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Diminished Circulating Monocytes after Peripheral Bypass Surgery for Critical Limb Ischemia

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Abstract:

Introduction:

Mononuclear cells (MNCs) have been shown to increase neovascularization and ulcer healing after direct injection into the ischemic limbs of patients with unreconstructable peripheral vascular disease (PVD). Circulating MNCs are composed of lymphocytes (85%), monocytes (15%) and endothelial progenitor cells (EPCs; 0.03%). It is thought that MNCs may be effective in ameliorating ischemia since EPCs are a component of the monocyte fraction, and EPCs have been shown to participate in vascular healing.

We hypothesized that ischemic areas secrete paracrine signals such as cytokines and growth factors that recruit bone marrow-derived monocytes into the circulation in order to augment vascular healing. For this reason we predicted that patients with critical limb ischemia (CLI) undergoing bypass surgery would have elevated preoperative monocyte counts compared to control subjects without CLI. In addition, since a successful surgical bypass procedure relieves ischemia, we expected a postoperative decrease in circulating monocyte numbers.

Methods:

We reviewed the records of all patients at the VA Connecticut Healthcare System undergoing lower extremity peripheral bypass surgery between 2002 and 2007. Patients were excluded if they did not have both preoperative and postoperative complete blood counts with differentials within a given time frame. Subjects were divided into two groups: those with preoperative critical limb ischemia (CLI) and those without. ANOVA and Chi-Square were

used to compare counts, and multivariable logistic regression was used to determine risk factors.

Results:

Patients with CLI (n=24) had elevated preoperative monocyte counts compared to control patients (n=8) undergoing bypass for claudication or asymptomatic popliteal aneurysm (0.753 ± 0.04 vs. 0.516 ± 0.05; p=0.0046), but the preoperative lymphocyte count was not significantly different (1.979 ± 0.14 vs. 1.912 ± 0.22; p=0.814). After revascularization, ischemic patients had decreased monocyte counts compared to control patients (-20% vs. +55%; p=.0003) although lymphocyte ratios were unchanged in both groups (-10% vs. +1%; p=0.404). Diabetic patients also had reduced postoperative monocyte counts (-32% vs. +13%; p=0.035), however multivariable analysis demonstrated that the only factor that independently predicted reduced postoperative monocyte count was preoperative critical limb ischemia (p=0.038).

Conclusions:

Diminished numbers of circulating monocytes correlate with relief of ischemia after surgical revascularization. Circulating monocytes may be a clinically useful surrogate marker of circulating stem cells for patients undergoing vascular surgery.

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Table of Contents:

Introduction and Background:

The introduction is divided into two chapters. The first chapter focuses on endothelial progenitor cells (EPCs), the newly described bone marrow-derived stem cells for the vascular system. The second chapter introduces peripheral vascular disease (PVD), and further describes the relevance of EPCs and mononuclear cells (MNCs) in the context of patients requiring lower extremity bypass surgery for critical limb ischemia (CLI). Monocytes are then introduced as a potential clinically-relevant surrogate marker for circulating EPCs.

Chapter I: Endothelial Progenitor Cells (EPCs)

EPC Significance:

EPCs have been shown to participate in vascular healing during both acute injury and chronic disease. The quantity and quality of circulating EPCs correlate inversely with the severity of vascular disease; a reduction in number or function of EPCs are significant independent risk factors for an impaired healing capacity, a dysfunctional endothelium, and progression of atherosclerosis and vascular disease. EPC therapy has been shown to assist in healing of cardiac and limb ischemia; this therapy has great potential for improving the quality of life and longevity of patients with severe cardiovascular and peripheral vascular disease (PVD) who are not candidates for conventional revascularization procedures. In addition, EPCs can be used to promote vascular graft patency. This chapter focuses on the characterization of EPCs, positive and negative regulators of EPCs, the role of EPCs in vascular disease, and the potential for EPC therapy to ameliorate the sequelae of severe PVD.

Introduction to EPCs:

The ability of endothelium to repair itself depends on recruitment, proliferation, and migration of surrounding mature endothelial cells as well as the mobilization and incorporation of circulating EPCs to the injured region. Recent studies have outlined the importance of EPCs with respect to atherosclerotic vascular disease, acute vessel injury, and tissue ischemia. EPCs are thought to promote healing of the endothelial monolayer and vessel wall primarily through processes of reendothelialization and neovascularization; these repair mechanisms have been shown to reduce the risk of atherosclerotic disease progression and minimize the negative sequelae of vessel wall injury and tissue ischemia.

Risk factors such as elevated cholesterol, hypertension, diabetes, smoking, physical inactivity and advanced age are associated with chronic damage to the endothelial monolayer [1]. In addition, vessel trauma or ischemia can acutely compromise the integrity of the endothelium and result in endothelial cell apoptosis [2]. Vascular damage is repaired by two synergistic mechanisms, mediated by two different cell populations. Repair by local proliferation and migration of resident mature endothelial cells can repopulate the endothelial layer, but these cells have a limited capacity for regeneration; they become senescent and insufficient for proper healing as successive replications result in telomere shortening. Alternatively, circulating bone-marrow derived EPCs have been shown to migrate towards sites of injury and ischemia, proliferate, differentiate, and incorporate into injured vessel walls as well as generate new vessels. This repair mechanism provides a source of healthy endothelium with longer telomeres and a renewed ability to respond to further vascular damage.

However, EPCs are also susceptible to senescence and dysfunction; normal aging in the presence of risk factors for vascular disease can lead to the progressive depletion of marrow cells that give rise to progenitors necessary for arterial repair [1,3]. EPC obsolescence implies insufficient circulating number and/or functional capacity of EPCs, and consequently a potential inability to maintain vascular integrity. In the setting of significant risk factors and a dysfunctional endothelium, which has a limited capacity to heal acute vessel injury, compensate for chronic vascular damage, and limit abnormalities in vasoreactivity, atherosclerotic disease will progress [3].

Pathophysiology of Atherosclerosis:

Endothelial damage results in the release of chemokines and cytokines which initiate the inflammatory cascade. Some of these paracrine factors promote mobilization of EPCs from the bone marrow, migration to the peripheral circulation, and homing to sites of injury or ischemia. This activity is coordinated by complex interactions involving: VEGF (vascular endothelial growth factor), a growth factor that responds to tissue ischemia, assists in the recruitment of EPC, promotes new vessel formation, and is correlated with rapid EPC mobilization after traumatic vascular injury [4]; G-CSF (granulocyte colony stimulating factor), a growth factor for neutrophils that also mobilizes EPCs from the bone marrow; MMP-9 (matrix metalloproteinase-9), a type 4 collagenase and gelatinase that cleaves the extracellular matrix to allow EPC migration; and SDF-1 (stromal cell-derived factor-1), a chemokine that attracts EPCs to the site of injury.

Under circumstances where a competent bone marrow is able to produce a sufficient quantity of functional EPCs, these cells arrive at the site of injury and proliferate, differentiate, and incorporate into the vessel wall. Vascular healing occurs via reendothelialization and neovascularization, and with healing, the local inflammatory response resolves. A negative feedback loop results in termination of cytokine release in the absence of inflammation [1].

If the bone marrow is not competent and produces an insufficient quantity of functional EPCs in response to the local inflammation at the site of vascular damage, then healing does not occur. This may result from ineffective recruitment or impaired function of senescent EPCs. In this setting cytokines continue to be released by local inflammatory cells, causing further inflammation and tissue damage via a positive feedback loop. The failure of aging organisms to renew endothelial cells exposed to noxious stimuli leads to endothelial dysfunction, inflammation, and atherosclerosis [1,3].

EPC Number is Inversely Correlated with Vascular Disease Severity:

Atherosclerosis is the result of chronic chemical or mechanical stresses that cause vascular damage in the setting of deficient repair mechanisms. It has been shown that the number of circulating EPCs is a significant independent predictor for the severity of atherosclerosis. Hill and colleagues measured the number of circulating EPCs in 45 men with a mean age of 50 years having various degrees of cardiovascular risk but no history of cardiovascular disease. Endothelium-dependent and endothelium-independent vascular function was assessed by ultrasonography of the brachial artery. Interestingly, the researchers observed a strong inverse correlation between the number of circulating EPCs and the combined Framingham

risk score, which predicts a subject's risk for cardiovascular disease. They also observed a significant positive correlation between the number of circulating EPCs and brachial artery endothelial function [5].

Werner and colleagues determined the number of EPCs in 519 patients with angiographically-proven coronary artery disease (CAD) and evaluated the association between baseline levels of EPCs and major cardiovascular events after 12 months. After adjustment for relevant variables, increased levels of EPCs were associated with a reduced risk of death from cardiovascular causes (hazard ratio 0.31), a first major cardiovascular event (hazard ratio 0.74), revascularization (hazard ratio 0.77), and hospitalization (hazard ratio 0.76) [6]. Thus these researchers showed that reduced levels of EPCs were a significant, independent predictor of poor prognosis even after adjustment for traditional cardiovascular risk factors and disease activity [7].

Characterization of EPCs: Lineage, Markers, and Abundance:

EPCs are rare bone marrow-derived mononuclear cells (MNCs) that are enriched in the bone marrow, but may also be found in the mononuclear cell fraction of peripheral blood. These cells were first isolated and induced to differentiate into endothelial cells in vitro by Asahara and colleagues in 1997 [8,9]. Asahara and colleagues also noted the capacity for these cells to contribute to vasculogenesis as well as angiogenesis in the setting of tissue ischemia. [10,11]. However, many aspects of EPC characterization are currently debated, including their lineage, markers, and the best methods for isolation and culture.

During embryonic development the mesodermal layer develops into bone marrow and resident stem cells, including hematopoietic and mesenchymal stem cells. It is likely that EPCs are derived from hemangioblasts, the multipotent stem cells that are a common precursor to hematopoietic stem cells and EPCs; however, EPCs may also be derived directly from hematopoietic stem cells [12-14]. Alternatively, there is evidence to show that myeloid cells, which are bone marrow-derived hematopoietic committed progenitor cells, may transdifferentiate into EPCs [15]. This proposed lineage is shown in Figure 1.

In addition, several other non-hematopoietic stem cell types have been induced to differentiate into mature endothelial cells. For example, multipotent adult progenitor cells isolated with mesenchymal stem cells and lacking hematopoietic stem cell markers can be cultured with VEGF and induced to differentiate into an endothelial phenotype that expresses endothelial cell markers, functions as a mature endothelial cell in vitro, and contributes to both wound healing and tumor angiogenesis in vivo [16].

Tissue-resident stem cells may also give rise to endothelial cells under certain conditions. For example, adult cardiac stem cells have been induced to differentiate into vascular tissues including vascular smooth muscle cells and endothelial cells. In one important study, Beltrami and colleagues demonstrated that an injection of clonally expanded tissue-resident cardiac stem cells into an ischemic heart resulted in regeneration of well-differentiated myocardium including new vessels and myocytes with the characteristics of young cells [17].

Side population (SP) cells, a heterogeneous cell population identified by a capacity to efflux

Hoechst-33342 and Rhodamine-123 dyes, are composed of hematopoietic stem cells and non-hematopoietic organ-specific multipotent stem cells [18]. SP stem cells are able to acquire the endothelial cell phenotype with VEGF stimulation, or acquire the smooth muscle cell phenotype with TGF-β1/PDGF-BB stimulation; in addition, SP cells can form branching vascular-like structures with evidence of both cell types in vitro [19]. Figure 1 shows several proposed routes for the derivation of endothelial cells from EPCs and other sources.

EPCs have also been defined by their functional capacity to form endothelial cell colonies in vitro, incorporate acetylated low-density lipoprotein (LDL), and exhibit enhanced endothelial nitric oxide synthase (eNOS) expression after shear stress exposure [20,21].

EPCs can be further characterized using cell surface markers, as depicted in Figure 1. Most researchers agree that EPCs express the following markers: CD34, which is present on EPCs, certain hematopoietic cells, and endothelial cells; vascular endothelial growth factor receptor 2 (VEGF-R2), which is essential for angiogenesis and vasculogenesis in conjunction with its ligand VEGF; and CD133, which is present on a variety of stem cells. It should be noted that EPCs in the bone marrow and early circulating EPCs express CD133, but after differentiation occurs circulating EPCs lose CD133 and gain several other surface markers, including platelet endothelial cell adhesion molecule (CD31), vascular endothelial cadherin (VE cadherin) and von Willebrand factor (vWF) [2,22].

Another important cell surface marker is CXCR4, which is the receptor for stromal cell-derived factor 1 (SDF-1). In the setting of tissue hypoxia, SDF-1 is upregulated by hypoxia

inducible factor-1 (HIF-1). The CXCR4/SDF-1 pair is known to be important in hematopoietic stem cell trafficking, including quiescence, homing to the bone marrow, and mobilization to sites of vascular injury and ischemia [23]. Of note, interfering with this receptor-ligand interaction can mobilize hematopoietic stem cells into the circulation by disrupting the CXCR4/SDF-1 bond that tethers these cells to the bone marrow.

CD133 and CD34 are often used as markers for selection and purification of EPCs since CD133 is not found on mature endothelial cells, and CD34 is not found on the undifferentiated stem cells from which EPCs derive [24]. However, there is significant overlap in terms of cell surface markers. For example, mature endothelial cells which are shed from the vessel wall may express VEGF-R2, VE cadherin, and CD34. In addition, certain hematopoietic stem cells may express CD34, CD31, VEGF-R2, and vWF [13]. CD133 is found on bone marrow EPCs and early circulating EPCs, but once in the circulation, EPCs lose expression of CD133 and gain expression of other less specific cell surface markers as described above; the specificity of this marker is further compromised because hematopoietic progenitor cells may also express CD133 [2,25].

The lack of a specific marker that is present on all EPCs implies a technical challenge with respect to isolating these cells from peripheral blood, and has hampered the development of an established protocol for isolating these cells. Lack of a specific marker has been cited as a potential source for conflicting evidence in studies evaluating EPC number [1]. In addition, the possibility that there may be subpopulations of EPCs having different functional characteristics and surface markers adds to the complexity of the issue [26]. One

consequence of this problem is that many of the clinical studies evaluating the therapeutic effects of EPCs have been performed using bone marrow samples that have not been purified to exclude other cell types, leading to the possibility that the functional effects ascribed to EPCs may not be due to these cells alone [13].

Another confounding factor is that other cell types bearing different cell surface markers and derived from bone marrow, peripheral blood, or other tissues may be precursors to endothelial cells, making the "true" EPC more elusive. As described above, this is further complicated by the fact that other stem cell types may be induced to transdifferentiate into EPCs, including mesenchymal stem cells as well as the SP phenotype of human stem cells (Figure 1) [9,13].

