Original Research

Electrical Muscle Stimulation Induces an Increase of VEGFR2 on Circulating Hematopoietic Stem Cells in Patients With Diabetes

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ABSTRACT

Purpose: External electric muscle stimulation (EMS) of the thigh muscles was found to reduce pain resulting from diabetic neuropathy (DN), a vascular complication of diabetes. This study investigated circulating hematopoietic stem cells (HSCs) after EMS treatment. Impaired function of HSCs and the subpopulation endothelial progenitor cells (EPCs), important for neovascularization and endothelial repair, has been associated with DN.

Methods: Twenty-four patients with painful DN were treated 3 times with EMS over a period of 1 week. Blood samples were collected before and after the first EMS treatment. Before a fourth treatment, neuropathic pain was evaluated and a third blood sample was collected. Cells were used for flow cytometry.

Findings: Patients with painful DN reported that the pain decreased after 3 times of 1-hour treatments with EMS (Neuropathy Symptom Score: from 8 to 6, $P = 0.001$; Neuropathy Disability Score: from 5.5 to 5, $P = 0.027$, n = 24). At the end of the study, diastolic blood pressure had decreased from 80 to 70 mm Hg ($P = 0.043$), and plasma adrenaline and noradrenaline metabolites metanephrine and normetanephrine were reduced (both $P \leq 0.01$; n = 21). A single EMS treatment caused an immediate and transient decrease in the frequency of $CD34^+$ HSCs in circulation (–20%; $P < 0.001$; n = 27). In 9 of the patients with DN, the proportion of HSCs expressing vascular endothelial growth factor receptor 2 (VEGFR2; defining the HSCs as EPCs) increased by 36% ($P = 0.011$) after EMS treatment. Proteins required for binding to endothelium (junctional adhesion molecule A and CD31), homing toward hypoxic tissue (C-X-C chemokine receptor type 4), and endothelial differentiation (CD31) were increased on HSCs immediately after EMS treatment. An increased frequency of VEGFR2 expression was also observed on HSCs of 6 healthy control volunteers (34%; $P = 0.046$) after EMS treatment, but not after sham treatment.

Implications: Three EMS treatments decreased symptoms of pain caused by DN and reduced diastolic

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blood pressure and biomarkers of stress. A single EMS treatment increased molecules mediating attachment and differentiation on the surface of HSCs in circulation. We hypothesize that the EMS-induced increase in surface attachment molecules on the HSCs caused the HSCs to leave circulation and that EMS treatment improves the function of HSCs and EPCs in vivo. (*Clin Ther*. 2017;39:1132–1144)

Key words: diabetes, neuropathy, pain, stem cells.

INTRODUCTION

Sensory diabetic neuropathy (DN) is a common late complication of diabetes, causing loss of sensation or neuropathic pain in approximately 30% of people with diabetes, making DN one of the most common complications of diabetes.[1] The strategies for treatment and prevention of DN, including lowering of blood glucose, were found to be inefficient, particularly for patients with type 2 diabetes.[2] DN is a microvascular disease, and there is evidence for accumulated damage of the vascular endothelium during diabetes, caused by the combination of reactive oxygen species,[3] inflammation,[4] and toxic metabolites that are increased in the diabetic condition.[5] Defects in endothelial repair mechanisms may also contribute to the development of both microvascular and macrovascular complications of diabetes. A low number of bone marrow–derived, circulating $CD34^+$ hematopoietic stem cells (HSCs) has been reported as a risk factor for cardiovascular complications.[6] Diabetes has been reported to be associated with impaired function and a reduced number of circulating HSCs.[7–12] The lower frequency of HSCs in persons with diabetes has been associated with a higher risk of progress and severity of diabetic vascular complications, including DN[8,13,14] and diabetic foot wounds.[15–18] Different types of HSCs have been used to successfully treat diabetic animal models of DN.[19–23]

