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

Animal studies have shown that diabetes mellitus, which is a major risk factor for cardiovascular disease and associated with impaired mobilization of PCs from the BM,1,3–5 induces pathological processes in the BM, including niche dysfunction,6 altered cytokine signaling,7 and BM vasculopathy8 and neuropathy,9 which relate to impaired PC function and mobilization. In a recent issue of Circulation Research, Spinetti et al10 confirmed and extend the observations of Spinetti et al by studying BM microvascular and neuropathic changes in patients with CLI with and without diabetes mellitus.

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Bone Marrow Microvascular and Neuropathic Alterations in Patients With Critical Limb Ischemia

Martin Teraa,* Joost O. Fledderus,* Ramil I. Rozbeh, Roos J. Leguit, Marianne C. Verhaar, on behalf of the Juventas Study Group†

Short Communication

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

Objective: To investigate microvascular and neuropathic changes in BM of patients with CLI.

Methods and Results: BM biopsies were obtained from patients with CLI (n=33) included in the Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) trial (NCT00371371) and controls (n=12). We performed immunohistochemistry and histomorphometry of the BM to assess microvascular density and to evaluate pan-neuronal and sympathetic innervation, which is involved in progenitor cell mobilization. Microvascular density was reduced significantly in CLI compared with controls (P=0.01), as was sympathetic (P=0.047) and pan-neuronal innervation (P=0.006). No differences in microvascular density and sympathetic or pan-neuronal innervation were observed between patients with CLI with and without diabetes mellitus.

Conclusions: CLI is associated with BM microvascular and neuropathic changes, both in patients with and without diabetes mellitus. (Circ Res. 2014;114:311-314.)

Key Words: bone marrow ▪ diabetic neuropathies ▪ microvasculature ▪ peripheral arterial disease

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quantified using CD34-based, α-smooth muscle actin–based, tyrosine hydroxylase–based, and protein gene product 9.5–based protocols, respectively. Scoring researchers were blinded to biopsy origin.

**Results**

**Subject Characteristics**

Groups did not differ with respect to age or sex (Table 1). Thirteen of the 33 patients with CLI had a history of diabetes mellitus and used glucose-lowering medication. Patients with CLI had a substantial burden of cardiovascular risk factors and comorbidities, which was not significantly different between patients with CLI with and without diabetes mellitus.

**CLI Induces Alterations in BM Vasculature and Innervation**

Less microvessels, that is, capillaries and sinusoids, were present in BM of patients with CLI compared with healthy controls (Table 2; Figure 1). BM arteriolar density was not different between patients with CLI and controls (P=0.86). The number of arterioles reached by protein gene product 9.5–positive nerve endings was less in CLI BM (P=0.006), as was the number of sympathetic nerve terminals in CLI BM, compared with controls (P=0.047; Figure 2).

**CLI Induces Alterations in BM Microvascular Density and Innervation Independent of Diabetes Mellitus Presence**

Between patients with CLI with (n=13) and without diabetes mellitus (n=20), no differences in BM microvascular density (P=0.87) and BM nerve density (P=0.37 and P=0.57 for tyrosine hydroxylase and protein gene product 9.5, respectively) were observed. Arteriolar density was higher in patients with CLI with versus without diabetes mellitus (P=0.008) and not influenced by other characteristics including cardiovascular risk factors and baseline characteristics. We found no significant correlations between presence of diabetes mellitus and the other BM characteristics. In patients with CLI without diabetes mellitus, numbers of microvessels (32.8±1.8 versus 40.2±1.7/ mm²; P=0.009), sympathetic nerve terminals (6.4±0.8 versus 10.1±1.8/mm²; P=0.01; Table 2; Figure 1), and number of arterioles reached by protein gene product 9.5–positive nerve endings (0.094±0.22 versus 0.20±0.03/mm²; P=0.003) were lower than in controls.

**Discussion**

In this Short Communication, we show for the first time that CLI, also in the absence of diabetes mellitus, is associated with structural changes in the BM, characterized by lower microvascular density and reduced general as well as sympathetic innervation. Our findings confirm and extend the observations of Spinetti et al8 recently published in this journal, who provided first human evidence for a damaging effect of type-2 diabetes mellitus on BM.