During steady-state conditions in healthy patients, circulating EPCs comprise approximately 0.01% of peripheral blood cells [13]. It has been repeatedly demonstrated that patients with chronic vascular disease have even fewer circulating EPCs. The rarity of this cell population is another factor that makes identification and isolation of these cells for therapy or for research very challenging.

Regulators of EPC Number and Function:

There are many positive and negative regulators of EPCs. These factors have a quantifiable effect on EPC quantity and quality, as described in the following studies.

Negative Regulators:

Aging

Heiss and colleagues evaluated circulating EPC number and function, as well as endothelial function in 20 aged and young subjects with no major cardiovascular risk factors. They reported that while older people had impaired endothelial function, there was no significant difference in the number of circulating EPCs between the two age groups. However, EPCs from older people had significantly decreased survival, migration, and proliferation rates, suggesting that the impaired maintenance of the endothelium in older subjects may correlate with functional EPC deficits rather than total EPC number [27,28].

Normal aging diminishes the potential for mature endothelial cells to participate in effective vascular repair, resulting in progression of atherosclerosis. Impaired endothelial cell function, bone marrow senescence, and limited availability or compromised function of circulating EPCs are all factors that may contribute to decreased reserve for repair of vascular injury with aging.

Hypercholesterolemia

Hypercholesterolemia is a significant risk factor for atherosclerosis. Rauscher and colleagues performed an elegant study demonstrating that long-term treatment with bone marrow-derived progenitor cells from young non-atherosclerotic ApoE-deficient mice was successful in preventing atherosclerosis progression in ApoE-deficient recipients despite persistent hypercholesterolemia. In contrast, treatment with bone marrow cells from older ApoE-deficient mice with atherosclerosis was much less effective. This study demonstrates the role

of competent EPCs in preventing atherosclerosis, as well as the decreased effectiveness of senescent EPCs to engraft on recipient arteries in areas at risk for atherosclerotic injury [3].

Diabetes

Fadini and colleagues analyzed the association between type 2 diabetes, peripheral vascular disease (PVD), and levels of circulating EPCs. These researchers used flow cytometry to quantify circulating progenitor cells (CD34-positive) and EPCs (CD34-positive and VEGF-R2-positive) in 51 diabetic patients and 17 control subjects. They reported that the number of circulating progenitor cells and EPCs from diabetic patients were reduced by 33% and 40%, respectively, compared with healthy subjects. An inverse correlation was found between the number of EPCs and the values of fasting glucose. In addition, they determined that PVD was associated with a 47% reduction in circulating EPCs, and that EPC number directly correlated with the ankle-brachial index [29].

In a second study, Fadini and colleagues quantified EPCs by flow cytometry in 127 diabetic patients with and without peripheral arterial disease (PAD). Diabetic patients with PAD displayed a 53% reduction in circulating EPCs compared to non-PAD patients, and EPC levels were negatively correlated with the degree of carotid stenosis and claudication symptoms. In addition, the proliferative and adhesion capacity of cultured EPCs were significantly lower in diabetic patients with PAD versus patients without PAD [30].

Tepper and colleagues expanded on these findings and noted that proliferation of diabetic EPCs relative to control subjects was decreased by 48% ($p<0.01$) and inversely correlated

with patient levels of hemoglobin A1c ($p<0.05$). In addition, diabetic EPCs demonstrated decreased adherence and were 2.5 times less likely to participate in tubule formation compared with controls ($p<0.05$) [31].

Smoking

Kondo and colleagues examined the effects of chronic smoking and smoking cessation on EPC levels. Circulating EPCs were quantified by flow cytometry in 14 nonsmokers and 15 smokers. The number of circulating EPCs was reduced in chronic smokers and inversely correlated with the number of cigarettes smoked. Circulating EPCs increased rapidly after smoking cessation ($p<0.0001$) and decreased again after resumption of smoking to a level similar to that before cessation ($p=0.0031$), suggesting that EPC levels directly respond to the effects of smoking [32].

Michaud and colleagues studied peripheral blood EPCs in 15 healthy smokers and 11 age-matched nonsmokers. The number of EPCs was significantly reduced in smokers versus control subjects, and the functional activities of EPCs isolated from smokers were severely compromised. The proliferative response was reduced by 75% and the migratory response was reduced by 19% ($p<0.05$). EPCs from smokers also showed decreased adherence and diminished capacity to form tubes in a matrigel assay. These researchers also found that EPCs from smokers had a significant reduction in the expression of the endothelial cell-specific markers (VE-cadherin, VEGF-R2, and vWF) [33].

Positive Regulators:

Exercise

It is generally accepted that exercise and physical training decrease the risk and severity of cardiovascular and peripheral vascular disease, but the molecular mechanisms for this protective effect have been elusive. Laufs and colleagues studied EPCs in mice randomized to either running wheels or no exercise. Numbers of EPCs circulating in the peripheral blood of trained mice were enhanced to 267%, 289%, and 280% of control levels after 7, 14, and 28 days, respectively. The researchers identified an exercise-induced nitric oxide-dependent mechanism which elevated serum levels of VEGF and reduced the rate of apoptosis in spleen-derived EPCs. In addition, running inhibited neointima formation after carotid artery injury by 22% and increased neoangiogenesis by 41% compared with control animals [34].

Steiner and colleagues examined the effect of exercise on circulating EPCs in patients with cardiovascular risk factors and/or coronary artery disease (CAD). Twenty patients with documented CAD and/or cardiovascular risk factors joined a 12-week supervised running program. After 12 weeks of exercise there was a significant 2.9-fold increase in circulating EPCs, which was positively correlated with both the change in flow-mediated vessel dilation and the increase in nitric oxide synthesis. Interestingly, plasma VEGF levels did not change in response to exercise [35]. Thus regular exercise training appears to augment the number of circulating EPCs in patients with cardiovascular risk factors.

Another study by Sandri and colleagues showed that ischemic exercise training in patients with PAD increased VEGF levels by 310% and the number of EPCs by 440% compared to

controls. However, subischemic exercise training in patients with PAD or CAD did not increase VEGF levels or EPC number, but was associated with improved integrative capacity of progenitor cells to organize into endothelial networks, as well as increased CXCR4 expression [36]. In this study ischemic exercise training increased EPC number and function, while non-ischemic exercise appeared to improve only EPC function.

Hyperbaric Treatment

EPC trafficking is generally thought to be regulated by hypoxic gradients and induced by VEGF-mediated increases in bone marrow nitric oxide. Interestingly, Goldstein and colleagues found that hyperoxia induced by hyperbaric oxygen (HBO_2) exposure paradoxically results in a signaling cascade similar to that induced by tissue hypoxia. In a hindlimb ischemia animal model, the researchers showed that the wound closure rate for a wound in the non-ischemic limb did not significantly improve in response to HBO_2 treatment, but a wound in the ischemic limb showed a significantly improved healing rate in the presence of HBO_2. The researchers proposed that HBO_2 treatment increases EPC mobilization from the bone marrow, but does not increase homing of EPCs into injured non-ischemic tissues [37]. Gallagher and colleagues proposed a mechanism of action; they showed that HBO_2 treatments increase nitric oxide synthase (NOS), which elevates NO in the blood. NO nitrosylates MMP-9 which cleaves membrane-bound stem cell factor (SCF), prompting EPC proliferation and migration [38]. Several other studies have also demonstrated that HBO_2 treatment mobilizes EPCs from the bone marrow and stimulates wound healing coincidently with the accumulation of EPCs in ischemic tissues [37,39].

Exogenous Drug Therapy

Many studies have investigated the effects of certain drug therapies for inducing recruitment, migration, proliferation, and incorporation of EPCs into target vascular epithelium. Many drugs have proven useful in this respect, both in vitro, in animal models, and in human clinical trials.

Vascular Endothelial Growth Factor (VEGF)

VEGF is a growth factor that is upregulated in hypoxic cells via hypoxia inducible factor-1 (HIF-1), and stimulates release of nitric oxide from endothelial cells. VEGF increases vascular permeability and exhibits a dose-dependent mitogenic and chemotactic effect on endothelial cells to promote new vessel formation [40]. VEGF is correlated with rapid but transient EPC recruitment after traumatic vascular injury [4], but has been tested more extensively in the context of therapeutic angiogenesis. Initial hindlimb ischemia studies evaluating VEGF therapy showed an increase in neovascularization and improvement in the hemodynamic deficit of the animal model [40]. Takeshita and colleagues demonstrated a significant dose-dependent augmentation in ischemic limb perfusion accompanied by increased collateral formation after intramuscular administration of VEGF [41].

However, VEGF does not seem to be a magic bullet. The Regional Angiogenesis with Vascular Endothelial Growth Factor (RAVE) trial was a randomized study of adenoviral VEGF (AdVEGF) gene transfer for the treatment of PAD. A total of 105 patients with unilateral exercise-limiting intermittent claudication were randomized to receive low-dose AdVEGF, high-dose AdVEGF, or placebo, administered as 20 intramuscular injections to the

index leg in a single session. The change in peak walking time, ankle-brachial index, claudication onset time, and quality-of-life measures were similar among all three groups at 12 and 26 weeks. In addition, AdVEGF administration was associated with increased peripheral edema, consistent with its known effect of increasing vascular permeability. The researchers were forced to conclude that VEGF-based treatment had limited utility since it was not associated with improved exercise performance or quality of life [42].

Granulocyte Colony Stimulating Factor (G-CSF)

G-CSF, a growth factor for neutrophils, interferes with the CXCR4/SDF-1 receptor-ligand interaction, mobilizing hematopoietic stem cells into the bloodstream. Degradation of SDF-1 in the bone marrow releases EPCs by disrupting the CXCR4/SDF-1 receptor-ligand interaction which sequesters these cells in the bone marrow. Upregulation of SDF-1 in peripheral tissues attracts EPCs from the bone marrow to the periphery, particularly to sites of tissue hypoxia and HIF-1 expression. In this way the CXCR4/SDF-1 receptor-chemokine interaction is instrumental for mobilizing and incorporating EPCs to sites of vessel injury and ischemia.

Takahashi and colleagues found that in rabbits with hindlimb ischemia, circulating EPCs were augmented after pretreatment with GM-CSF, with a corresponding improvement in hindlimb neovascularization [43]. However, in a pilot study on STimulation of ARTeriogenesis (the START Trial), GM-CSF or placebo was delivered subcutaneously to patients with intermediate to severe intermittent claudication as treatment for PAD. This randomized controlled trial found no difference in the treatment and placebo groups in terms

of the primary outcome, walking time, or the secondary outcome, ankle-brachial index [44].

Notably, the CXCR4/SDF-1 interaction has been shown to simultaneously recruit bone marrow-derived smooth muscle cell progenitors to regions of vessel injury where medial smooth muscle cells undergoing apoptosis express SDF-1. Smooth muscle cell precursors may assist in vascular repair; however, this response has also been shown to result in pathological healing and neointimal hyperplasia [45].

HMG CoA Reductase Inhibitors

Known primarily for their lipid-lowering activity, HMG-CoA reductase inhibitors (statins) increase neovascularization by increasing circulating EPC number and/or augmenting EPC function. Vasa and colleagues studied 15 patients with angiographically documented stable CAD to determine the effect of statin therapy on circulating EPC number. These patients were prospectively treated with 40 mg of atorvastatin per day for 4 weeks. Statin treatment was associated with an approximately 1.5-fold increase in the number of circulating EPCs after one week, followed by sustained increased levels to approximately 3-fold throughout the 4-week study period [46]. Walter and colleagues demonstrated that statins increased circulating rat EPCs by 2.5-fold at 4 weeks [47]. Dimmeler and colleagues used a mouse model to show that statins potently augment EPC differentiation from mononuclear cells and CD34-positive hematopoietic stem cells isolated from peripheral blood [48].

Interestingly, statins, VEGF, erythropoietin, estrogen, and exercise all exert effects on EPCs via the PI3K/Akt signal transduction pathway. This observation suggests an essential role for

Akt in regulating hematopoietic progenitor cell mobilization, which is likely mediated through the eNOS pathway [49].

EPCs and Peripheral Vascular Disease (PVD):

Most studies evaluating the effectiveness of stem cell therapy have used unpurified bone marrow containing a combination of EPCs, hematopoietic stem cells, and other cells. Asahara and colleagues originally described the incorporation of autologous CD34-positive mononuclear cells into foci of neovascularization in the rabbit hindlimb ischemia model [8]. Kalka and colleagues confirmed these studies using human EPCs transplanted into a nude mouse model, and found that blood flow recovery and capillary density in the ischemic hindlimb were markedly improved, and the rate of limb loss was significantly reduced. The rate of limb necrosis and auto-amputation was reduced by 50% compared to controls [15,50].

Tateishi-Yuyama and colleagues investigated the efficacy of autologous implantation of bone marrow-derived mononuclear cells (BMCs), including EPCs, into the ischemic limbs of patients with PAD. Twenty-two patients with bilateral leg ischemia were injected with BMCs in one leg and peripheral blood-derived mononuclear cells in the other. At four weeks, the ankle-brachial index was significantly improved in legs injected with BMCs compared with those injected with peripheral blood-derived mononuclear cells. Similar improvements were seen for transcutaneous oxygen pressure, rest pain, and pain-free walking time. These improvements were sustained at 24 weeks. The authors concluded that autologous implantation of BMCs could be safe and effective for achievement of therapeutic angiogenesis because of the natural ability of marrow cells to supply EPCs and to secrete

various angiogenic factors or cytokines [51]. Esato and colleagues demonstrated similar results in patients with PAD who had failed traditional medical treatment and/or surgical bypass procedures. After BMC transplantation, patients reported improved subjective symptoms [52].

Bartsch and colleagues reported early results of the TAM-PAD study (Transplantation of Autologous Mononuclear bone marrow stem cells in patients with Peripheral Arterial Disease). This study was designed to evaluate the use of combined intraarterial and intramuscular bone marrow-derived mononuclear cell (BMC) therapy for patients with moderate to severe intermittent claudication. They reported that two months after harvesting and delivering BMCs to the ischemic limb, pain-free walking distance increased 3.7-fold and the ABI was significantly improved after exercise and at rest [53]. Notably, after 13 months these positive effects persisted at their improved level [54].

