken of strategieën die gebruik maken van geselecteerde subpopulaties van cellen, methoden om beenmergcelfunctie te optimaliseren, of andere toedieningswegen of combinaties hiervan effectief kunnen zijn bij kritieke ischemie. Het feit dat in onze studie (**Hoofdstuk 6**) in beide groepen, dus ook in de placebogroep, een algehele verbetering wordt waargenomen gedurende follow-up benadrukt de noodzaak voor toekomstige studies om een dubbelblind en placebogecontroleerd design toe te passen.

Factoren die de celopbrengst beïnvloeden

Beenmergpuncties worden al sinds de jaren 60 van de vorige eeuw toegepast om stam- en progenitorcellen te verkrijgen voor beenmergtransplantaties voor de behandeling van hematologische aandoeningen (ziektes van de bloedcellen). Het succes van deze behandelingen hangt vaak samen met de hoeveelheid donorcellen die worden getransplanteerd. Er zijn aanwijzingen dat dit ook een rol speelt wanneer deze cellen toegepast worden in het kader van de regeneratieve geneeskunde. In **hoofdstuk 7** laten we zien dat de diameter van de naald die gebruikt wordt om het beenmerg af te nemen een belangrijke bepalende factor is voor de samenstelling van het afgenomen beenmerg. Wanneer een naald met een grotere diameter wordt gebruikt is de opbrengst van het aantal witte bloedcellen in het aspiraat circa 30% hoger en het aantal hematopoietische stamcellen tot 60% hoger. Hoewel de resultaten van de Juventas-trial geen directe aanleiding geven tot het introduceren van beenmergceltherapie voor de behandeling van kritieke ischemie, zijn onze bevindingen mogelijk relevant voor toekomstige studies en voor andere toepassingsgebieden van beenmergcellen.

BEENMERG, PROGENITORCEL, EN PLAATJES GERELATEERDE DYSFUNCTIE IN CARDIOVASCULAIRE ZIEKTEN

Verstoorde beenmerg- en progenitorcelfunctie

De aanwezigheid van cardiovasculaire risicofactoren en daadwerkelijke cardiovasculaire ziekten geven aanleiding tot een beperking van kwantiteit en kwaliteit van uit beenmerg afkomstige progenitorcellen die betrokken zijn bij herstel van vaatschade en vaatnieuwvorming. In **hoofdstuk 8** wordt de beschikbare literatuur beschreven omtrent

de invloed van diabetes, één van de belangrijkste cardiovasculaire risicofactoren, op EPC-aantallen en de onderliggende mechanismen die aanleiding geven tot de gereduceerde progenitorcelaantallen. Diverse processen in het beenmerg, de circulatie, en op weefselniveau geven gezamenlijk aanleiding tot gereduceerde circulerende EPC-aantallen bij diabetes. Dit draagt mogelijk bij aan de beperkte capaciteit tot cardiovasculaire regeneratie, zoals dat het geval is bij patiënten met diabetes. In **hoofdstuk 9** bevestigen we in een diermodel met diabetes de observaties uit eerdere publicaties van anderen dat de mobilisatie van progenitorcellen uit het beenmerg naar de bloedbaan is verstoord. Daarenboven laten onze resultaten zien dat diabetes een verandering in de interactie tussen progenitorcellen en het ondersteunende weefsel in het beenmerg teweegbrengt en het regeneratieve vermogen van het beenmerg beperkt. Deze studies onderstrepen dat een verstoring van het beenmergmileu een rol speelt bij de afgenomen capaciteit voor vaatherstel en vaatnieuwvorming bij diabeten.