![Table 1. Demographic and Clinical Characteristics](image-url)
Spinetti et al\textsuperscript{8} reported microvascular changes, alterations in BM composition and PC content in patients with diabetes mellitus and showed most striking microvascular remodeling in patients with CLI and diabetes mellitus. However, they performed their analyses in patients with CLI on the proximal part of the amputated femoral bone, which may have influenced their results. We obtained BM from the iliac crest in patients with CLI and controls, excluding effects from variances in BM structure in different anatomic areas and confirm the microvascular changes observed by Spinetti et al. Moreover, our study included patients with CLI with and without diabetes mellitus, showing similar changes in microvasculature, suggesting that CLI in itself, independent of diabetes mellitus, induces microvascular changes in the human BM.

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

Altogether, our observational study shows that CLI, irrespective of diabetes mellitus, is associated with microvascular rarefaction and impairment of sympathetic innervation in the human BM. Our data do not allow conclusions on causality or underlying mechanisms but suggest that CLI induces alterations in BM structure which may contribute to the reduced

### Table 2. Bone Marrow Histomorphometry and Immunohistochemistry

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<tbody>
<tr>
<td>Microvessels, n/mm\textsuperscript{2}</td>
<td>32.3±3.0</td>
<td>32.8±1.8</td>
<td>40.2±1.7</td>
<td>0.010</td>
<td>0.87</td>
<td>0.009</td>
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<tr>
<td>Arterioles, n/mm\textsuperscript{2}</td>
<td>2.9±0.6</td>
<td>1.4±0.2</td>
<td>2.1±0.5</td>
<td>0.86</td>
<td>0.008</td>
<td>0.09</td>
</tr>
<tr>
<td>PGP9.5\textsuperscript{+} arterioles, n/mm\textsuperscript{2}</td>
<td>0.12±0.04</td>
<td>0.09±0.02</td>
<td>0.20±0.03</td>
<td>0.006</td>
<td>0.57</td>
<td>0.003</td>
</tr>
<tr>
<td>Tyrosine hydroxylase\textsuperscript{–} fibers, n/mm\textsuperscript{2}</td>
<td>7.6±1.0</td>
<td>6.4±0.8</td>
<td>10.1±1.8</td>
<td>0.047</td>
<td>0.37</td>
<td>0.067</td>
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Numbers are mean±SEM. CLI indicates critical limb ischemia; CLI-DM, critical limb ischemia with diabetes mellitus; and PGP9.5, protein gene product 9.5.

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Figure 1. Critical limb ischemia (CLI) is associated with decreased microvascular density. Microscopic photographs of human bone marrow from control (A), CLI-diabetes mellitus (DM)+ (B), and CLI-DM– (C) donors, stained for microvascular marker CD34. A distinction was made between microvessels (MV) and nonvessel structures, most likely hematopoietic stem cells (HSCs).

Figure 2. Sympathetic nerve terminals in bone marrow (BM). Microscopic photographs of human BM from control (A), CLI-diabetes mellitus (DM)+ (B), and CLI-DM– (C) donors, stained for sympathetic nerve marker tyrosine hydroxylase (arrows).
circulating PC counts in CLI and reflect impaired vasculoregenerative potential.

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We acknowledge Drs Liane C. te Boome, Dr Sofie L.J. Verbeke, Krista den Ouden, and Charlotte Vial for performing immunohistochemical stainings and histomorphometric analyses of the bone marrow biopsies.

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Disclosures
None.

References

Novelty and Significance

What Is Known?
- Bone marrow (BM)–derived progenitor cells (PCs) are involved in neovascularization of ischemic tissue and their numbers are reduced in patients with cardiovascular disease.
- PC egress from the BM is influenced by chemokine and neuronal signaling, sympathetic signaling, in particular.
- In diabetic patients, BM architecture is altered, that is, reduction of hematopoietic tissue, increased fat deposition, and microvascular rarefaction, especially when associated with critical limb ischemia (CLI).