Two additional studies evaluating BMCs in patients with PVD are worth mentioning. Higashi and colleagues evaluated BMC transplantation and its effect on endothelial dysfunction. They found that BMC implantation significantly improved the transcutaneous oxygen pressure, pain-free walking time, and endothelium-dependent vasodilation in patients with limb ischemia [55]. Lastly, Saigawa and colleagues noted that the clinical effectiveness of autologous bone marrow implantation, as measured by an improvement in ankle-brachial index, was strongly correlated with the number of CD34-positive cells delivered to the ischemic limb [56].

In contrast to the studies evaluating BMC therapy, Ishida and colleagues used only peripheral blood-derived mononuclear cells in treating patients with PAD. These cells were mobilized with G-CSF and then harvested and injected intramuscularly. After four weeks the researchers observed a significant improvement in ankle-brachial index, healing of ischemic ulcers, and increased mean maximum walking distance [57].

As a group, these studies suggest that the specific cell type, or the combination of cells and cytokines, required to generate beneficial healing effects in patients with PAD are still incompletely understood.

Mononuclear Cells and Graft Patency:

Although studies have not been performed with isolated EPCs, several studies have shown that some bone marrow-derived stem cells can colonize and epithelialize synthetic and biological vascular grafts and devices to render the foreign surface less thrombogenic. Shi and colleagues used a canine bone marrow transplantation model in which the marrow cells from the donor and recipient are genetically distinct. Between 6 and 8 months after transplantation, a Dacron graft, made impervious to prevent capillary ingrowth from the surrounding perigraft tissue, was implanted in the descending thoracic aorta. After 12 weeks the graft was retrieved, and cells with endothelial morphology were identified by silver nitrate staining. Using repeat polymorphisms to distinguish between the donor and recipient DNA, Shi and colleagues observed that only donor alleles were detected in DNA from positively stained cells on the impervious Dacron graft. These results strongly suggest that a subset of CD34-positive cells localized in the bone marrow can be mobilized to the

peripheral circulation and can colonize endothelial flow surfaces of vascular prostheses [20].

In a similar study, Bhattacharya and colleagues demonstrated accelerated endothelialization on polyethylene terephthalate (PET) grafts treated with enriched CD34-positive autologous bone marrow cells in a canine model. The authors used composite grafts implanted in the descending thoracic aorta for 4 weeks. The composite grafts had a 4-cm PET graft in the center flanked by two 4-cm standard PTFE grafts; the entire composite was coated with silicone rubber to make it impervious, and the PET segment was shielded from perigraft and pannus ingrowth. On the day before surgery, 120 mL of bone marrow was aspirated, and the CD34-positive cells were enriched. During surgery, these cells were mixed with venous blood and seeded onto the PET segment of composite study grafts; the control grafts were treated with venous blood only. After harvesting, there was a significant increase in surface endothelialization on the seeded grafts (approximately 92% vs. 27%) with markedly increased microvessels in the neointima, graft wall, and external area compared with control grafts [58].

In humans, Peichev and colleagues found that the neointima formed on the surface of left ventricular assist devices was colonized with cells expressing the markers CD34, CD133, and VEGF-R2, consistent with the EPC phenotype [22]. Furthermore, Matsuo and colleagues showed that senescent EPCs may be a risk factor for in-stent restenosis in a study of 46 patients who underwent coronary stenting. Blood samples were collected at the time of follow-up coronary angiography after stent placement. Patients (n=16) with in-stent restenosis, defined as greater than 40% stenosis, had decreased EPC numbers and increased

senescent cells compared to patients without restenosis (n=30). There was no significant difference in angiogenic growth factors secreted by EPCs between the two groups. On multivariate analysis, an increased number of senescent EPCs was the independent factor associated with in-stent restenosis (odds ratio 1.10) [59].

Shin'oka and colleagues have pioneered the exciting field of tissue-engineered biodegradable grafts seeded with autologous bone marrow cells for the repair of cardiovascular defects in children. Previous studies showed that bone marrow cells contribute to the construction of tissue-engineered vascular autografts (TEVA) in vivo, and since these constructs contain living cells, they may have the ability to grow, self-repair, and self-remodel. These characteristics are especially desirable in growing children to decrease the number of necessary revision surgeries. Biodegradable conduits (n=23) and patches (n=19) were implanted in children for extracardiac total cavopulmonary connection and congenital heart defects, respectively. TEVA were only implanted in low pressure (venous or pulmonary artery) systems. Patients received anticoagulation therapy for 3 to 6 months post-operatively. Intermediate-term follow-up after a median of 16.7 months showed no complications such as thrombosis, stenosis, or obstruction of TEVA. There was no evidence of aneurysm formation or calcification. All tube grafts were patent, and the diameter of the tube grafts increased to approximately 110% of the implanted size over time. These results show that after intermediate-term follow-up, TEVA are safe and feasible in pediatric cardiovascular surgery and have excellent hemodynamic performance, even after cessation of anticoagulation therapy [60-62]. However, the implications for adult patients with PAD are not yet known.

EPC Therapy:

EPC therapy refers to an intervention that targets the recruitment, mobilization, proliferation, and incorporation of EPCs into injured vessels and ischemic tissues with the purpose of augmenting healing and tissue regeneration in animal models and in patients with severe vascular disease.

In addition to autologous transplantation of EPCs as described previously, certain factors may be important in augmenting EPC function and increasing ischemic limb perfusion. Murohara and colleagues showed a significant role for nitric oxide and endothelial nitric oxide synthase (eNOS) in revascularization of ischemic limbs using L-arginine dietary supplementation [49]. Notably Takahashi and colleagues found that the development of regional ischemia in animal models increased the frequency of circulating EPCs, and that pretreatment with GM-CSF further increased EPC numbers with a corresponding improvement in hindlimb neovascularization. These findings indicate that circulating EPCs may be mobilized endogenously in response to tissue ischemia or exogenously by cytokine therapy to assist in neovascularization of ischemic tissues [43].

As these therapies become established, three categories of patients may be considered for therapy with EPCs. The first category includes patients with severe ischemic peripheral vascular disease, a threatened limb, and no surgical or endovascular options for reconstruction. The second category includes patients with severe comorbid disease who are not operative candidates for potential procedures. The third category includes patients with bypass grafts or stents in place; under these circumstances EPC therapy may improve conduit

survival by preventing restenosis [15]. However, EPC therapy has the potential to stimulate neovascularization in tumors, and therefore is not indicated for patients with cancer or at high risk for acquiring cancer [63,64].

Another important consideration in EPC therapy is the possibility of resultant neointimal hyperplasia after arterial injury due to the contribution of smooth muscle cells stimulated by SDF-1. Zernecke and colleagues describe the involvement of local SDF-1alpha and its receptor CXCR4 in neointimal hyperplasia via recruitment of bone marrow-derived smooth muscle cell progenitors. After arterial injury, SDF-1alpha is expressed in medial smooth muscle cells. SDF-1alpha binds to platelets at the site of injury, triggers CXCR4- and P-selectin-dependent arrest of progenitor cells on injured arteries or matrix-adherent platelets, and preferentially mobilizes and recruits progenitors for neointimal smooth muscle cells [45].

In patients that are selected as candidates for EPC therapy, several methods for augmentation of EPC number and function exist. Strategies include improving the intrinsic function of a patient's native cells, processing of precursor cells, providing allogeneic progenitors, or using a combination of these approaches.

Most studies evaluating EPC therapy for patients with vascular disease used either unpurified bone marrow samples or a combination of EPCs, hematopoietic stem cells, and other cells derived from bone marrow. Thus it is unclear whether EPCs alone are sufficient to repair vascular injury, or whether other supporting cells or components of the bone marrow are also necessary for a therapeutic effect. This is a critical point, as introduction of nonessential cells

may be associated with aggravation of inflammation and vascular injury, thereby exacerbating the problem for which the patient seeks treatment [13].

Chapter II: Monocytes and Peripheral Vascular Disease (PVD)

Overview of PVD:

Vascular disease has a very high prevalence and mortality rate in the United States. According to the Centers for Disease Control and Prevention (CDC), cardiovascular and cerebrovascular disease are the first and third leading causes of death in the United States, accounting for approximately 40% of all deaths in 2004. The American Heart Association analysis of 2004 Heart Disease and Stroke Statistics categorizes coronary artery disease (CAD), stroke, hypertension, heart failure, and peripheral vascular disease (PVD) as subsets of Cardiovascular Disease. They estimate that in the United States the total cost of these diseases, which includes funds spent on healthcare as well as indirect costs associated with loss of work, will be $432 billion in 2007 [65,66].

These vascular conditions are interrelated due to the fact that vascular disease is often systemic. Events such as myocardial infarction, stroke, and acute ischemia of the lower extremities may be a manifestation of the same disease process in different organs or vascular beds. Since atherosclerosis and other risk factors for PVD also predispose to CAD and stroke, these patients often have coexistent cardiovascular and cerebrovascular disease. The Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) trial showed that 41.1% of patients with PVD had concurrent CAD or cerebrovascular disease,

and 8.6% had disease in all three vascular beds [67]. Patients with symptomatic PVD have a 5-year mortality of almost 30% due to cardiovascular and cerebrovascular events, and those with large-vessel PVD have a relative risk of 5.9 (95% CI) for death from cardiovascular disease within ten years [68-71].

Peripheral arterial disease (PAD) is a subset of PVD. In the lower extremities, PAD involves obstruction of blood flow in the major arteries that supply the lower limbs. Atherosclerotic disease is the most common culprit, leading to stenosis and partial or total occlusion of these large arteries, resulting in various degrees of ischemia. PAD causes significant morbidity by adversely affecting mobility, function, and the ability to perform activities of daily living.

The clinical severity of PAD is commonly classified using two different modalities, namely the ankle-brachial index (ABI) and the Fontaine Stages. The ABI is calculated by dividing the systolic blood pressure at the ankle by the higher of the two systolic brachial blood pressures. A normal ABI is approximately 1.0-1.1, and increasing severity of PAD is associated with decreasing ABI. Fontaine Stages are defined as follows: Stage I is asymptomatic (ABI approximately 0.8-0.9), Stage IIa corresponds to the presence of mild claudication and Stage IIb represents moderate to severe claudication (ABI approximately 0.5-0.8, depending on severity of claudication), Stage III corresponds to the presence of ischemic rest pain (ABI approximately 0.3-0.5), and Stage IV to the presence of tissue loss (ulceration or gangrene, ABI approximately 0.3-0.5) [72,73]. Notably, patients may have absent palpable pedal pulses irrespective of the severity of PAD.

The initial signs of lower extremity PAD (Fontaine Stage I) include trophic changes, where a chronically diminished blood supply results in thickened skin and toenails, as well as loss of hair growth on the toes and feet. Patients are usually asymptomatic at this point. Intermittent claudication is the next stage in severity (Fontaine Stage II), and is the earliest and most frequent presenting symptom [73]. Patients typically complain of pain in the calf or thigh muscles upon ambulation, due to the increased demand for oxygen by the working muscles and the inability of the vessels to increase the supply of blood to the ischemic tissue. Importantly, intermittent claudication is reproducibly produced by exercise and relieved within 10 minutes after exercise is discontinued. As the disease progresses in severity patients may develop rest pain (Fontaine Stage III), which is typically described as a sharp pain in the foot at rest, most commonly when lying flat in bed at night. This pain is commonly relieved by dependency, and patients may dangle their feet off the bed for relief. In the late stages of PAD, tissue hypoperfusion may lead to tissue loss (Fontaine Stage IV), where the blood supply is insufficient to sustain the basic metabolic requirements of the distal tissues such that ulceration and gangrene result. Major amputation is eventually required in more than a third of patients with tissue loss [73]. Patients with rest pain and/or tissue loss are identified as having critical limb ischemia (CLI) which is an important predictor of poor prognosis, with a 2-year mortality rate of 31.6% according to a study by the I.C.A.I. Group (Gruppo di Studio dell'Ischemia Cronica Critica degli Arti Inferiori) [74].

Screening for PAD in the absence of symptoms utilizes the ABI measurement. A resting ABI of ≤ 0.90 is caused by hemodynamically significant lower extremity arterial stenosis and is commonly used as a threshold below which a patient is given the diagnosis of PAD. In

symptomatic individuals, a resting ABI of ≤ 0.90 is approximately 95% sensitive for detecting angiographically significant PAD and almost 100% specific in identifying healthy individuals [72]. Interestingly the Edinburgh Artery Study, using duplex scanning, found that one-third of patients with asymptomatic PAD had complete occlusion of a major artery to the leg [75]. The Rotterdam Study documented the frequency of intermittent claudication and PAD in a large study group of 7715 patients. They found that although the frequency of intermittent claudication was between 1% and 4.6% depending on the age group, the actual prevalence of PAD (as defined by an ABI of ≤ 0.9) was 16.9% in men and 20.5% in women over age 55 [76]. The Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II) agreed with this assessment, stating that "For every patient with symptomatic PAD there are another three to four subjects with PAD who do not meet the clinical criteria for intermittent claudication" [72].

In addition, the TASC II Consensus notes that although intermittent claudication is an important finding in many patients with PAD, the presence or absence of this symptom does not always predict the presence or absence of PAD. This is due to the fact that other disease processes, such as spinal stenosis, can produce symptoms that mimic intermittent claudication in a patient without PAD. Additionally, patients with sedentary lifestyles, severe deconditioning, heart disease or other comorbidities which limit exercise may not report symptoms of intermittent claudication because they do not ambulate far enough to experience them [72].

Since the majority of patients with significant PAD are asymptomatic, and since it is uncommon to screen an asymptomatic patient, the exact prevalence of PAD is difficult to estimate [73]. However, studies have suggested that, when defined as an ABI of ≤ 0.9, PAD has a prevalence of approximately 3% in patients under 60 yrs old to approximately 20% in patients over 75 yrs old [69,75,77].

PAD is usually diagnosed by history and physical exam alone. A high index of suspicion in patients with multiple risk factors is recommended, even if the patient does not complain of lower extremity symptoms. The diagnosis of asymptomatic PAD has little clinical relevance with respect to the lower extremities, but as a strong marker for future cardiovascular events, early diagnosis and intervention are an important aspect of preventative treatment [71]. As alluded to previously, CAD is the most common cause of death in PAD patients, accounting for 40% to 60% of deaths, with cerebral artery disease accounting for 10% to 20% of deaths, and only 20% to 30% of patients dying of non-cardiovascular causes [72].