Aanwezigheid van cardiovasculaire risicofactoren en cardiovasculaire ziekten geven aanleiding tot gereduceerde EPC-aantallen, terwijl acute doorbloedingsstoornissen leiden tot een toename van EPC-aantallen in de bloedbaan. Studies naar EPC-aantallen en -functie bij patiënten met chronische continue doorbloedingsstoornissen als gevolg van voortschrijdende cardiovasculaire ziekten, zoals bij kritieke ischemie het geval is, zijn zeldzaam. In **hoofdstuk 10** hebben wij de aantallen van verschillende progenitorcellen in het beenmerg en in de bloedbaan van patiënten met kritieke ischemie bestudeerd. We laten zien dat het aantal circulerende EPCs in de bloedbaan en een specifieke primitieve voorlopercel in het beenmerg bij patiënten met kritieke ischemie verlaagd zijn ten opzichte van een gezonde controlegroep. Verder is de (paracriene) functie van een uit het bloed gekweekte progenitorcel afgenomen. In het beenmerg en het bloed zijn bepaalde factoren verstoord die een rol spelen bij de mobilisatie van progenitorcellen uit het beenmerg, hetgeen aangeeft dat kritieke ischemie niet alleen een lokaal probleem, maar vooral een systemische ziekte is, die de balans tussen vaatschade en -herstel verstoort. Dit zien we wederom terug in **hoofdstuk 11**, waar we beschrijven dat de vaat- en zenuwstructuur in het beenmerg, welke een belangrijke rol spelen bij de mobilisatie van progenitorcellen uit het beenmerg, van patiënten met kritieke ischemie afwijkend is.

Verandering van de bloedplaatjesreactiviteit bij kritieke ischemie

Bloedplaatjes zijn betrokken bij het vormen van stolsels na en herstel van weefselschade. De aanwezigheid van een abnormale bloedplaatjesfunctie met een toegenomen neiging tot activatie en samenklontering speelt ook een belangrijke rol bij het ontstaan van atherosclerose en de daarmee geassocieerde acute doorbloedingscomplicaties. Voor perifeer arterieel vaatlijden zijn er conflicterende resultaten met betrekking tot bloedplaatjesactiviteit, welke mogelijk gerelateerd zijn aan het stadium van de ziekte waarin deze wordt bestudeerd. In **hoofdstuk 12** laten we zien dat bij patiënten met kritieke ischemie de bloedplaatjesactiviteit is toegenomen, maar dat de reactiviteit, dus de mogelijkheid tot aanvullende activatie, is afgenomen. Verder blijkt de aanwezigheid van cardiovasculaire

risicofactoren gepaard te gaan met een afgenomen plaatjesreactiviteit. Toekomstige studies moeten deze bevindingen bevestigen en onderzoeken of de plaatjesreactiviteit in deze populatie voorspellend is voor toekomstige cardiovasculaire problematiek.