HSCs can differentiate into hepatocytes, epithelial cells,[24] cardiomyocytes, smooth muscle cells, or endothelial cells.[25] Differentiation into endothelial cells is associated with the up-regulation of vascular endothelial growth factor receptor 2 (VEGFR2; KDR), after which the HSCs are considered endothelial progenitor cells (EPCs).[26,27] The importance of EPCs for cardiovascular health in diabetes has recently been reviewed elsewhere.[27] Patients with type 1 or type 2 diabetes have been reported to have reduced EPC numbers.[10,28,29] Transferring EPCs can regenerate vessels in animal models with vascular disorders,[30] because of the ability of EPCs to incorporate into hypoxic endothelium and to participate in vessel repair.[31] Hypoxia leads to stabilization of transcription factors, inducing the expression of stromal-derived factor (SDF)-1 from the hypoxic endothelium, which binds C-X-C chemokine receptor type 4 (CXCR4) on the surface of the EPC, leading to adhesion to endothelial cells and new vessel formation.[32] In diabetes, circulating EPCs were found to lack a sufficient responsiveness to hypoxia.[12,33,34]

Another molecule controlling HSC adhesion to the endothelium is the junctional adhesion molecule A (JAM-A).[35–38] JAM-A has two immunoglobulin-like domains and is localized at the tight junction between endothelial cells,[39] where homodimers bind JAM-A on the surface of neighboring cells.[40] On inflammation, JAM-A is distributed to the surface of the endothelium which faces the blood stream and binds infiltrating cells.[41] It was found that JAM-A mediates the binding of HSCs to inflamed shear-stressed endothelium, with differentiation into EPCs in vitro and re-endothelialization in vivo.[35] Expression of JAM-A on HSCs, therefore, provides a hypoxia-independent ability of these cells to adhere to inflamed endothelium.

Platelet endothelial cell adhesion molecule, CD31, is expressed on the surface of endothelial and several hematopoietic cells. CD31 mediates adhesion between adjacent endothelial cells and between endothelium and leukocytes and supports the endothelial transmigration of HSCs.[42] Kim et al[43,44] have suggested that $CD31^+$ cells enhance the vessel-forming activities of HSCs through several different mechanisms, in addition to being just a marker of endothelial differentiation.

It was previously found that external electrical muscle stimulation (EMS) of the thigh muscles leads to reduced pain in patients experiencing painful DN.[45] The aim of this prospective study was to determine the possible underlying mechanisms for the improvement in neuropathic symptoms after EMS treatment. Because low numbers of HSCs are associated with DN, it was hypothesized that the decrease of painful symptoms of DN was due to an improvement of endothelial repair functions by HSCs. A protocol of 4 EMS treatments was performed over 2 weeks, and HSC numbers were assayed immediately before and after an initial EMS treatment and again before the last treatment. It was found that

EMS treatment of patients with diabetes caused a rapid but transient disappearance of HSCs from circulation. This observation would suggest that EMS had affected the ability of the HSC to attach to the endothelial wall. The remaining HSCs indicated an increase in expression of VEGFR2 and molecules mediating adhesion and differentiation on the surface of HSCs. Thus, EMS had an immediate effect on surface markers on HSCs, related to HSC function in patients with diabetes.

PATIENTS AND METHODS

Study Participants

This study was performed in accordance with the Declaration of Helsinki. The study protocol was approved by the University of Heidelberg's Ethics Committee (number S-107/2014), and all participants gave written informed consent to participate in the study. Twenty-eight type 1 and 2 diabetic patients with painful DN were included (see Table I for participant characteristics). Patients with implanted pacemaker or defibrillator or other common causes of neuropathy (ie, alcohol abuse, peripheral artery disease, tumors, or neuropathy-causing medication) were excluded from the study. Healthy control volunteers (5 men and 4 women, 23–40 years of age, with no recorded medical conditions, a body mass index <25), who reported regular physical exercise, received one treatment. The study design was prospective and uncontrolled.

EMS Treatment Protocol

Treatment consisted of four 60-minute treatments with high-frequency external electrical stimulation of the thigh muscles (referred to as EMS) of both legs using the HiTop184 (gbo Medizintechnik, Rimbach, Germany), as previously described.[45] EMS was performed twice per week over a period of 2 weeks between 8 and 9 AM, while the patient was fasting, with at least 2 days between each treatment. For the patients with diabetes, a clinical assessment was made, and blood was drawn directly before the first EMS (baseline), immediately after the first EMS, and before the fourth EMS. The blood samples from the baseline and before the fourth EMS treatment were assessed for changes in clinical parameters as described below. Healthy control volunteers received only the first EMS, according to the same treatment protocol as the patients with diabetes, but no blood samples were taken for assessment of the clinical parameters. All blood samples collected from the healthy control volunteers and patients with diabetes were analysed by flow cytometry.