What New Information Does This Article Contribute?
- Presence of CLI is related with alterations in BM architecture, even in the absence of diabetes mellitus.
- CLI is associated with a marked reduction in general and sympathetic innervation of the BM, independent of diabetes mellitus.
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**SUPPLEMENTAL MATERIAL**

Bone marrow microvascular and neuropathic alterations in critical limb ischemia patients

Short Communication

**First authors:** Teraa and Fledderus  
**Short title:** Bone marrow rarefaction in CLI

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Detailed Methods

**Study population**  
Patients with documented CLI (n=33; 20 without diabetes and 13 with diabetes, respectively), participating in the Juventas-trial; a clinical trial evaluating the clinical effects of intra-arterial infusion of BM mononuclear cells in CLI (clinicaltrials.gov NCT00371371), were included for the present study. Identification of diabetics and non-diabetics was performed at study inclusion and based on medical history and medication use. Patients were classified as diabetic based on a known history of diabetes and/or self-reported diabetes (use of glucose-lowering agents: oral anti-diabetic medication or insulin). Patients were identified as non-diabetic if there was no history of diabetes, no self-reported diabetes, no use of glucose lowering medication and a non-fasting plasma glucose below 200 mg/dL (11.1 mmol/L). In short, the Juventas-trial included patients with chronic CLI, an ankle-brachial index (ABI) of 0.6 or less, or an unreliable index (non-compressible or not in proportion to the Fontaine classification), who were not candidate for conventional revascularization. Exclusion criteria were a history of neoplasm or malignancy in the past 10 years, concomitant disease with life expectancy of less than one year, inability to obtain sufficient BM aspirate, known infection with human immunodeficiency virus, hepatitis B or C virus, and an impossibility to complete follow-up. All patients underwent a BM aspiration procedure, during which approximately 100mL of BM was harvested according to the trial protocol. In patients included in the current study an additional BM biopsy was taken from the iliac crest distant to the site of the BM aspiration. The control BM samples (n=12) consisted of routine iliac crest biopsies, performed as part of routine work-up in patients with suspected lymphoma, selected to match age and sex of the patient population. Only biopsies of patients with localized disease and without BM involvement were included. Of this control population only age, gender and absence of cardiovascular history, including diabetes, were available. Since biopsies were part of
standard medical care, other characteristics, such as BMI and HbA1c were not measured routinely.

The study was performed according to the Declaration of Helsinki, the study protocol was approved by the institutional review board of the University Medical Center Utrecht, The Netherlands, and patients provided written informed consent.

**Histomorphometry and immunohistochemistry**

Biopsy samples were fixed in buffered formalin for 24h and subsequently decalcified in saturated Na₂EDTA (125 g/L) solution in distilled water for 2 days under constant agitation. Tissues were embedded in paraffin. 3 µm sections were mounted on 3-aminopropyl-3-triethoxysilane/bovine serum albumin coated slides and deparaffinized in xylene, ethanol, to distilled water.

For histomorphometry slides were stained with conventional hematoxylin-eosin (H&E). Cellularity (percentage area comprised of hematopoietic tissue) was estimated by two experienced independent pathologists, and results were averaged (Pearson’s correlation of both pathologists 0.97, P<0.001). To identify vascular structures in the BM CD34-antibody was used,² alpha smooth muscle actin (α-SMA) antibody was used to identify arterioles, tyrosine hydroxylase (TH) and protein gene product 9.5 (PGP9.5) antibodies were used for sympathetic and pan-nerve characterization, respectively.

**Immunohistochemical staining** for CD34 (mouse anti-human antibody, 1:800 dilution; Immunotech, Beckman Coulter Inc, Marseille, France; 0786) and alpha-SMA (mouse anti-human antibody, 1:32,000 dilution; Sigma-Aldrich Corp, St. Louis, MO,USA; A2457) were performed using the Bond automated staining machine (Leica Microsystems GmbH, Wetzlar, Germany) with the Bond Polymer Refine Detection kit (Leica Microsystems GmbH). Antigen retrieval was performed with Bond Epitope Retrieval Solution 2 (Leica Microsystems GmbH) for 20 min at 99°C (only for CD34), then slides were incubated with the primary antibody for 15 min, the Bond Polymer Detection kit for 8 min, and 10 min with 3,3'-diaminobenzidine (DAB) all at room temperature (RT).