Conclusies en een blik op de toekomst

De slechte kwaliteit van leven van patiënten met kritieke ischemie (**Hoofdstuk 4**) en het aanzienlijke aantal patiënten dat niet in aanmerking komt voor de standaard behandelingsmogelijkheden (**Hoofdstuk 2 en 3**), zoals besproken in het eerste deel van dit proefschrift, onderstrepen dat er een noodzaak is voor nieuwe therapeutische opties voor patiënten met kritieke ischemie. De kern van dit proefschrift (**Deel II**), bestaat uit studies die zich concentreren op celtherapie bij kritieke ischemie. Deze benadering lijkt, ondanks de resultaten van de Juventas-trial, nog altijd een veelbelovende nieuwe behandelingsoptie voor uitbehandelde kritieke ischemie (**Hoofdstuk 5**). In de grote gerandomiseerde, dubbelblinde, placebogecontroleerde, Juventas-trial, besproken in **hoofdstuk 6**, zagen we geen positieve resultaten van herhaaldelijke intra-arteriële toediening van beenmergcellen in de liesslagader ten opzichte van placebo bij patiënten met uitbehandelde kritieke ischemie. Terwijl deze resultaten, die van eerdere kleine, niet-geblindeerde, niet-placebogecontroleerde studies, lijken tegen te spreken, passen ze in de lijn van meer recent gepubliceerde en goed ontworpen, geblindeerde en placebo-gecontroleerde studies die meer gematigde resultaten laten zien, zoals besproken in de meta-analyse in **hoofdstuk 5**. De afwezigheid van effect van beenmergceltoediening in de Juventas-trial zou het gevolg kunnen zijn van onze bevindingen van gereduceerde progenitorcelaantallen en -functie bij patiënten met kritieke ischemie als gevolg van atherosclerotisch vaatlijden en de daaraan gerelateerde risicofactoren (**Hoofdstuk 8, 9, en 10**), wat het effect van autologe celtherapie (cellen afkomstig van de patiënt zelf) in deze patiëntenpopulatie ernstig zou kunnen beperken. Toekomstige studies zijn vereist om te bestuderen of gemodificeerde celtherapeutische benaderingen, zoals middels celselectie of methoden gericht op optimalisatie van beenmergceltherapie met behulp van pre-treatment strategieën, effectief zouden kunnen zijn bij kritieke ischemie. We zijn deze mogelijkheden momenteel aan het exploreren in een translationeel onderzoeksproject gesponsord door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO; ZonMw-TAS grant 116001026). Gezien het feit dat in de Juventas-trial in beide groepen, dus ook de placebogroep, een verbetering wordt waargenomen over een grote verscheidenheid aan uitkomsten gedurende follow-up, is het essentieel dat toekomstige gerandomiseerde studies bij patiënten met uitbehandelde kritieke ischemie een gedegen placebogecontroleerd en dubbelblind design implementeren.

PART FOUR

Discussion and summary

CHAPTER 15

Review committee
Dankwoord
Curriculum vitae
List of publications

REVIEW COMMITTEE

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DANKWOORD

Het proefschrift, zoals dat nu voor u ligt, is primair uit mijn naam geschreven, maar dit dankwoord biedt me de mogelijkheid om te benadrukken dat dit proefschrift nooit voor u had gelegen zonder de hulp van een grote groep mensen. De welwillendheid, flexibiliteit, kritische blik en vooral ook het harde werk van velen heeft mij in de gelegenheid gesteld om mijn proefschrift succesvol af te ronden. Ik wil iedereen waarop ik de afgelopen jaren heb kunnen rekenen niet alleen bedanken voor dit tastbare bewijs van drieënhalf jaar hard werken, maar vooral ook voor alle niet direct zichtbare bagage die ik mijn leven mee zal dragen. Het is bijna inherent aan een dankwoord dat je personen vergeet, waarvoor bij voorbaat excuses.

Prof. Dr. M.C. Verhaar, beste Marianne, allereerst dank voor het vertrouwen al die jaren! Ik heb grote bewondering voor de wijze waarop jij mensen weet aan te voelen en te motiveren. Ook heel prettig dat er altijd ruimte was om dingen te bespreken die niet direct werkgerelateerd waren en dat je ook altijd aandacht hebt voor de persoon achter de promovendus. Jouw kritische blik en ons gezamenlijk perfectionisme hebben dit proefschrift ongetwijfeld naar een hoger niveau getild. Ik hoop nog vaak en lang met je samen te mogen werken. Dat is een absoluut voorrecht!

Prof. Dr. F.L. Moll, beste Frans, de dag waarop we, bij de koffieautomaat op de stafgang, tijdens mijn ASAS Chirurgie, aan de praat raakten over promotieonderzoek, herinner ik me nog als de dag van gisteren. Het begin van leerzame, succesvolle en leuke jaren. Hoe jij mensen weet te inspireren vind ik absoluut benijdenswaardig. Je deskundige adviezen op klinisch, onderzoeks-, en politiek gebied hebben mij mede gebracht waar ik nu sta, dit boekje in mijn hand en een aanstelling als ANIOS bij de heelkunde in het UMC. Ik hoop nog heel veel van je te mogen leren en nog veelvuldig met je samen te mogen werken zowel in de kliniek als daarbuiten! Naast alle professionele adviezen, ook heel erg bedankt voor de gezelligheid en wijze lessen!