Laboratory Parameters

Analysis of blood glucose (stored in sodium fluoride), glycosylated hemoglobin (HbA_{1c}), HDL, LDL, triglycerides, creatinine, metanephrine, normetanephrine, complete blood count, urine albumin, and creatinine was performed in the central laboratory at the University Clinic of Heidelberg before the first EMS and before the fourth EMS.

Grading of Neuropathic Symptoms

Neuropathic symptoms were assessed using the Neuropathy Symptom Score and Neuropathy Disability Score.[46] For the numerical rating scale, participants were asked to rate their pain on a scale from 0 (no pain) to 10 (worst pain possible). Neuropathy symptom scoring was assessed before the first treatment and before the fourth treatment (Figure 1).

Isolation of PBMCs

Blood samples (9-mL EDTA tubes) from before and after the first 1-hour treatment were immediately stored at 4°C and the peripheral blood mononuclear cells (PBMCs) were separated, within 2 hours of the first sample drawn, using Biocoll 1.077 g/mL (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. After separation the cells were kept on ice, washed twice in ice-cold saline solution, resuspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing 10% fetal calf serum and 1 mM EDTA), and counted (using a COULTER AcT diff Analyzer; Beckman Coulter, Krefeld, Germany). Cells (10^7) of each sample were stained with antibodies for flow cytometry.

Flow Cytometry

PBMCs of all study participants were incubated on ice with an antibody against CD34- phycoerythrin (PE). PBMCs of a subset of 9 patients with diabetes and 6 healthy control volunteers were also stained with CD184-APC (CXCR4), CD31-BrilliantViolet, CD309-PE/Cy7 (VEGFR2), JAM-A–FITC, or respective isotype controls in FACS buffer. All antibodies were from BioLegend (San Diego, California) and were used at concentrations according to the manufacturer's instructions, and staining was performed within 30 minutes of isolation of the PBMCs. Flow cytometry was performed

Table I. Characteristics of the patients with diabetes and values for clinical parameters at baseline (before first EMS) and at the end of the study (before fourth EMS).

Variable	Before First EMS	Before Fourth EMS	P^*	no.
Age, y	70.5 (15)			
Sex, no.				
Female	12			
Male	16			
Diabetes, no.				
Type 1	4			
Type 2	24			
Years since initial diagnosis	14.5 (13)			
Body mass index, kg/m^2	31.9 (7.1)	32.3 (6.5)	NS	22
Fasting glucose, mg/dL	142.0 (124)	134.0 (92)	NS	24
HbA_{1c}			NS	24
%	7.1	7.1		
mmol/mol	54	54		
GFR, mL/min	79.1 (25.1)	76.6 (21.2)	NS	24
Neuropathy Symptom Score	8 (3)	6 (4)	0.001	24
Neuropathy Disability Score	5.5 (4)	5.0 (4)	0.027	24
Pain (verbal NRS)	6 (4)	4 (3)	0.026	18
Diastolic blood pressure, mm Hg	80 (15)	70 (16)	0.043	21
Triglycerides, mg/dL	122.5 (89)	146.5 (117)	NS	24
HDL, mg/dL	49.5 (28)	47.0 (27)	NS	24
LDL, mg/dL	101 (41)	99 (36)	0.033	23
Metanephrine, pg/mL	34.3 (14.4)	28.0 (18.6)	0.006	24
Normetanephrine, pg/mL	71.6 (48.6)	52.5 (32.2)	0.007	24
Albumin (urine), mg/L	26.1 (60.1)	20.2 (30.7)	NS	23

Values are median (IQR; quartile 3 - quartile 1, i.e. the range within which 50% of the values are found).
EMS = electric muscle stimulation; GFR = glomerular filtration rate; HbA_{1c} = glycosylated hemoglobin; NRS = numerical rating scale.
*Statistical error analysis was performed with 2-tailed Wilcoxon's signed-rank test.

the same day on either BD FACS LSR II or FACS Aria II SORP using Diva software (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Cytometer Setup & Tracking Beads Kit (Becton, Dickinson and Company) was used before each analysis, according to the manufacturer's instructions.