There are many risk factors for PAD, only the most significant of which are presented here. All of the following data and statistics are derived from the TASC II Consensus. As suggested previously, the prevalence of symptomatic PAD increases with age, following a smooth curve from approximately 0.5% in patients 30-34 years old to approximately 6.5% in patients 70-74 years old. There also seems to be a correlation between PAD and race: an ABI ≤ 0.9 was more common in non-Hispanic blacks (7.8%) than whites (4.4%). The prevalence is also slightly greater in men than women, and this difference increases with the severity of

disease: in patients with intermittent claudication the ratio of men to women is between 1:1 and 2:1, but in patients with CLI the ratio increases to 3:1 [72].

Smoking is one of the most significant and longest-known risk factors for PAD, recognized since 1911 when it was documented that symptoms of intermittent claudication were three times more common in smokers compared to nonsmokers. Furthermore, smoking has a dose-dependent effect on PAD; there is a direct correlation between the number of cigarettes smoked and the severity of disease. Heavy smokers have a four-fold risk of developing intermittent claudication compared to nonsmokers, and smoking cessation is associated with a decline in the incidence of PAD symptoms [72]. The Edinburgh Artery Study found that the relative risk of intermittent claudication was 3.7 in smokers compared to 3.0 in ex-smokers who had abstained for five years [75].

Overall, PAD is twice as common in patients with diabetes that in patients without. The correlation is impressive: in patients with diabetes, for every 1% increase in hemoglobin A1c, there is a 26% increase in risk of PAD. The presence of peripheral neuropathy and decreased resistance to infection in diabetic patients complicates the picture, and the subsequent need for a major amputation is five to ten times more common in patients with diabetes than in patients without. Notably, insulin resistance is a risk factor even in patients without diabetes [72].

Hypertension is associated with all types of vascular disease but does not predict the risk of developing PAD as highly as smoking or diabetes. Hyperlipidemia, on the other hand, has a

stronger correlation with PAD. The Framingham Study showed that a fasting cholesterol level of greater than 270 mg/dL was associated with a doubling of the incidence of intermittent claudication, but it was the ratio of total to high density lipoprotein (HDL) cholesterol that was the best predictor of occurrence of PAD. Other factors such as markers of inflammation, hyperviscosity, hypercoagulable states, and hyperhomocysteinemia may also be risk factors for a poor prognosis [72].

Treatment options for PAD are aimed at two targets: decreasing the risk factors for atherosclerosis and improving the lower extremity symptoms of PAD. In all cases except for acute ischemic events, it is advisable to begin treatment with efforts to decrease the risk factors associated with atherosclerotic disease, with the intent of reducing the risk of life-threatening thrombotic events such as myocardial infarction and stroke, as well as improving the symptoms of limb ischemia. Decreasing risk factors involves lifestyle modification and pharmacotherapy. A cornerstone of the management for PAD involves smoking cessation. Other important lifestyle modifications include increased exercise and improved diet. In addition, a combination of lifestyle modification and pharmacotherapy to control diabetes, hyperlipidemia, and hypertension is recommended. Administration of an antiplatelet agent such as aspirin or clopidogrel is now a common practice [72].

For patients with intermittent claudication, exercise rehabilitation has been shown to provide significant clinical benefit, improving exercise performance and community-based walking ability. It is interesting to note that the benefits correlate more strongly with supervised vs. unsupervised programs, with treadmill exercise vs. weight training, with experience of severe

claudication pain during exercise, and with 6 months or more of training [72]. In addition, agents such as cilostazol (a phosphodiesterase III inhibitor with vasodilator, metabolic, and antiplatelet activity) as well as naftidrofuryl (a 5-hydroxytryptamine antagonist which may improve muscle metabolism) have been shown to improve peak treadmill performance and quality of life in some studies [72].

As mentioned previously, the trials evaluating the use of VEGF and GM-CSF in patients with intermittent claudication showed no beneficial effects, so these agents are not recommended for treatment. To summarize, the RAVE trial randomized patients with intermittent claudication to receive adenoviral VEGF (AdVEGF) gene transfer or placebo for the treatment of PAD. The researchers concluded that VEGF-based treatment had limited utility since it was not associated with improved exercise performance or quality of life [42]. In the START Trial, GM-CSF or placebo was delivered subcutaneously to patients with intermediate to severe intermittent claudication as treatment for PAD. This randomized controlled trial found no difference in the treatment and placebo groups in terms of the primary outcome, walking time, or the secondary outcome, ABI [44].

In severe cases refractory to conservative treatment, where intermittent claudication interferes significantly with a patient's lifestyle or work and adversely impacts quality of life, revascularization surgery may be considered for symptoms of claudication alone.

For patients with critical limb ischemia (CLI), the primary goals of treatment include the following: relief of ischemic rest pain, healing of ulceration, prevention of limb loss, and

improvement of patient quality of life, function, and survival. Most patients with CLI will ultimately require a revascularization procedure. However, some patients with severe comorbidities or poor predicted post-revascularization outcome in terms of quality of life or function may be treated with a primary amputation. For patients with CLI who are awaiting a revascularization procedure, who decline amputation, or who have no surgical options for reconstruction, pain control with regularly-dosed narcotics is often required. Pressure relief and antibiotics may assist in ulcer healing. In addition, intermittent IV infusion of prostanoids (prostaglandin-E1 or iloprost) may help reduce rest pain and heal ischemic ulcers, though these agents are more commonly used in Japan than in the United States. Notably, recent trials do not support the benefit of prostanoids in promoting amputation-free survival [72]. Hyperbaric oxygen therapy may be beneficial in reducing the risk of major amputation in patients with diabetic foot ulceration according to a Cochrane review, but methodological issues and the studied populations limit its generalizability to all patients with CLI [72].

For patients with CLI who are surgical candidates, several options exist. For localized disease, techniques include percutaneous angioplasty and stenting or open surgical endarterectomy. The exact distribution and severity of the lesions, as well as individual patient factors, dictate the recommended intervention. For more extensive disease, open peripheral bypass procedures are recommended, provided there is an appropriate target vessel for reconstruction [72]. It is important to note that for patients undergoing angioplasty or surgery it is necessary to obtain an angiogram to localize and characterize the lesions, and to identify inflow and outflow vessels as necessary. Angiography requires that a patient have

acceptable renal function or the option for hemodialysis.

Percutaneous angioplasty and stenting has the distinct advantage that it is minimally invasive and thus less stressful than an open surgical procedure, making it attractive for patients with comorbidities and high risk for complications. However, the long-term patency rates are inferior to that of open surgical procedures. For infrainguinal stenting, the 3-year patency rates are below 60%; depending on the study quoted, they range from 42% to 72% [73]. As expected, however, the TASC II Consensus reports that perioperative complication and mortality rate are superior to those of open surgical procedures [72]. Importantly, percutaneous angioplasty and stenting interventions have dismal results when used distal to the knee, so for patients with infrapopliteal disease and CLI, autogenous vein bypass is the first line of reconstructive therapy [73].

In general, endarterectomy is performed for local disease in patients who are appropriate candidates, and infrainguinal bypass procedures are performed for more extensive disease. Notably, patients with CLI tend to have more extensive disease and often require open bypass reconstruction, provided there is an adequate target vessel. Typically autogenous vein grafts are used for bypass procedures if they are available and of sufficient caliber. When using a non-diseased saphenous vein graft of adequate caliber, the patency rate is approx 70-80% at 5 years irrespective of whether the vessel is reversed or incorporated in situ. In patients without an available autologous vein for use as a graft, a prosthetic graft may also be used. These grafts have better results when the outflow is to the above-knee (AK) popliteal artery, as patency rates drop sharply when the outflow is infrapopliteal. For this reason the

use of a native vessel is recommended if the distal anastomosis is below the knee. According to the TASC Consensus, autologous vein grafts spanning from the femoral to the below-knee (BK) popliteal artery have a patency rate of approximately 70% at 5 years. In contrast, prosthetic grafts spanning from the femoral to the BK popliteal artery have a patency rate of approximately 30% at 5 years [72].

As mentioned previously, the goals of peripheral bypass surgery include resolution of ischemia, healing of ulcers and wounds, improvement in patient symptoms, function, and quality of life, and preservation of the extremity. However, bypass grafts are not without their problems. In patients with preoperative limited healing potential, the risk of postoperative delayed wound healing and infection are very real. In addition, grafts may thrombose acutely or after several months or years. Patients must remain on anticoagulation and must have a high index of suspicion for complication so that if problems arise they can be treated emergently.

Graft patency is intimately correlated with graft incorporation and the natural healing process. These processes are different depending on the type of graft; specifically, whether a native vein or a prosthetic graft is used. During vein graft incorporation there is smooth muscle infiltration and extracellular matrix deposition as the vein graft adapts to the arterial environment. In some cases exuberant healing may be pathologic, resulting in neointimal hyperplasia, vein graft stenosis, and subsequent graft failure. In prosthetic grafts, incorporation involves the deposition of a layer of endothelial cells on the flow surface of the grafts, which assists in making the graft less thrombogenic. However, if there is a disruption

or failure of this process, the graft may become thrombogenic, predisposing to occlusion and failure [78-80].

Cell-Based Therapy for PVD:

As previously described, mononuclear cells (MNCs) and endothelial progenitor cells (EPCs) are two populations of cells found in the bone marrow and periphery which are associated with vascular protection and healing. The circulating MNC fraction consists of approximately 85% lymphocytes, 15% monocytes, and 0.03% EPCs [13]. EPCs are thought to assist with reendothelialization and neovascularization in response to acute or chronic vascular injury; in addition, EPC numbers directly correlate with vascular protection and healing, and inversely correlate with the severity of vascular disease.

Prior studies have used bone marrow-derived MNCs to increase neovascularization and ulcer healing by direct injection into the limbs of patients with unreconstructable limb ischemia. In addition, circulating peripheral MNCs have been investigated with similar beneficial effects. The protective and healing properties of the MNC fraction have been attributed to EPC activity; however it is possible that the monocyte component plays an active role in vascular healing and that monocytes are a clinically-relevant surrogate marker for EPCs.

In summary, Tateishi-Yuyama and colleagues investigated the efficacy of autologous implantation of bone marrow-derived mononuclear cells (BMCs), including EPCs, into the ischemic limbs of patients with PAD. At four weeks, they noted significant improvement in ABI, transcutaneous oxygen pressure, rest pain, and pain-free walking time [51]. Bartsch and

colleagues reported early results of combined intraarterial and intramuscular BMC therapy for patients with moderate to severe intermittent claudication. Two months after harvesting and delivering BMCs to the ischemic limb, pain-free walking distance increased 3.7-fold and the ABI was significantly improved after exercise and at rest [53]. Notably, after 13 months these positive effects persisted at their improved level [54]. In addition, Saigawa and colleagues noted that the clinical effectiveness of autologous bone marrow implantation, as measured by an improvement in ABI, was strongly correlated with the number of CD34-positive cells delivered to the ischemic limb [56]. In contrast to the studies evaluating BMC therapy, Ishida and colleagues used only peripheral blood mononuclear cells in patients with PAD. These cells were mobilized with G-CSF and then harvested and injected intramuscularly. After four weeks the researchers observed a significant improvement in ABI, healing of ischemic ulcers, and increased mean maximum walking distance [57].

These studies suggest that the specific cell type, or the combination of cells and cytokines, required to generate beneficial healing effects in patients with PAD are still incompletely understood. However, the implications of these studies for patients with unreconstructable lower extremity critical limb ischemia are very exciting. The patients most likely to benefit from cell-based therapy include those patients with CLI and no surgical or endovascular options for reconstruction, as well as patients with severe comorbid disease who are not operative candidates for potential bypass procedures.

Monocytes and Peripheral Arterial Disease (PAD):

The relationship between monocytes and advanced atherosclerosis is well established, since monocytes are known to contribute to the foam cells of the lesions. The following key processes outline the importance of monocytes in the development of atherosclerosis [81]: 1) endothelial injury; 2) intimal cholesterol accumulation and monocyte invasion with subsequent foam cell formation; 3) migration and proliferation of smooth muscle cells with expression of extracellular matrix; 4) local thrombus formation with secondary organization; 5) calcification and /or plaque rupture; and 6) final occlusion due to plaque rupture or thrombus formation [81].

The fact that EPCs are a component of the circulating monocyte fraction adds relevance to the study of monocytic populations in the setting of vascular disease. However, only a few studies have specifically addressed circulating monocytes in patients with PAD. Several studies have shown that patients with PAD have increased inflammatory markers, including monocyte chemoattractant protein-1 (MCP-1), a paracrine factor which mediates the recruitment of monocytes. Nylaende and colleagues studied the relationship between biochemical markers of inflammation and the diagnostic measures of PAD, including ABI, maximum treadmill walking distance, and angiographic score. In 127 patients with angiographically verified PAD, MCP-1 and CD40L were independently and significantly correlated with the angiographic score [82]. A study by Petrkova and colleagues also found that MCP-1 is elevated in patients with PAD compared to controls [83].

Hoogeveen and colleagues, as part of the Atherosclerosis Risk in Communities study,

demonstrated that there was a significant association of MCP-1 with PAD, independent of traditional coronary heart disease risk factors, with an odds ratio of 2.14 (95% CI) for the highest MCP-1 tertile compared to the lowest. Incident coronary heart disease risk increased significantly for each standard deviation difference in MCP-1 level, independently of other cardiovascular risk factors, including inflammatory markers. These data show that MCP-1 is associated with atherosclerotic disease. [81].

The correlation between PAD and inflammation is known, but what makes these studies interesting is that MCP-1 has also been associated with angiogenesis. A study by Muhs and colleagues in 2004 followed up prior observations that local infusion of recombinant MCP-1 could enhance collateral artery formation in rabbit and pig hindlimb models. Owing to the clinical disadvantages of protein infusion, Muhs and colleagues developed a nonviral, liposome-based MCP-1 gene transfer in a pig hindlimb ischemia model. Development of conductance as a measure of functionally relevant collateralization was evaluated in occluded as well as untreated limbs of each treatment animal and compared to control animals. The MCP-1 and control liposomes were locally delivered at the time of femoral artery occlusion. Two weeks after occlusion, collateralization was determined as changes in peripheral hemodynamic conductance, peripheral over aortic blood pressure ratio, and angiographically visible morphology of the peripheral vessel tree. Nonviral MCP-1 gene transfer significantly improved peripheral conductance (MCP-1 = 23.81 ± 2.81% and control = 11.69 ± 2.78%) as well as the ratio of peripheral over aortic blood pressure (MCP-1 = 0.75 ± 0.02% and control = 0.64 ± 0.03%) compared to controls [84].