Leden van de leescommissie, Prof. Dr. W.P.Th.H. Mali, Prof. Dr. I.H.M. Borel Rinkes, Prof. Dr. A. Algra, en Prof. Dr. W.J.A. Dhert, hartelijk dank voor het lezen en beoordelen van mijn manuscript.

Behalve Prof. Dr. M.C. Verhaar, Prof. Dr. F.L. Moll en ikzelf, bestaat het Juventas-studieteam uit nog een aantal personen zonder wie de Juventas-studie niet tot een succes gemaakt had kunnen worden. Allereerst, Dr. R.W. Sprengers, beste Ralf, zonder jouw zorgvuldige en gestructureerde opzet en start van de Juventas was het nooit gelukt om de inclusie te voltooien. Tevens bedankt voor het feit dat ook jij me het vertrouwen hebt gegeven om je op te volgen als coordinating investigator van de Juventas. Dr. R.E.G. Schutgens, beste Roger, dank voor je flexibiliteit en het verrichten van de beenmergpuncties, ondanks je eigen drukke agenda. Er waren gedurende mijn promotie een paar zekerheden

en één daarvan was dat ik altijd op je kon rekenen! Dr. I.C.M. Slaper-Cortenbach, beste Ineke, zonder jouw inzet en die van de stamcellabmedewerkers, was de uitvoer van de Juventas niet mogelijk geweest. Dank! Prof. Dr. Y. Van der Graaf, beste Yolanda, hartelijk dank voor je betrokkenheid en professionele adviezen tijdens de opzet en uitvoer van de Juventas-trial. Prof. Dr. A. Algra, beste Ale, en Dr. I. Van der Tweel, beste Ingeborg, hartelijk dank voor jullie goede raad omtrent de uitvoer en analyse van de resultaten van de Juventas-trial. Prof. Dr. W.P.Th.H. Mali en Prof. Dr. P.A. Doevendans, hartelijk dank voor jullie advies en wijze woorden.

Grote dank aan alle vaatchirurgen in het UMC Utrecht, Gert Jan de Borst, Raechel Toorop, Joost van Herwaarden, Paul Berger, Stijn Hazenberg en Evert Waasdorp. Hartelijk dank voor de laagdrempelige mogelijkheid tot overleg, bereidheid mee te denken en het verwijzen van patiënten. Mede dankzij jullie heb ik me thuisgevoeld en voel ik me thuis bij de vaatchirurgie!
Uiteraard ook dank aan de rest van Vaatchirurgisch Nederland, want een studie als de Juventas is alleen uit te voeren wanneer er voldoende steun is van de perifere ziekenhuizen. Door de handen in één te slaan kunnen we de wetenschap, en uiteindelijk de patiënt, vooruit helpen. Dank voor alle steun!

Ik wil ook de collega's van de radiologie bedanken, die in het geval van een onmogelijk aan te prikken AFC, nooit te beroerd waren om een handje te helpen. In het bijzonder ook Evert-Jan Vonken, dank voor je laagdrempelige bereikbaarheid voor overleg.

Beste Liane en Peter, hartelijk dank voor de beenmergpuncties op de momenten waarop Roger echt niet kon.

Beste Susan en Cobie, dank voor alle gezelligheid en de fijne samenwerking. Ik kon bij jullie altijd even crashen voor een bak "echte" koffie en een praatje. Wat kan de vaatchirurgie zich gelukkig prijzen met jullie!
Medewerksters van het vaatcentrum, hartelijk dank voor alle inzet. Suzanne, Erica, Jaenette, Yvonne, Wilma, Rachma en Monique hartelijk dank voor de gezelligheid en al het regelwerk! Veerle, Anne-Marieke en Marlous, ik kan jullie op geen enkele wijze voldoende bedanken, want zonder jullie ondersteuning bij het regelen en plannen van alle logistiek rondom de aanvullende onderzoeken, beenmergpuncties en opnames, was het uitvoeren van de Juventas ondoenlijk geweest. Wanneer ik jullie iets vroeg wist ik dat het goed geregeld werd. Jullie zijn echt TOPPERS!