Flow Cytometric Analysis

Flow cytometric detector voltage settings were kept constant throughout the experiments. Samples before and after the first EMS treatments were stained in parallel, using the same antibody master mix and analyzed consecutively in the same session, using the same gates, and gates were adjusted for the sample taken before the fourth EMS treatment, if needed. Compensation was adjusted to fit all three time points for each study participant, based on single stains done at each flow cytometric session. EPCs were defined as $CD34^+VEGFR2^+$.[27] At least 5×10^6 leukocytes were recorded per sample.

Statistical Analysis

GraphPad Prism version 6.05 for Macintosh (GraphPad Software, San Diego, California) was used to visualize experimental data and to calculate variation. Outliers (4 × interquartile range) were included in all

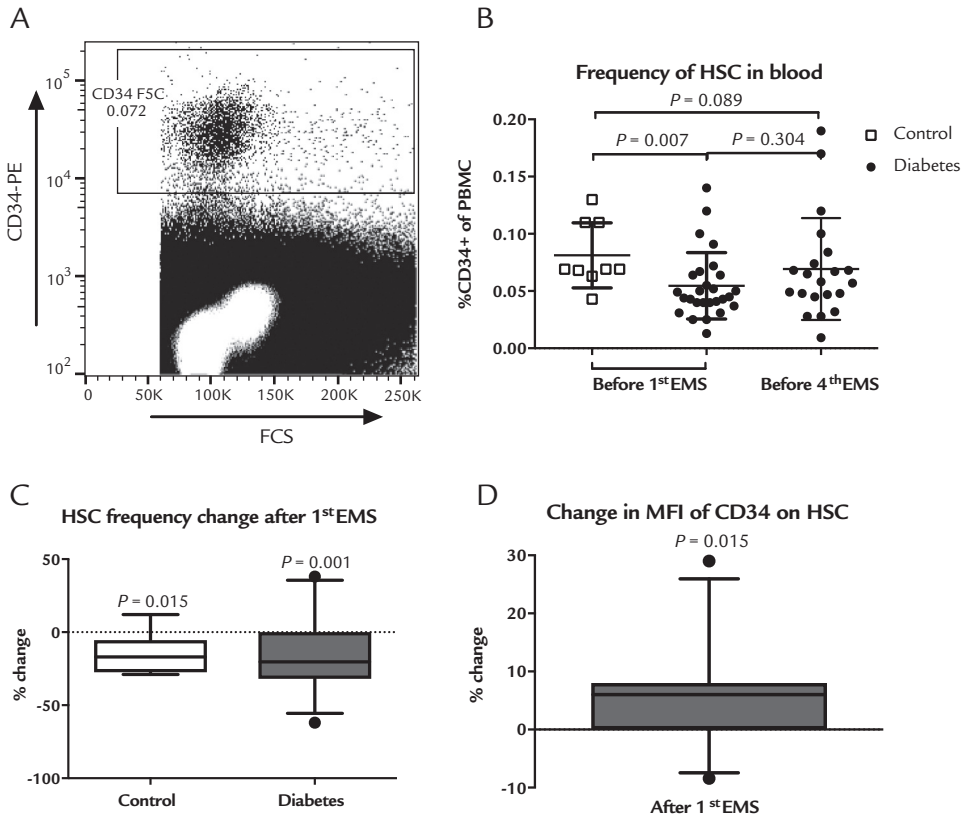

Figure 1. EMS treatment resulted in an immediate and transient reduction in the frequency of HSCs in blood. (A) Representative flow cytometric dot plot of $CD34^+$ HSCs in leukocytes versus forward scatter (FSC). (B) Frequency of HSCs in circulating leukocytes in 9 healthy control volunteers (white boxes) and in patients with diabetes (black circles) before the first EMS treatment (n = 27) and in patients with diabetes before the fourth EMS treatment (n = 22). (Statistical error analysis was performed with 2-tailed Mann-Whitney *U* test.) (C) Change in frequency of HSCs between samples taken immediately before and after the first EMS treatment of 9 healthy control volunteers (white box) and 27 patients with diabetes (gray box). (D) Change in mean fluorescence intensity (MFI) of CD34 on HSCs of patients with diabetes after the first EMS treatment (n = 27). Boxes indicate 25th to 75th percentile, whiskers indicate 5th to 95th percentile, and the line indicates median. Statistical error analysis was performed with 2-tailed Wilcoxon's signed-rank test. EMS = electric muscle stimulation; HSC = hematopoietic stem cell; PBMC = peripheral blood mononuclear cell; PE = phycoerythrin.