Another relevant study by Seidler and colleagues reported that intra-arterial infusion of MCP-1 in a pig model after unilateral femoral artery occlusion stimulated collateral artery growth. Specifically, infusion of two micrograms per minute of MCP-1 for six hours was sufficient to double the arterial conductance at two weeks and sustain a significant increase in arterial conductance after six weeks [85].

These studies suggest that ischemic areas are capable of angiogenesis and are responsive to monocytes, stimulating a monocyte influx by release of the chemoattractant MCP-1. Since human patients with PAD and CAD have elevated plasma MCP-1, the connection between monocytes and relief of ischemia is plausible. However, the preceding studies did not correlate the levels of MCP-1 or the severity of PAD with numbers of circulating monocytes. This data would be helpful with respect to the determination of the effector cell.

To our knowledge, there is only one study in the literature that evaluated patients with PAD and circulating monocyte number. In 2005 Nasir and colleagues reported the results of a study in 3949 patients followed as part of the National Health and Nutrition Examination Survey (NHANES). The objective of the study was to assess the independent association of white blood cell (WBC) types and other inflammatory markers with the presence of reduced ABI, a marker of subclinical PAD. All subjects had no known vascular disease, and subclinical PAD was defined as an ABI < 0.9 in at least one leg. The authors found that elevated monocytes were the only specific WBC type that independently predicted the presence of atherosclerotic PAD. Even after adjustment for traditional cardiovascular risk factors, the odds ratio of PAD when comparing the highest to the lowest quartiles was 2.24

(95% CI). When WBC types and inflammatory markers were simultaneously included in the full model, the corresponding odds ratio was 1.91 (95% CI). Neutrophil counts, C-reactive protein (CRP) levels, and fibrinogen levels did not have significant odds ratios. Nasir and colleagues thus showed that elevated monocytes were significantly and independently associated with PAD in a representative sample of the U.S. population after adjustment for other inflammatory markers [86]. Notably, all of these patients had subclinical PAD, suggesting that even patients with minimal atherosclerosis and no clinical ischemia may activate the monocyte fraction of the WBC population.

Together these studies suggest that elevated MCP-1 and increased monocyte numbers correlate with tissue ischemia in patients with PAD. It is plausible that ischemic tissues release MCP-1 into the bloodstream to recruit monocytes into ischemic areas and promote vascular healing. Since monocytes and EPCs are components of the mononuclear cell fraction which has been shown to be responsible for vascular healing in many prior studies, this healing process may involve EPCs, monocytes, or both. Although this theory explains many observations, it may further blur the distinction between EPCs and monocytes. On the other hand, it may also provide a novel and clinically-relevant surrogate marker for circulating EPCs.

Distinguishing EPCs and Monocytes:

As suggested earlier, the lineage of EPCs is not entirely known. In addition, many types of cells may be precursors to endothelial cells. Monocytes are closely related to EPCs; they are thought to be derived from common precursors and to give rise to similar daughter cells. This

is shown in Figure 1, where it is clear that the lineage of EPCs and monocytes intersect; however, Figure 1 is an oversimplified representation of EPC and monocyte lineage.

In fact, a very recent study by Sieveking and colleagues isolated and defined different populations of putative EPCs; they have called them early endothelial progenitor cells (early EPCs) and late outgrowth endothelial cells (late OECs). Early EPCs, which appear in culture after 4 to 7 days, are similar to those originally described by Asahara and colleagues [8] and have been used in therapeutic studies [50]. In contrast, late OECs appear in culture after 14 to 21 days, and form colonies with high proliferation rates. These two different populations have been classified as EPCs because they both express endothelial markers [87].

Sieveking and colleagues identified these subpopulations by using a novel endothelial cell-specific angiogenesis assay that highlights strikingly different angiogenic properties of different putative EPCs and permits detailed functional characterization of EPCs. The researchers found that late OECs, but not early EPCs, form vascular networks in vivo and incorporate into vascular networks. In contrast, early EPCs, but not late OECs, augment angiogenesis in a paracrine fashion [87].

According to Sieveking and colleagues, since the differentiated fate of any putative endothelial progenitor cell is necessarily an endothelial cell, de novo tubulogenesis and incorporation into established vascular networks are important functional criteria by which putative EPCs should be assessed. Thus the researchers suggest that early EPCs are not true progenitors of endothelial cells but a monocytic cell capable of indirectly facilitating

angiogenesis in a paracrine fashion [87]. Figure 2 shows a proposed lineage diagram for EPCs that incorporates these new findings.

The results generated by Sieveking and colleagues confirm an important study by Yoder and colleagues, which reported that early EPCs are cells of low proliferative potential that are hematopoietic in origin and differentiate into macrophages rather than endothelial cells in culture [26]. In addition, a study by Yoon and colleagues suggested that the paracrine factors secreted by the monocytic early EPCs include VEGF and IL-8, and that both early EPCs and late OECs have receptors for these cytokines [88]. Notably, there was no mention of MCP-1 in any of these studies.

Although the connections are as yet undefined, there is a wide array of literature that suggests that the interplay between the following three related concepts may be significant: the known capability of the mononuclear cell fraction, composed of lymphocytes, monocytes, and EPCs, to participate in vascular healing with clinically relevant effects; the synergistic relationship between EPCs and monocytes in terms of paracrine stimulation, which orchestrates incorporation of EPCs and formation of vascular networks; and the evidence that patients with PAD have higher levels of paracrine factors (MCP-1) and greater numbers of circulating monocytes than controls. These findings support a unifying theory where monocyte activity contributes to relief of ischemia in patients with PAD. While the exact mechanism is unclear, it is plausible that increased circulating monocytes, recruited from the bone marrow to ischemic areas via MCP-1, stimulate EPCs that subsequently contribute to vascular healing.

Statement of Purpose/Hypothesis/Specific Aims:

Monocytes may contribute to the healing process in patients with peripheral vascular disease. Since ischemia appears to increase numbers of circulating monocytes, which in turn may stimulate EPC activity, we hypothesize that ischemic areas secrete paracrine factors such as MCP-1 to recruit bone marrow-derived monocytes into the circulation. These monocytes may then recruit EPCs that participate in angiogenesis and resolution of ischemia. In patients with critical limb ischemia requiring peripheral arterial bypass surgery, the relief of ischemia by endogenous mechanisms is insufficient despite elevated levels of monocytes and paracrine factors, possibly due to an impaired EPC response. We hypothesize that successful surgical revascularization resolves ischemia and diminishes the stimulus for monocyte recruitment, consequently decreasing the number of monocytes in the peripheral circulation.

Methods:

The records of all consecutive lower extremity peripheral bypass surgery cases performed at the VA Connecticut Healthcare Systems (West Haven, CT) between July 2002 and June 2007 were reviewed.

Patients were included in the study if they had a preoperative complete blood count (CBC) with differential recorded within one year prior to bypass surgery as well as a postoperative CBC with differential recorded between four months and 1.5 years after performance of the bypass surgery. The primary study time frame is defined as the time bounded by the dates of these lab tests.

Patients were excluded from the study if the surgery was a revision, in cases where staged surgeries for bilateral lower extremities resulted in overlapping lab tests, and if the bypass surgery was an inflow procedure (with target vessel proximal to the AK popliteal artery). In addition, procedures complicated by limb-threatening graft failure within the study time frame were excluded; for example, if graft thrombosis required graft revision or amputation, or if persistent graft infection required graft removal or lifetime suppressive antibiotics.

Patient risk factors were determined by thorough chart review. Demographic variables included the following: age, sex, diabetes, statin therapy, smoking status (never, prior, or current smoker), and affected extremity (left or right). Operative data included the type of graft (native or prosthetic) and the outflow vessel (AK popliteal, BK popliteal or tibial).

Outcome variables included the following: graft status (patent or thrombosed), limb status (intact or amputation), and mortality. Patient information with respect to the outcome variables was gathered until the date of last patient contact, which extended beyond the primary study time frame.

The primary dependent variable of interest in this study was the monocyte count ratio, which was used to determine whether there was a change in monocyte count with any given risk factor. Each cell count was derived from the CBC by multiplying the absolute white blood cell (WBC) count by the percent of cells in the differential. Each cell ratio was calculated by dividing the postoperative cell count by the preoperative cell count; a ratio greater than one corresponds to an increase in circulating cells postoperatively, whereas a ratio less than one corresponds to a decrease in circulating cells postoperatively.

Results are reported as mean ± SEM. Categorical variables were analyzed using Pearson's Chi-Square or the Fisher exact test. Continuous variables were analyzed using ANOVA. Survival data was analyzed using Kaplan-Meier statistics, and the curves were compared using the Log Rank (Mantel-Cox) test. The effect of multiple patient risk factors on the dependent variable of interest was analyzed by multivariable logistic regression. All tests were 2-tailed and p values ≤ 0.05 were considered statistically significant. (Statview 5.0, SAS Institute, Cary, NC).

Results:

Demographics

There were a total of 110 lower extremity peripheral bypass surgery cases performed at the VA Connecticut Healthcare System between July 2002 and June 2007. A total of 32 cases from the initial 110 charts reviewed met the inclusion, but not the exclusion, criteria and were the subject of this study.

Three-fourths of our study population (n=24) had baseline critical limb ischemia (CLI; Fontaine Stage III-IV), and presented with rest pain, chronic foot ulceration, and/or gangrene requiring operative intervention. One-fourth of our study population (n=8) did not present with signs of CLI, i.e. had no baseline ischemia, and were designated the control group. Half of these patients (n=4) had bypass surgery for exclusion of asymptomatic popliteal aneurysm and the other half (n=4) had surgery for relief of intermittent claudication (Fontaine Stage II).

The demographics of these patients are listed in Table 1. The mean age of CLI patients was 67.6 ± 1.7 years and the mean age of controls was 72.6 ± 5.1 years; there was no significant difference in age between the two groups (p=0.242). All of the patients were men, as this study was conducted within the VA system (Table 1).

Diabetes (n=10) was more prevalent in the group with CLI as compared to control patients (41.7% vs. 0%; p=0.035). Smoking was also distributed unequally; of the patients who had never smoked (n=3) none were in the CLI group (0% of CLI patients vs. 37.5% of controls;

p=0.003). Of the patients who were prior smokers but had quit before the surgery (n=14) there was little difference between groups (41.7% of CLI patients vs. 50% of controls); and of the patients who were current smokers at the time of the surgery (n=15) the majority were in the CLI group (58.3% of CLI patients vs. 12.5% of controls; p=0.003). There was no significant difference between the two groups in terms of age, sex, statin therapy, type of graft, outflow vessel, or operative extremity (Table 1).

Patients with CLI had increased preoperative monocyte counts compared to controls (0.753 ± 0.04 vs. 0.516 ± 0.05; p=0.0046) but there was no difference in preoperative lymphocyte counts between patients with CLI and controls (1.979 ± 0.14 vs. 1.912 ± 0.22; p=0.814). Patients with CLI also had elevated preoperative WBC counts (9.517 ± 0.56 vs. 7.225 ± 1.10; p=0.055) and neutrophil counts (6.458 ± 0.53 vs. 4.612 ± 0.93; p=0.092) compared to control patients, but these differences were not significant (Table 1).

Outcomes

Postoperative outcomes are presented in Table 2. Follow-up was complete in all patients; however, the mean follow-up time was slightly shorter in patients with ischemia compared to control patients (2.14 years vs. 3.26 years; p=0.042). Consistent with the study design, all deaths and amputations occurred outside of the study time frame, which was defined as the time bounded by the dates of the preoperative and postoperative CBC. Death prior to a postoperative CBC would exclude the patient due to insufficient data, and amputation would exclude the patient due to graft failure and unsuccessful resolution of limb ischemia at the time of the postoperative CBC. Deaths (n=9) occurred only in CLI patients (37.5% vs. 0%;

p=0.070) and amputations (n=4) also occurred only in CLI patients (16.7% vs. 0%; p=0.550). These results are notable but not statistically significant (Table 2).

Thrombosed grafts were grouped by the date of occurrence (within or outside the study time frame). Of the patients who had thrombosed grafts within the study time frame (n=4) none were in the CLI group (16.7% of CLI patients vs. 0% of controls; p=0.550). After the study time frame, the majority of patients who had thrombosed grafts (total n=9) were in the CLI group (n=8 or 33.3% of CLI patients vs. n=1 or 12.5% of controls; p=0.386; Table 2).

The survival curves for mortality rate, amputation rate, and thrombosis rate are shown in Figure 3. Survival data was analyzed using Kaplan-Meier statistics, and the curves were compared using the Log Rank (Mantel-Cox) test. These curves demonstrate the percent of patients in the CLI group and the control group who remained free of any given event (death, amputation, or thrombosis) over time. Patients dropped out of the analysis either on the date of last follow-up or on the date of the index event (Figure 3).

The mortality curve (Figure 3A) shows a significant difference between the groups. All deaths (n=9) occurred in CLI patients within three years of the surgery. Approximately 91% of the CLI patients were alive at one year, 73% at two years, and 47% at three years. This is statistically significant compared to control patients, who had a 100% survival rate (p=0.032; Figure 3A).

The amputation curve (Figure 3B) shows no significant difference between groups. All amputations (n=4) occurred in CLI patients within two years of the surgery. Approximately 92% of the CLI patients were intact at one year, and 76% at two years. These results are not statistically significant despite the fact that control patients remained 100% intact (p=0.168; Figure 3B).

The thrombosis curve (Figure 3C) shows no significant difference between groups. There were a total of n=9 thrombotic events; n=8 in the CLI group and n=1 in the control group. Thrombosis occurred within two years of the surgery. Approximately 83% of the CLI patients had patent grafts at one year, and 58% at two years. All of the control patients had patent grafts at one year, and 87.5% were patent at two years. These results are not statistically significant (p=0.186; Figure 3C).

Monocyte Counts

The primary dependent variable of interest was the monocyte ratio, and on univariable analysis only two risk factors had a significant effect on this ratio. The univariable analyses are summarized in Table 3. This table shows that patients with preoperative CLI had decreased monocyte counts after revascularization compared to control patients without preoperative CLI (-20% vs. +55%; p=.0003). In addition, diabetic patients also had significantly reduced postoperative monocyte counts compared to patients without diabetes (-32% vs. +13%; p=0.035). Age greater than or less than 70, statin therapy, smoking status, type of graft, outflow vessel, operative extremity, graft status, and mortality had no significant effect on the postoperative monocyte count (Table 3).