Iedereen op de afdelingen D3 West en D4 Oost hartelijk dank voor de hulp bij de vele infusies en opnames. En daarnaast uiteraard ook voor alle gezelligheid!

Medewerkers van het stamcellab, Kasper, Ruud, Lex, Paula, Simone en Marcelle, het moet voor jullie ook niet altijd even eenvoudig zijn geweest om je mond niet voorbij te praten

wanneer jullie me de studiemedicatie aanleverden. Toch bijzonder dat het voor geen enkele patiënt is uitgelekt, dat getuigd van discipline!

Dames van het vaatlab, hartelijk dank voor alle enkel/arm-indexmetingen.

Ik wil alle (oud) collega's van het lab experimentele nefrologie en experimentele cardiologie bedanken voor de gezelligheid en alle adviezen omtrent laboratoriumzaken, want daar ben je namelijk als arts, met een enkele uitzondering (lees Hendrik), niet voor in de wieg gelegd. Kamergenoten, Petra, Arianne, Diana en Dries, ondanks het feit dat we elkaar soms meer dan 40 uur per week zagen raakten we niet uitgepraat. Met jullie leken de lange dagen een stuk korter. Hendrik, waar de Juventas ophoudt, ga jij verder. Als er iemand is die in staat om celtherapie 3.0 werkelijkheid te maken, dan ben jij het wel. Bas, Joost, Dimitri, Gisela, Olivier, Maarten, Krista, Marloes, Chantal dank voor de fijne samenwerking en succes. Krijn en Alain jullie waren prettige labburen. Alle andere promovendi en medewerkers van het cardiolab dank voor jullie gezellgheid en de prettige samenwerking.

Promovendi van de chirurgie, Dominique, Lutske, Stefanie, Amir, Bas, Kari, Okan, Pieter van der Sluis, Marjolein, Margriet, Joyce, Teus, Leonie, Samira, Herman, Peter Paul, Claire, Anne, Jakob, Pieter Leliefeld, Femke, en Querine, allemaal bedankt voor de gezellige momenten tijdens borrels, trips en chirurgencups. Succes met het vervolg en het is echt een onwijs goed gevoel wanneer je het boekje in je handen hebt! Hou vol! Alle oud-promovendi, Benjamin, Jasper, Ernst, Stijn, Klaas, Emily, Roy, Dave en vele anderen eveneens dank voor de gezelligheid en we komen elkaar vast nog regelmatig tegen.

Dank aan alle arts-assistenten van de chirurgie in het UMC, Cas, Hjalmar, Anneriet, Falco, Marjolein, Guus, Bob, Winan, Olaf, Charlotte, Pieter, Henk, Cees, Jacqueline, Rosa en Vanessa, voor de hulp en wijze adviezen. Ondanks het feit dat we met zijn allen vreselijk hard werken, is er toch tijd voor gezelligheid en wordt er naar elkaar geluisterd. Ik voel me in de korte tijd dat ik nu als ANIOS aan de slag ben erg op mijn plek, hopelijk mag dat op niet al te lange termijn als AIOS voortduren.

Dank ook aan alle stafleden van de chirurgie in het UMC Utrecht voor het begrip, de hulp en adviezen. In het bijzonder ook Dr. M.R. Vriens, beste Menno, bedankt voor het vertrouwen.

Wendy ik wil je ontzettend bedanken voor het grandioze werk dat je geleverd hebt. Het is echt een bijzondere kwaliteit hoe je met enkele woorden en aanwijzingen exact weet op te pikken wat de wensen van iemand zijn. Jouw vormgeving van omslag en binnenwerk zorgen ervoor dat ik nog veel trotser ben met wat ik in mijn handen heb!