For TH and PGP9.5 manual staining protocols were applied. Sections were treated with peroxidase block for 15 min and incubated in citrate-HCl buffer at 100°C for 20 min. For TH sections were stained with rabbit anti-human antibody to TH (1:100 dilution; Abcamplc, Cambridge, UK; ab59276) for 1 h at RT. For PGP9.5 sections were stained with rabbit anti-human antibody to PGP9.5 (1:400 dilution; Abcamplc; ab15503) for 1 h at RT. Sections were incubated with Bright Vision Polyhorseradish peroxidase (HRP)-anti rabbit IgG (Immunologic BV, Duiven, The Netherlands; DPVR55HRP) for 1h at RT. Finally, incubation with the NovaRED Peroxidase substrate kit (Vector Laboratories Inc, Burlingame, CA, USA; SK-4800) for 10 min was applied and sections were counterstained with hematoxylin.

All sections were scanned and converted to digital images. Analyses and scoring of all sections was performed using Aperio ImageScope software (Aperio Technologies Inc, Vista, CA, USA) by one investigator (RR) blinded for the origin of the biopsies and cross-checked by a second investigator (MT).

Vascularity of the BM was assessed using the “hot spot” method as previously described.³,⁴ Briefly, three microvascular hot spots were identified at 100x magnification in each CD34-stained section” and counted at 400x magnification. Structures staining positive for CD34 were classified as being a microvessel if they either had a clearly discernible lumen or a vessel-like structure. Results were expressed as the average of the three hot spots. Arterioles were identified in the α-SMA-stained sections. The total area of hematopoietic tissue was measured. In the complete section the number of arterioles was scored at 70x magnification and divided by the total area of hematopoietic tissue.

Sympathetic nerve fibers were scored in three random high-power fields identified at 100x magnification and nerve fibers were subsequently counted at 400x magnification. Results were expressed as the average of the three fields. The pan-nerve staining with PGP9.5 was scored by counting the number of arterioles (based on α-SMA expression in consecutive slides) reached by PGP9.5-positively staining nerve terminals, since aspecific staining of especially megakaryocytes made it necessary to count structures that clearly classify as PGP9.5 positive nerve terminals, such as in the walls of arterioles.
Statistical Analyses
Continuous data are expressed as means±SEM or medians with 25th and 75th percentiles (P25-P75), depending on the normality of the data. Normality of variables was tested using the Kolmogorov-Smirnov test. A Levene's test was used to test for equality of variances between the groups. Independent samples t-tests were used to test for differences of parametric variables between groups. The Mann-Whitney U-test was used to test for differences of non-parametric variables between groups. Categorical variables were compared using the Fisher’s exact test. Univariate analyses for correlations were performed by calculating the Pearson’s R. Two-sided P-values <0.05 were considered statistically significant. All analyses were performed using IBM SPSS Statistics for Windows version 20.0 (IBM Corp, Armonk, NY, USA).
Supplemental Figures and Figure Legends

Online Figure I. Arteriolar staining in BM.

Microscopic photographs of human BM from control (A), CLI-DM+ (B) and CLI-DM- (C) donors, stained for arteriolar marker α-SMA.

Online Figure II. PGP9.5-positive arterioles in BM.

Microscopic photographs of human control (A, C) and CLI (B, D) BM samples with α-SMA-positive arterioles (insets in A and B), showing PGP9.5-positive nerve terminals in the arteriolar walls (arrows in C and D). Magnification 100x (A, B) or 400x (C, D).
**Online Figure III.** Positive control stainings for TH and PGP9.5 in spinal marrow (nerve tissue).

Microscopic photographs of human spinal marrow stained for sympathetic nerve marker TH (A) or pan-neuronal marker PGP9.5 (B).

**Online Figure IV.** Negative control stainings for TH and PGP9.5 in BM.

Microscopic photographs of human CLI BM stained with secondary antibody only (A, C) or with antibodies against sympathetic nerve marker TH (B, arrows) or pan-neuronal marker PGP9.5 (D). MK: megakaryocytes.
Supplemental References


Additional supplemental information

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