Since mononuclear cells are represented in the peripheral blood by both monocytes and lymphocytes, we determined the effect of CLI on both the monocyte and the lymphocyte counts. Figure 4 shows the effect of CLI on various peripheral blood cell populations. In patients with CLI, the monocyte ratio decreased significantly after bypass surgery (-20% vs. +55%; p=.0003; Figure 4A); however, the lymphocyte ratio was unchanged after revascularization in both ischemic and control patients (-10% vs. +1%; p=0.404; Figure 4B). In patients with CLI, both the WBC and neutrophil counts were also significantly decreased after revascularization (Figure 4). The diminished WBC ratio in patients with CLI (-14% vs. +26%; p=0.008; Figure 4C) paralleled the diminished neutrophil ratio (-9% vs. +39%; p=0.037; Figure 4D).

To determine the significance of the risk factors identified by univariable analysis, we performed multivariable logistic regression to identify which of these risk factors was relevant (Table 4). Our results demonstrate that the only factor that independently predicts reduced postoperative monocyte count is preoperative CLI (p=0.038). Age, thrombosed graft, native graft, smoking status, diabetes, statin therapy, tibial outflow, and mortality do not predict a diminished postoperative monocyte count (Table 4).

Discussion:

We report two findings consistent with our hypothesis regarding patients with CLI undergoing lower extremity peripheral bypass surgery. First, we report that the preoperative monocyte count was elevated in patients with CLI compared to control patients with no baseline critical ischemia. Second, we report that the monocyte count decreased significantly after successful revascularization in patients with CLI compared to control patients. Diabetes was also associated with a decreased monocyte count after peripheral bypass surgery; however, using multivariable analysis, only CLI independently predicted a decreased monocyte count after successful revascularization.

We believe that one of our most significant findings was that the preoperative monocyte count was elevated in patients with CLI compared to control patients. Interestingly, the difference was not apparent on the WBC differential, which only lists individual WBC populations as percentages of the total WBC count. It is likely that the poor sensitivity for differences in the low number of circulating monocytes resulted in a lack of significance when percentages were compared. However, normalization to the absolute WBC count revealed the greater preoperative absolute number of circulating monocytes in patients with CLI (Table 1). This observation is consistent with our hypothesis that patients with baseline CLI secrete paracrine factors such as MCP-1 to stimulate monocyte recruitment from the bone marrow, promoting angiogenesis and resolution of ischemia. Notably, monocytes were the only WBC component that had significantly different preoperative values when comparing patients with CLI to control patients ($p=0.0046$; Table 1). These results also agree

with the report from Nasir and colleagues which demonstrated that elevated circulating monocytes were the only WBC fraction that correlated significantly and independently with the presence of PAD [86]. Further studies that quantify the severity of ischemia may lead to a quantitative correlation between the magnitude of ischemia and the degree of elevation of the preoperative monocyte count.

We believe that increased recruitment of monocytes in patients with CLI is intimately related to recruitment of EPCs, and that circulating numbers of EPCs correlate inversely with the severity of PAD. We propose that monocytes are recruited in parallel with EPCs, perhaps via the same paracrine mechanism. This possibility is supported by the recent study by Sieveking and colleagues, who isolated and defined two different populations of putative EPCs, early endothelial progenitor cells (early EPCs) and late outgrowth endothelial cells (late OECs) [87]. Early EPCs augment angiogenesis in a paracrine fashion, and have been used in therapeutic studies [10,11,50]. In contrast, late OECs form colonies with high proliferation rates, and are capable of forming vascular networks in vitro. This EPC and monocyte lineage is represented in Figure 2. It has been proposed that early EPCs are not true progenitors of endothelial cells but are actually monocytic cells capable of indirectly facilitating angiogenesis in a paracrine fashion, while late OECs are the true EPCs [87].

Thus it is entirely possible that monocytic cells (called “early EPCs” by Sieveking, which may not be progenitor cells, despite the nomenclature) stimulate true EPCs (called “late OECs” by Sieveking) to respond to ischemia. This possibility correlates with our observation that monocytes are increased in patients with ischemia and decreased after resolution of

ischemia, since recruitment of EPCs depends on both monocytes and on the severity of vascular injury.

Furthermore, in patients with CLI that is severe enough to require peripheral arterial bypass surgery, endogenous mechanisms are necessarily insufficient to relieve the ischemia, despite elevated levels of MNCs, monocytes, and other paracrine factors. This suggests that the monocyte may not be the ultimate effector cell responsible for healing, and reinforces the theory that the EPC is the effector cell. It is possible that elderly and chronically ill patients with deficient reserves of EPCs may experience greater increases in monocytes in the setting of ischemia due to ineffective EPC recruitment, failure of EPCs to repair vascular injury, or other monocyte-EPC interactions. This theory explains the simultaneous existence of two findings that seem contradictory: how elevated monocyte counts are present in patients with CLI, despite the fact that monocytes participate in vascular healing and resolution of ischemia.

The only two demographic variables that were significantly different in ischemic patients compared to controls were diabetes and smoking. Diabetes was significantly more prevalent in the group with CLI as compared to control patients, and smoking status was also distributed unequally, with smokers being at greater risk for more severe disease. For these variables, our results parallel the known risk factors for PAD and CLI with remarkable accuracy [72].

In addition, we also found that monocytes, but not lymphocytes, decreased in patients with CLI after successful revascularization with a surgical bypass procedure (Figure 4A and 4B). This decrease in monocytes after resolution of ischemia is consistent with our hypothesis that monocytes recruit EPCs to promote healing in the setting of ischemia. These results are also consistent with the report from Seidler and colleagues describing the angiogenesis response to MCP-1 in ischemic tissue [85]. When resolution of ischemia is achieved, the demand for active cell populations that target vessel healing is diminished, decreasing both circulating MCP-1 and monocytes.

Importantly, the fact that the lymphocyte ratio did not change postoperatively in either the CLI patients or the control patients strongly suggests that it is the monocyte fraction, not the lymphocyte fraction, of the mononuclear cell population which is responsive to ischemia.

Notably, we measured the postoperative CBC at a minimum of four months after surgery to minimize effects of transient postoperative changes associated with injury, inflammation, and stress of the surgical procedure. Although we were limited to data obtained in a retrospective format, and therefore had no control over the dates of test selection, we believe that the random sampling of both preoperative and postoperative tests increases the power of findings, as there is no test selection bias.

In addition to decreased postoperative monocyte count in patients with CLI, we found that the postoperative WBC and neutrophil counts were also diminished in these patients (Figure 4C and 4D). Since neutrophils comprise approximately 85% of circulating WBCs, we

believe that the diminished postoperative neutrophil count is primarily responsible for the diminished WBC count. However, since neutrophils are not mononuclear cells, they cannot account for the decrease in monocyte count. We speculate that the diminished WBC and neutrophil counts in patients with successful limb revascularization reflects the resolution of preoperative inflammation present in these critically ill patients. This is consistent with the slightly elevated pre-operative WBC counts in patients with CLI, as well as elevated rates of smoking and diabetes, compared to control patients (Table 1). In addition, we believe that the increased inflammation in patients with CLI may account for their diminished long-term survival (Figure 3A).

One distinct advantage of our control group is that it is composed of patients without baseline ischemia who underwent peripheral bypass surgery. This is an unusual patient population, since the vast majority of patients who undergo open limb revascularization procedures have CLI. The incidental discovery of asymptomatic popliteal aneurysms (n=4 in our control group) is also rare. In addition, surgery is not the first line treatment for patients with claudication (n=4 in our control group), and is recommended only in patients whose claudication interferes significantly with their activities or with their work. The advantage of our control group is that it allows comparison of patients with no baseline ischemia to patients with critical ischemia before and after the same intervention. We believe that the small increases in monocyte, WBC, and neutrophil ratios in control patients (Figure 4) may reflect natural variation in this small number of patients, and additional studies with larger numbers of patients will clarify whether these increases are truly significant.

A major limitation of this study is the small number of patients. This is primarily due to the exclusion of large numbers of patients because they did not have a CBC with differential performed as part of their perioperative laboratory studies. However, enough patients were present for us to detect a decrease in postoperative monocyte count. Since laboratory tests may be ordered more frequently for patients admitted to the hospital, selection of an asymptomatic control group may be difficult in retrospective studies. In addition, our study design selected control patients without limb-threatening graft failure, so our survival data (Figure 3) was biased towards patients with better outcomes and is not generalizable to all patients with limb revascularization in our institution; on the other hand, selection of patients with patent grafts, or only mild graft failure, was necessary to determine whether relief of ischemia influenced the monocyte count. Additional studies that are not retrospective must be conducted to confirm the results of this study. In addition, further studies may allow correlation of circulating cytokines and other factors to the level of ischemia and to the circulating monocyte count.

Conclusions:

Our retrospective study suggests that resolution of CLI after surgical revascularization results in diminished numbers of circulating monocytes. We believe that this is due to the relief of ischemia and a subsequent decrease in the demand for regenerative stem cells recruited from the bone marrow. Our theory presupposes that the resolution of ischemia and associated inflammation causes a concomitant decrease in circulating cytokines or growth factors, resulting in decreased mobilization of stem cells and monocytes from the bone marrow. However, the true effector cell remains unclear: of the mononuclear cell fraction, our results strongly suggest a role for monocytes rather than lymphocytes. However, as EPCs are thought to be a component of the monocyte population, it remains to be determined whether the protective and healing effect is due to monocyte activity, EPC activity, or the activity of both cell types. It is reasonable to conclude, however, that circulating monocytes may be a clinically useful surrogate marker of circulating stem cells in patients with peripheral arterial disease.

References:

1 Dong C, Crawford LE, Goldschmidt-Clermont PJ: Endothelial progenitor obsolescence and atherosclerotic inflammation. J Am Coll Cardiol 2005;45:1458-1460.
2 Hristov M, Erl W, Weber PC: Endothelial progenitor cells: Mobilization, differentiation, and homing. Arterioscler Thromb Vasc Biol 2003;23:1185-1189.
3 Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P, Pippen AM, Annex BH, Dong C, Taylor DA: Aging, progenitor cell exhaustion, and atherosclerosis. Circulation 2003;108:457-463.
4 Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S: Vascular trauma induces rapid but transient mobilization of vegfr2(+)ac133(+) endothelial precursor cells. Circ Res 2001;88:167-174.
5 Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003;348:593-600.
6 Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G: Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005;353:999-1007.
7 Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S, Zeiher AM: Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: Proof of concept for the clinical importance of endogenous vascular repair. Circulation 2005;111:2981-2987.
8 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-967.
9 Doyle B, Metharom P, Caplice NM: Endothelial progenitor cells. Endothelium 2006;13:403-410.
10 Asahara T, Isner JM: Endothelial progenitor cells for vascular regeneration. J Hematother Stem Cell Res 2002;11:171-178.
11 Asahara T, Kawamoto A: Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 2004;287:C572-579.
12 Urbich C, Dimmeler S: Endothelial progenitor cells: Characterization and role in vascular biology. Circ Res 2004;95:343-353.
13 Dong C, Goldschmidt-Clermont PJ: Endothelial progenitor cells: A promising therapeutic alternative for cardiovascular disease. J Interv Cardiol 2007;20:93-99.
14 Loges S, Fehse B, Brockmann MA, Lamszus K, Butzal M, Guckenbiehl M, Schuch G, Ergun S, Fischer U, Zander AR, Hossfeld DK, Fiedler W, Gehling UM: Identification of the adult human hemangioblast. Stem Cells Dev 2004;13:229-242.
15 Eguchi M, Masuda H, Asahara T: Endothelial progenitor cells for postnatal vasculogenesis. Clin Exp Nephrol 2007;11:18-25.
16 Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM: Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002;109:337-346.
17 Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P: Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114:763-776.

18 Challen GA, Little MH: A side order of stem cells: The sp phenotype. Stem Cells 2006;24:3-12.
19 Sainz J, Al Haj Zen A, Caligiuri G, Demerens C, Urbain D, Lemitre M, Lafont A: Isolation of "Side population" Progenitor cells from healthy arteries of adult mice. Arterioscler Thromb Vasc Biol 2006;26:281-286.
20 Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP: Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362-367.
21 Urbich C, Dimmeler S: Risk factors for coronary artery disease, circulating endothelial progenitor cells, and the role of hmg-coa reductase inhibitors. Kidney Int 2005;67:1672-1676.
22 Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S: Expression of vegfr-2 and ac133 by circulating human cd34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952-958.
23 Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, Bosch-Marce M, Masuda H, Losordo DW, Isner JM, Asahara T: Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 2003;107:1322-1328.
24 Hristov M, Erl W, Weber PC: Endothelial progenitor cells: Isolation and characterization. Trends Cardiovasc Med 2003;13:201-206.
25 Shmelkov SV, St Clair R, Lyden D, Rafii S: Ac133/cd133/prominin-1. Int J Biochem Cell Biol 2005;37:715-719.
26 Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA: Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007;109:1801-1809.
27 Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M, Kalka C: Impaired progenitor cell activity in age-related endothelial dysfunction. J Am Coll Cardiol 2005;45:1441-1448.
28 Shaffer RG, Greene S, Arshi A, Supple G, Bantly A, Moore JS, Mohler ER, 3rd: Flow cytometric measurement of circulating endothelial cells: The effect of age and peripheral arterial disease on baseline levels of mature and progenitor populations. Cytometry B Clin Cytom 2006;70:56-62.
29 Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, Menegolo M, de Kreutzenberg SV, Tiengo A, Agostini C, Avogaro A: Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol 2005;45:1449-1457.
30 Fadini GP, Sartore S, Albiero M, Baesso I, Murphy E, Menegolo M, Grego F, Vigili de Kreutzenberg S, Tiengo A, Agostini C, Avogaro A: Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. Arterioscler Thromb Vasc Biol 2006;26:2140-2146.
31 Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC: Human endothelial progenitor cells from type ii diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002;106:2781-2786.
32 Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y, Murohara T: Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 2004;24:1442-1447.