Naast steun, gezelligheid en begrip van collega's, is dat van vrienden en familieleden

eveneens onmisbaar! Het is ondoenlijk om iedereen persoonlijk te bedanken, dus bij deze allemaal bedankt! Maar enkele personen verdienen een aparte vermelding.

Allereerst de paranimfen. Lieve Maud, een beetje een ongebruikelijke keus is het misschien wel, aangezien paranimfen veelal uit eenzelfde generatie afkomstig zijn, dat scheelt er bij ons wel één. Ik ben vreselijk blij en trots dat jij mijn paranimf bent en me op de dag van mijn promotie wilt bijstaan. Er zijn meerdere redenen waarom ik jou als paranimf heb gevraagd, maar bovenal je voortdurende betrokkenheid bij zowel mijn promotie als bij ons persoonlijke leven hebben daarbij een rol gespeeld. Daarnaast heb je toch ook wel een beetje een voorbeeldfunctie voor zowel mij als Chris. Dank voor alle fijne adviezen en wijze raad en dat geldt uiteraard ook voor Tom.
Lieve Tavie (Taaf voor intimi), je bent geweldig als persoon en als vriend! Jullie betrokkenheid, die van jouw en Thomas, bij mijn promotie en met name ook de laatste hectische maanden daarvan, zijn voor zowel mij als Chris een grote steun geweest. Echte vrienden heb je maar weinig, maar daar horen jullie zeker bij! We hebben al veel mooie en leuke momenten met elkaar meegemaakt en er zullen er vast nog vele volgen. Taaf, dank dat je me ook op deze dag wilt steunen. Daarnaast is het voor de foto's ook een leuke bijkomstigheid zo'n schoonheid aan mijn zijde ;) Taaf en Thomas bedankt!

Beste ome Maart, van sommige mensen leer je wijze lessen in de professionele sfeer en van anderen wijze levenslessen, die je op alle vlakken een completer persoon maken. Onze gesprekken en lange wandelingen, daar komt het niet meer zo van, maar ik denk er nog vaak aan terug. Ik heb veel van je geleerd en jullie, jij, Astrid, Melis en Milou, hebben een apart plekje bij Chris en mij. Bedankt!

Lieve oma, Christa en ik willen je graag bedanken voor alle lieve woorden en gebaren en het begrip voor het feit dat we niet bij alle familiefeestjes en dergelijke konden zijn, vaak vanwege mijn werk. Ik hoop dat we het met twee unieke gebeurtenissen binnen een half jaar, voor de eerste keer overgrootoma worden en de eerste promotie in de familie, een beetje goedmaken. Lieve oma, bedankt!

Lieve Rob en Annemiek, je hoort vaak dat de relatie met schoonouders niet ideaal is. Gelukkig kan ik daar niet over meepraten. Jullie zijn super! We hopen nog veel met jullie te ondernemen en nog vaak met jullie op de lange latten te mogen staan! Bedankt voor alles!

Liefste papa en mama, kleine jongens worden groot! Jullie zijn er altijd voor mij geweest en zijn er nog altijd voor ons. Zonder jullie steun, hulp en af en toe een strenge blik, was ik niet geweest wie ik nu ben en waar ik nu ben. Ik had me geen betere ouders kunnen wensen en hoop jullie nog heel lang in goede gezondheid bij me te hebben! Geniet van deze dag, want die is ook een beetje van jullie! Bedankt voor alles!!!