33 Michaud SE, Dussault S, Haddad P, Groleau J, Rivard A: Circulating endothelial progenitor cells from healthy smokers exhibit impaired functional activities. Atherosclerosis 2006;187:423-432.
34 Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, Nickenig G: Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. Circulation 2004;109:220-226.
35 Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J, Penka M, Wolzt M, Huber K, Wojta J, Minar E, Kopp CW: Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. Atherosclerosis 2005;181:305-310.
36 Sandri M, Adams V, Gielen S, Linke A, Lenk K, Krankel N, Lenz D, Erbs S, Scheinert D, Mohr FW, Schuler G, Hambrecht R: Effects of exercise and ischemia on mobilization and functional activation of blood-derived progenitor cells in patients with ischemic syndromes: Results of 3 randomized studies. Circulation 2005;111:3391-3399.
37 Goldstein LJ, Gallagher KA, Bauer SM, Bauer RJ, Baireddy V, Liu ZJ, Buerk DG, Thom SR, Velazquez OC: Endothelial progenitor cell release into circulation is triggered by hyperoxia-induced increases in bone marrow nitric oxide. Stem Cells 2006;24:2309-2318.
38 Gallagher KA, Goldstein LJ, Thom SR, Velazquez OC: Hyperbaric oxygen and bone marrow-derived endothelial progenitor cells in diabetic wound healing. Vascular 2006;14:328-337.
39 Thom SR, Bhopale VM, Velazquez OC, Goldstein LJ, Thom LH, Buerk DG: Stem cell mobilization by hyperbaric oxygen. Am J Physiol Heart Circ Physiol 2006;290:H1378-1386.
40 Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS, Isner JM: Vascular endothelial growth factor-c (vegf-c/vegf-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol 1998;153:381-394.
41 Takeshita S, Pu LQ, Stein LA, Sniderman AD, Bunting S, Ferrara N, Isner JM, Symes JF: Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. Circulation 1994;90:II228-234.
42 Rajagopalan S, Mohler ER, 3rd, Lederman RJ, Mendelsohn FO, Saucedo JF, Goldman CK, Blebea J, Macko J, Kessler PD, Rasmussen HS, Annex BH: Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: A phase ii randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. Circulation 2003;108:1933-1938.
43 Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434-438.
44 van Royen N, Schirmer SH, Atasever B, Behrens CY, Ubbink D, Buschmann EE, Voskuil M, Bot P, Hoefer I, Schlingemann RO, Biemond BJ, Tijssen JG, Bode C, Schaper W, Oskam J, Legemate DA, Piek JJ, Buschmann I: Start trial: A pilot study on stimulation of arteriogenesis using subcutaneous application of granulocyte-macrophage colony-stimulating factor as a new treatment for peripheral vascular disease. Circulation 2005;112:1040-1046.

45 Zernecke A, Schober A, Bot I, von Hundelshausen P, Liehn EA, Mopps B, Mericskay M, Gierschik P, Biessen EA, Weber C: Sdf-1alpha/cxcr4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ Res 2005;96:784-791.
46 Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, Dimmeler S: Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001;103:2885-2890.
47 Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T, Isner JM: Statin therapy accelerates reendothelialization: A novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002;105:3017-3024.
48 Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM: Hmg-coa reductase inhibitors (statins) increase endothelial progenitor cells via the pi 3-kinase/akt pathway. J Clin Invest 2001;108:391-397.
49 Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM: Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 1998;101:2567-2578.
50 Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T: Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97:3422-3427.
51 Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T: Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: A pilot study and a randomised controlled trial. Lancet 2002;360:427-435.
52 Esato K, Hamano K, Li TS, Furutani A, Seyama A, Takenaka H, Zempo N: Neovascularization induced by autologous bone marrow cell implantation in peripheral arterial disease. Cell Transplant 2002;11:747-752.
53 Bartsch T, Brehm M, Zeus T, Strauer BE: Autologous mononuclear stem cell transplantation in patients with peripheral occlusive arterial disease. J Cardiovasc Nurs 2006;21:430-432.
54 Bartsch T, Brehm M, Zeus T, Kogler G, Wernet P, Strauer BE: Transplantation of autologous mononuclear bone marrow stem cells in patients with peripheral arterial disease (the tam-pad study). Clin Res Cardiol 2007;96:891-899.
55 Higashi Y, Kimura M, Hara K, Noma K, Jitsuiki D, Nakagawa K, Oshima T, Chayama K, Sueda T, Goto C, Matsubara H, Murohara T, Yoshizumi M: Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. Circulation 2004;109:1215-1218.
56 Saigawa T, Kato K, Ozawa T, Toba K, Makiyama Y, Minagawa S, Hashimoto S, Furukawa T, Nakamura Y, Hanawa H, Kodama M, Yoshimura N, Fujiwara H, Namura O, Sogawa M, Hayashi J, Aizawa Y: Clinical application of bone marrow implantation in patients with arteriosclerosis obliterans, and the association between efficacy and the number of implanted bone marrow cells. Circ J 2004;68:1189-1193.
57 Ishida A, Ohya Y, Sakuda H, Ohshiro K, Higashiuesato Y, Nakaema M, Matsubara S, Yakabi S, Kakihana A, Ueda M, Miyagi C, Yamane N, Koja K, Komori K, Takishita S: Autologous peripheral blood mononuclear cell implantation for patients with peripheral arterial disease improves limb ischemia. Circ J 2005;69:1260-1265.

58 Bhattacharya V, McSweeney PA, Shi Q, Bruno B, Ishida A, Nash R, Storb RF, Sauvage LR, Hammond WP, Wu MH: Enhanced endothelialization and microvessel formation in polyester grafts seeded with cd34(+) bone marrow cells. Blood 2000;95:581-585.
59 Matsuo Y, Imanishi T, Hayashi Y, Tomobuchi Y, Kubo T, Hano T, Akasaka T: The effect of senescence of endothelial progenitor cells on in-stent restenosis in patients undergoing coronary stenting. Intern Med 2006;45:581-587.
60 Shin'oka T, Matsumura G, Hibino N, Naito Y, Watanabe M, Konuma T, Sakamoto T, Nagatsu M, Kurosawa H: Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. J Thorac Cardiovasc Surg 2005;129:1330-1338.
61 Matsumura G, Ishihara Y, Miyagawa-Tomita S, Ikada Y, Matsuda S, Kurosawa H, Shin'oka T: Evaluation of tissue-engineered vascular autografts. Tissue Eng 2006;12:3075-3083.
62 Matsumura G, Miyagawa-Tomita S, Shin'oka T, Ikada Y, Kurosawa H: First evidence that bone marrow cells contribute to the construction of tissue-engineered vascular autografts in vivo. Circulation 2003;108:1729-1734.
63 Kopp HG, Ramos CA, Rafii S: Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. Curr Opin Hematol 2006;13:175-181.
64 Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, Young PP: Vegf and plgf promote adult vasculogenesis by enhancing epc recruitment and vessel formation at the site of tumor neovascularization. Faseb J 2006;20:1495-1497.
65 Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y: Heart disease and stroke statistics--2007 update: A report from the american heart association statistics committee and stroke statistics subcommittee. Circulation 2007;115:e69-171.
66 Minino AM, Heron MP, Smith BL: Deaths: Preliminary data for 2004. Natl Vital Stat Rep 2006;54:1-49.
67 A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (caprie). Caprie steering committee. Lancet 1996;348:1329-1339.
68 Criqui MH, Fronek A, Barrett-Connor E, Klauber MR, Gabriel S, Goodman D: The prevalence of peripheral arterial disease in a defined population. Circulation 1985;71:510-515.
69 Leng GC, Lee AJ, Fowkes FG, Whiteman M, Dunbar J, Housley E, Ruckley CV: Incidence, natural history and cardiovascular events in symptomatic and asymptomatic peripheral arterial disease in the general population. Int J Epidemiol 1996;25:1172-1181.
70 Criqui MH: Peripheral arterial disease and subsequent cardiovascular mortality. A strong and consistent association. Circulation 1990;82:2246-2247.
71 Criqui MH, Langer RD, Fronek A, Feigelson HS, Klauber MR, McCann TJ, Browner D: Mortality over a period of 10 years in patients with peripheral arterial disease. N Engl J Med 1992;326:381-386.
72 Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG: Inter-society consensus for the management of peripheral arterial disease (tasc ii). J Vasc Surg 2007;45 Suppl S:S5-67.

73 Ouriel K: Peripheral arterial disease. Lancet 2001;358:1257-1264.
74 Long-term mortality and its predictors in patients with critical leg ischaemia. The i.C.A.I. Group (gruppo di studio dell'ischemia cronica critica degli arti inferiori). The study group of criticial chronic ischemia of the lower exremities. Eur J Vasc Endovasc Surg 1997;14:91-95.
75 Fowkes FG, Housley E, Cawood EH, Macintyre CC, Ruckley CV, Prescott RJ: Edinburgh artery study: Prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population. Int J Epidemiol 1991;20:384-392.
76 Meijer WT, Hoes AW, Rutgers D, Bots ML, Hofman A, Grobbee DE: Peripheral arterial disease in the elderly: The rotterdam study. Arterioscler Thromb Vasc Biol 1998;18:185-192.
77 Hirsch AT, Criqui MH, Treat-Jacobson D, Regensteiner JG, Creager MA, Olin JW, Krook SH, Hunninghake DB, Comerota AJ, Walsh ME, McDermott MM, Hiatt WR: Peripheral arterial disease detection, awareness, and treatment in primary care. Jama 2001;286:1317-1324.
78 Schanzer A, Hevelone N, Owens CD, Belkin M, Bandyk DF, Clowes AW, Moneta GL, Conte MS: Technical factors affecting autogenous vein graft failure: Observations from a large multicenter trial. J Vasc Surg 2007;46:1180-1190; discussion 1190.
79 Zhang L, Hagen PO, Kisslo J, Peppel K, Freedman NJ: Neointimal hyperplasia rapidly reaches steady state in a novel murine vein graft model. J Vasc Surg 2002;36:824-832.
80 Ramdev P, Rayan SS, Sheahan M, Hamdan AD, Logerfo FW, Akbari CM, Campbell DR, Pomposelli FB, Jr.: A decade experience with infrainguinal revascularization in a dialysis-dependent patient population. J Vasc Surg 2002;36:969-974.
81 Hoogeveen RC, Morrison A, Boerwinkle E, Miles JS, Rhodes CE, Sharrett AR, Ballantyne CM: Plasma mcp-1 level and risk for peripheral arterial disease and incident coronary heart disease: Atherosclerosis risk in communities study. Atherosclerosis 2005;183:301-307.
82 Nylaende M, Kroese A, Stranden E, Morken B, Sandbaek G, Lindahl AK, Arnesen H, Seljeflot I: Markers of vascular inflammation are associated with the extent of atherosclerosis assessed as angiographic score and treadmill walking distances in patients with peripheral arterial occlusive disease. Vasc Med 2006;11:21-28.
83 Petrkova J, Szotkowska J, Hermanova Z, Lukl J, Petrek M: Monocyte chemoattractant protein-1 in patients with peripheral arterial disease. Mediators Inflamm 2004;13:39-43.
84 Muhs A, Lenter MC, Seidler RW, Zweigerdt R, Kirchengast M, Weser R, Ruediger M, Guth B: Nonviral monocyte chemoattractant protein-1 gene transfer improves arteriogenesis after femoral artery occlusion. Gene Ther 2004;11:1685-1693.
85 Seidler RW, Lenter MC, Guth BD, Doods H: Short-term intra-arterial infusion of monocyte chemoattractant protein-1 results in sustained collateral artery growth. J Cardiovasc Pharmacol Ther 2007;12:61-68.
86 Nasir K, Guallar E, Navas-Acien A, Criqui MH, Lima JA: Relationship of monocyte count and peripheral arterial disease: Results from the national health and nutrition examination survey 1999-2002. Arterioscler Thromb Vasc Biol 2005;25:1966-1971.

87 Sieveking DP, Buckle A, Celermajer DS, Ng MK: Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: Insights from a novel human angiogenesis assay. J Am Coll Cardiol 2008;51:660-668.
88 Yoon CH, Hur J, Park KW, Kim JH, Lee CS, Oh IY, Kim TY, Cho HJ, Kang HJ, Chae IH, Yang HK, Oh BH, Park YB, Kim HS: Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: The role of angiogenic cytokines and matrix metalloproteinases. Circulation 2005;112:1618-1627.
89 Shantsila E, Watson T, Tse HF, Lip GY: New insights on endothelial progenitor cell subpopulations and their angiogenic properties. J Am Coll Cardiol 2008;51:669-671.

Table 1: Demographic Variables

Variable	Total Number	Percent of Total		Control	Control (%)	Ischemic	Ischemic (%)	P value
Total patients	32			8		24		
Age (years)	68.9 ± 1.8	-		72.6 ± 5.1	-	67.6 ± 1.7	-	0.2423
Sex								
Male	32	100.0%		8	100.0%	24	100.0%	>0.9999
Female	0	0.0%		0	0.0%	0	0.0%	
Diabetes								
No	22	68.8%		8	100.0%	14	58.3%	0.0353
Yes	10	31.3%		0	0.0%	10	41.7%	
Statin Therapy								
No	12	37.5%		3	37.5%	9	37.5%	> 0.9999
Yes	20	62.5%		5	62.5%	15	62.5%	
Smoking Status								
Never a Smoker	3	9.4%		3	37.5%	0	0.0%	0.0028
Prior Smoker	14	43.8%		4	50.0%	10	41.7%	
Current Smoker	15	46.9%		1	12.5%	14	58.3%	
Type of Graft								
Native Vein	19	59.4%		4	50.0%	15	62.5%	0.6838
Prosthetic	13	40.6%		4	50.0%	9	37.5%	
Outflow Vessel								
AK Popliteal	10	31.3%		4	50.0%	6	25.0%	0.1091
BK Popliteal	13	40.6%		4	50.0%	9	37.5%	
Tibial	9	28.1%		0	0.0%	9	37.5%	
Operative Extremity								
Left	10	31.3%		1	12.5%	9	37.5%	0.3803
Right	22	68.8%		7	87.5%	15	62.5%	
Preoperative WBC Count (cells per HPF)				7.225 ± 1.10		9.517 ± 0.56		0.0554
Preoperative Lymphocyte Count				1.912 ± 0.22		1.979 ± 0.14		0.8143
Preoperative Neutrophil Count				4.612 ± 0.93		6.458 ± 0.53		0.0923
Preoperative Monocyte Count				0.516 ± 0.05		0.753 ± 0.04		0.0046
Preoperative Eosinophil Count				0.147 ± 0.03		0.254 ± 0.05		0.2627
Preoperative Basophil Count				0.032 ± 0.01		0.063 ± 0.01		0.1902

Table 2: Outcome Variables

Variable	Total Number	Percent of Total		Control	Control (%)	Ischemic	Ischemic (%)	P value
Total patients	32			8		24		
Mean Follow-Up Time (years)	2.42			3.26		2.14		0.0421
Mortality:								
Alive	23	71.9%		8	100.0%	15	62.5%	0.0699
Dead	9	28.1%		0	0.0%	9	37.5%	
Limb Status:								
Intact	28	87.5%		8	100.0%	20	83.3%	0.5497
AKA or BKA	4	12.5%		0	0.0%	4	16.7%	
Graft Status:								
Within study time frame								
Patent	28	87.5%		8	100.0%	20	83.3%	0.5497
Thrombosed	4	12.5%		0	0.0%	4	16.7%	
Through Follow-Up								
Patent	23	71.9%		7	87.5%	16	66.7%	0.3858
Thrombosed	9	28.1%		1	12.5%	8	33.3%	