Lieve Chris, mijn steun en toeverlaat, wat jij voor mij betekent is in geen woorden uit te drukken. Dit is niet alleen mijn proefschrift, maar ons proefschrift! Je klaagde nooit wanneer ik weer eens om negen uur thuiskwam voor het eten, wanneer ik zei "ik vertrek over vijf minuten", maar dat er standaard altijd 50 werden, wanneer ik weer eens een heel weekend bezig was met een manuscript, wanneer ik met je verjaardag in Chicago was, het was altijd goed, ook al was het niet altijd leuk. Ik ben trots op jou! Je bent geweldig als persoon, als vrouw en als moeder! Ik hou van je! 1+1=1! Dat laatste klopt alleen niet helemaal meer, want inmiddels is ons leven verrijkt met onze kleine man, Stijn, wat een geweldig kereltje. Stijn, excuses dat papa je wat weinig aandacht heeft gegeven, maar dat gaan we helemaal goedmaken. Ik kijk uit naar de toekomst samen!

CURRICULUM VITAE

Martin Teraa was born on October 13, 1983 in Boxtel, The Netherlands. After he graduated from secondary school in 2002 (Jacob Roelandslyceum, Boxtel) he joined the Royal Netherlands Air Force for a short period of time, after which he realized that a different uniform would suit him better. In 2003 he started studying Medicine at Utrecht University, The Netherlands, and graduated with honor in 2009. During his regular internships he developed his interest in surgery, which was further fueled by his clinical and research internship under the supervision of Prof. Dr. L.P.H. Leenen, which ultimately resulted in two publications. After graduating he was offered the opportunity to work as a PhD candidate with Prof. Dr. M.C. Verhaar and Prof. Dr. F.L. Moll, coordinating a large randomized, double-blind, placebo-controlled trial studying the clinical effectiveness of repetitive intra-arterial infusion of bone marrow-derived cells in patients with no-option critical limb ischemia (the Juventas-trial; NCT00371371), which has been the foundation of this thesis. Study results have been presented at large international congresses. During the last part of his PhD project he started working as a resident at the Department of Surgery of the University Medical Center Utrecht. In the near future he plans to apply for a position to start surgical training.

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* Shared first authorship

Stellingen behorende bij het proefschrift

Treatment of critical limb ischemia

A SHIFTING PARADIGM

Door Martin Teraa

During the past decade treatment and prognosis of critical limb ischemia (CLI) have evolved, which should be taken into account when interpreting somewhat older studies in this population. (dit proefschrift)

High-level evidence, typically level I, to guide evidence-based clinical decision making in CLI is limited. (dit proefschrift)

Transatlantic or large continental collaborative efforts may be needed to guarantee sufficiently powered trials in CLI. (dit proefschrift)

Despite a positive trend, there is room for improvement of secondary prevention strategies in peripheral artery disease (PAD) and CLI patients. (dit proefschrift)

No-option CLI results in substantial impairment of physical functioning and quality of life, underlining the need for novel interventions in this population. (dit proefschrift)

Stem and progenitor cell-based interventions in PAD should still be confined to a research setting and not be implemented as a standard of care. (dit proefschrift)

Results of trials that focus on cell-based interventions in CLI should be interpreted with caution. (dit proefschrift)

The results of the Juventas-trial show no differential effects in the bone marrow-derived cell and the placebo treated group. (dit proefschrift)

Future trials that study cell-based interventions in CLI should implement a double-blind and placebo-controlled design in order to provide sound and reasonable conclusions. (dit proefschrift)

Cardiovascular diseases and risk factors induce bone marrow and progenitor cell dysfunction, which might contribute to cardiovascular complications and impaired efficacy of autologous cell-based approaches. (dit proefschrift)

Cell-based interventions in CLI are still promising, however future research should focus on selecting the most effective approach with respect to cell subtype and origin, administration route, and pretreatment strategies. (dit proefschrift)

Those who dare to fail miserably can achieve greatly. (John F. Kennedy)

What we know is a drop of water, what we do not know is the ocean. (Isaac Newton)

Find a job you love and you'll never work a day in your life. (Confucius)

It's supposed to be hard. If it wasn't hard, everyone would do it. The hard is what makes it great. (Tom Hanks in A League of Their Own)

Simpel is het moeilijkst. (Johan Cruijff)