Table 3: Univariable Analysis for Factors Affecting Monocyte Ratio

Variable	Risk Factor	Mean Monocyte Ratio	P-value
Age (years)	< 70	1.077	0.3726
	≥ 70	0.898	
Preoperative CLI	No	1.554	0.0003
	Yes	0.799	
Diabetes	No	1.127	0.0348
	Yes	0.682	
Statin Therapy	No	0.952	0.7858
	Yes	1.009	
Smoking Status	Never a Smoker	1.182	0.5655
	Prior Smoker	1.063	
	Current Smoker	0.878	
Type of Graft	Native Vein	0.940	0.5693
	Prosthetic	1.057	
Outflow Vessel	AK Popliteal	1.192	0.0740
	BK Popliteal	1.071	
	Tibial	0.641	
Operative Extremity	Left	0.996	0.9552
	Right	0.984	
Graft Status	Patent	1.002	0.7126
(within study time frame)	Thrombosed	0.889	
Mortality	Alive	1.088	0.1055
(after study time frame)	Dead	0.732	

Table 4: Multivariable Logistic Regression Analysis

Variable	P-Value	Exp (Coef)	95% Lower	95% Upper
Age	0.5506	1.046	0.903	1.211
Thrombosed Graft	0.1212	0.021	1.62E-04	2.775
Native Graft	0.2664	0.186	0.01	3.619
Preoperative CLI	0.0377	91.883	1.291	6538
Smoking Status	0.9155	1.245	0.022	71.239
Diabetes	0.6373	0.423	0.012	15.169
Statin Therapy	0.5023	0.348	0.016	7.596
Tibial Outflow	0.9962	4.77E+08	0	∞
Mortality	0.534	0.317	0.008	11.852

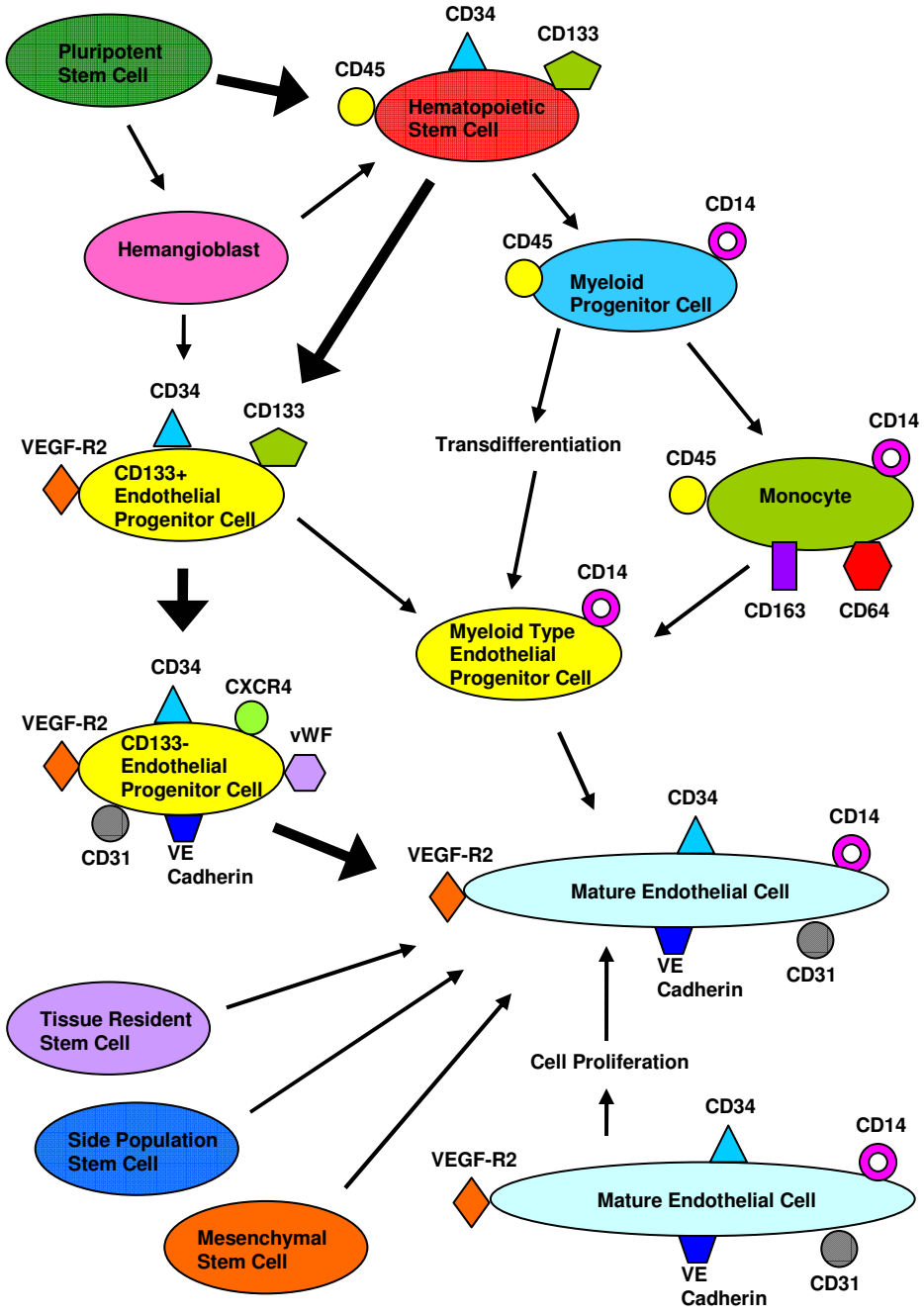

Figure 1: Endothelial Cell Derivation Diagram

Multiple cell types are known to give rise to endothelial cells. Bold arrows show the pathway most commonly cited. Adapted from Urbich [12] and colleagues. VE cadeherin = vascular endothelial cadherin; VEGF-R2 = vascular endothelial growth factor receptor 2; vWF = von Willebrand factor.

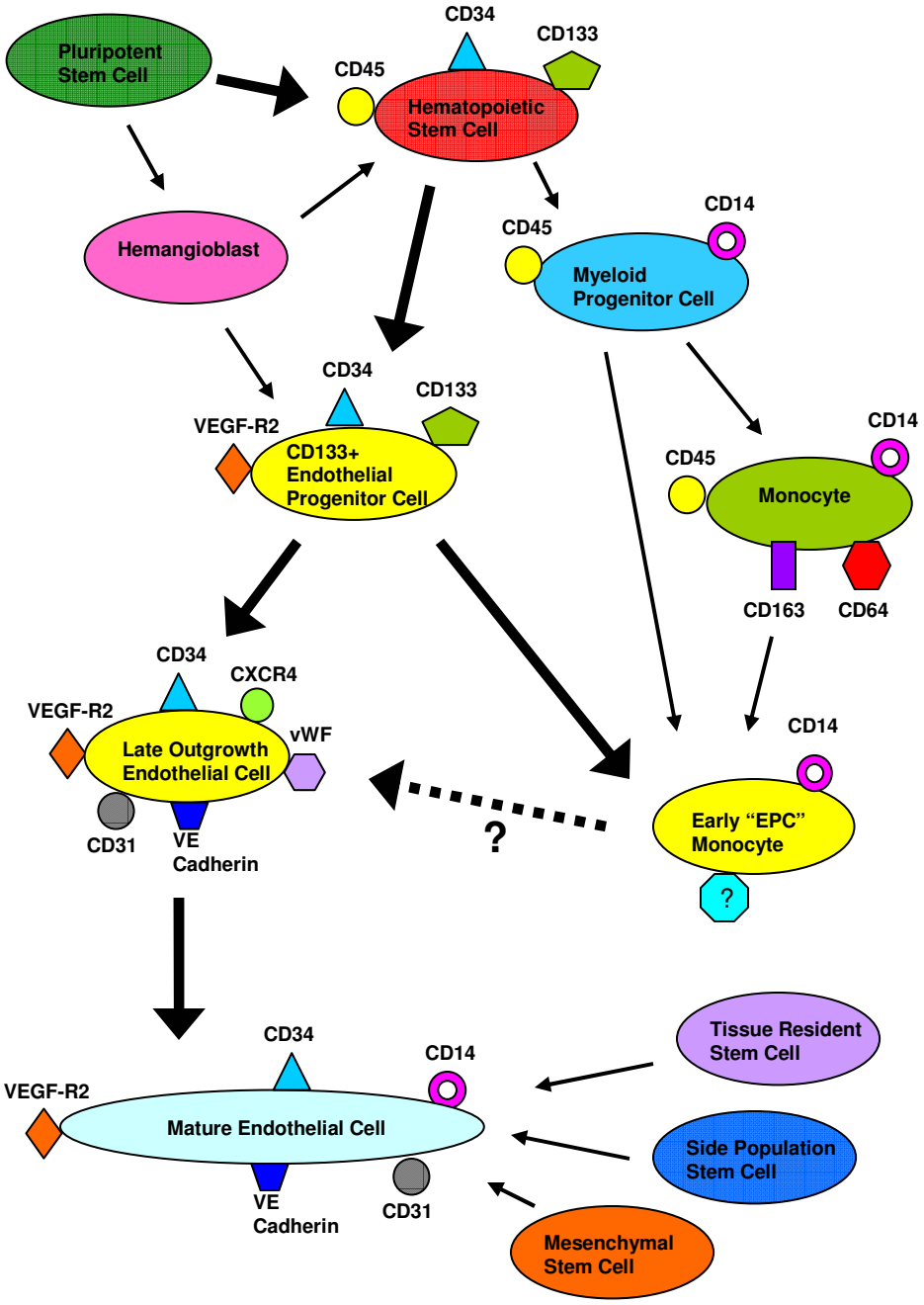

Figure 2: EPC and Monocyte Lineage Diagram

Bold arrows show the pathways most commonly cited. Dashed arrow shows the pathway for paracrine stimulation. Adapted from Urbich [12] and Shantsila [89]. VE cadeherin = vascular endothelial cadherin; VEGF-R2 = vascular endothelial growth factor receptor 2; vWF = von Willebrand factor; early "EPC" = early endothelial progenitor cell, found to be a monocyte, not a progenitor cell, by Sieveking and colleagues [87]; question mark on early "EPC" monocyte cell membrane suggests specific markers are unknown; late outgrowth endothelial cell, found to be a true endothelial progenitor cell by Sieveking and colleagues [87].

Figure 3: Survival Curves

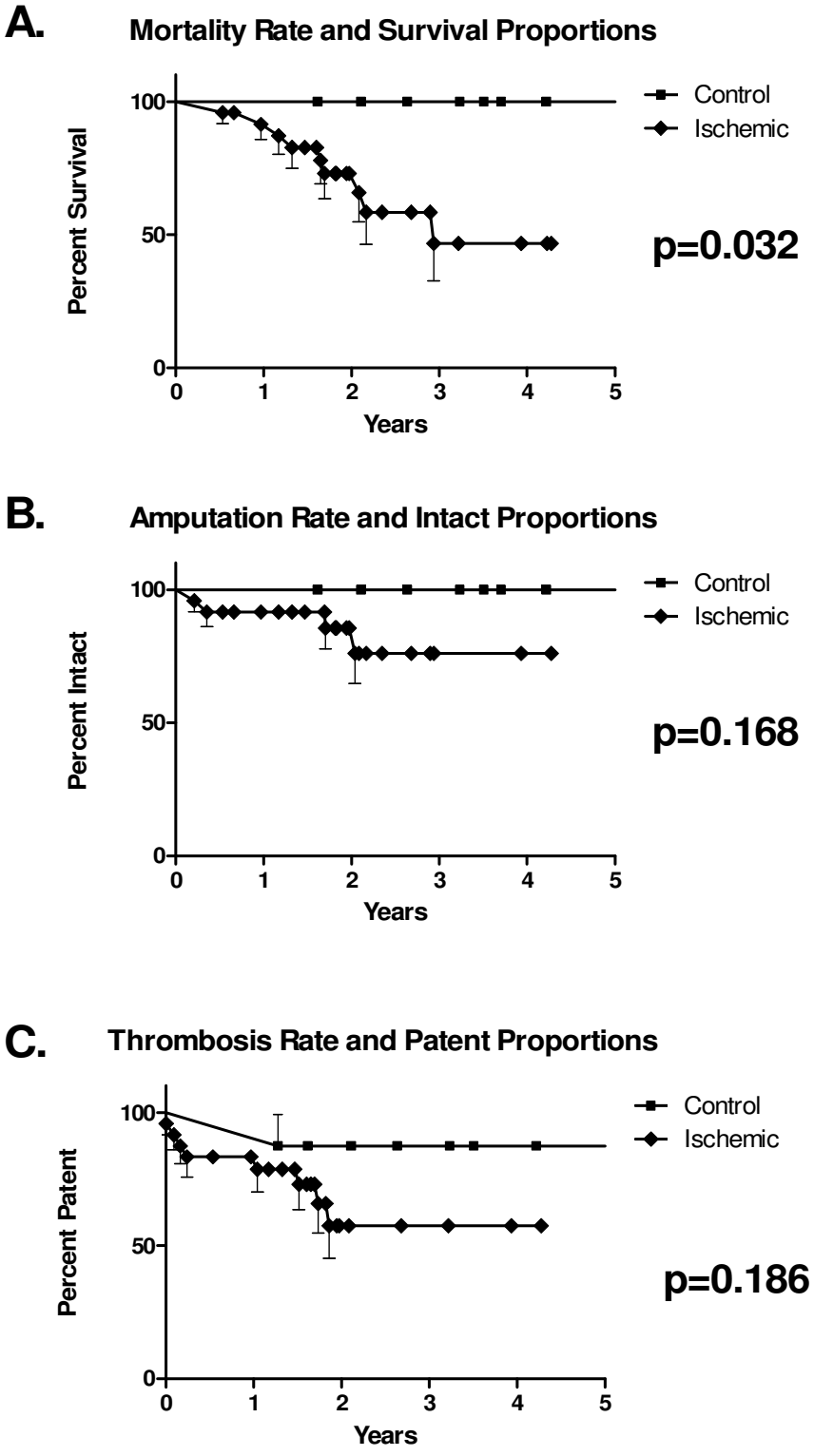

Figure 4: Effect of Critical Limb Ischemia on Peripheral Blood Cell Populations