statistical analysis but excluded from some graphs according to figure legends (outlier exclusion or inclusion did not alter statistical significances). IBM SPSS statistics version 22 (IBM Inc, Armonk, New York) was used for statistical analysis. Changes over time were calculated using 2-tailed Wilcoxon's signed-rank test for paired samples. Two-tailed Mann-Whitney *U* test was used for comparison of unpaired samples to analyze differences between the control and the diabetic group. Correlations were analyzed using the nonparametric Spearman's ρ model for bivariate correlations.

RESULTS

Of the 28 study participants with painful DN recruited, 24 completed the four EMS treatments, and 23 of the patients exhibited an improvement in at least one measure of neuropathic symptoms (Table I).

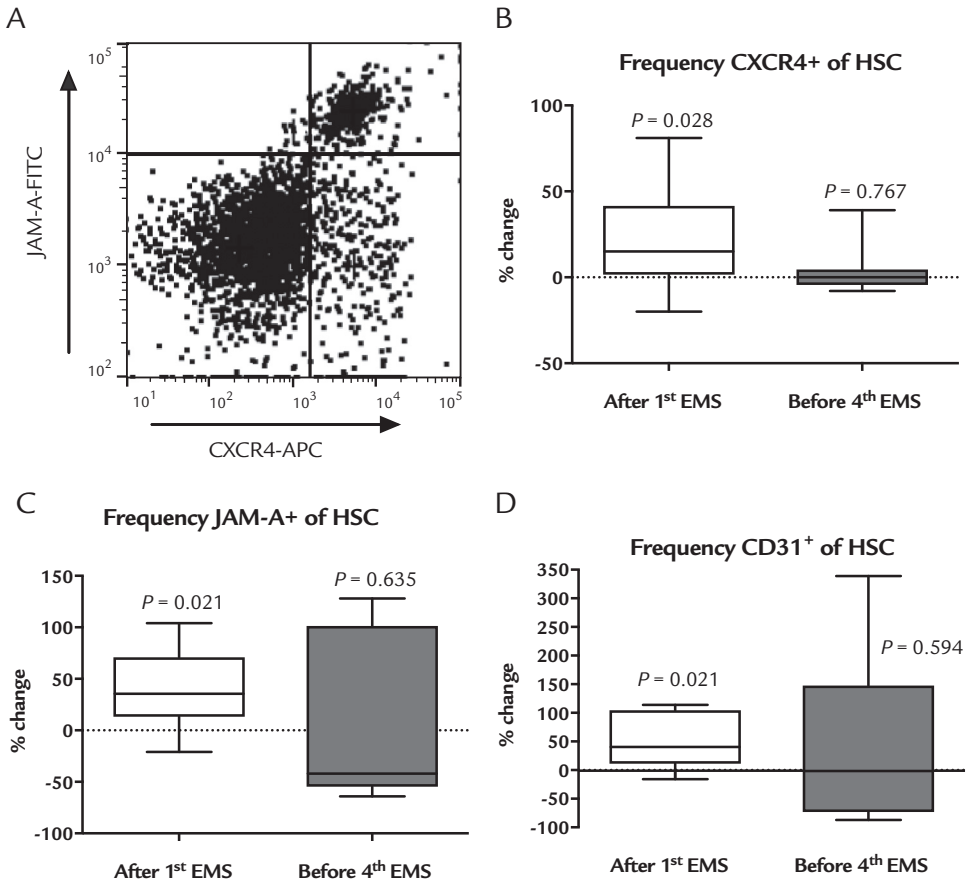

Figure 2. JAM-A and CXCR4 were up-regulated on the surface of HSCs in circulation after EMS treatment of patients with diabetes. (A) Representative flow cytometric dot plot of JAM-A and CXCR4 on HSCs in PBMC. Percentage of change was calculated between the baseline value (before first EMS) and after first EMS (white boxes) or before fourth EMS (gray boxes): change in frequency of HSCs expressing (B) CXCR4, (C) JAM-A (1 outlier excluded from after first treatment and before fourth treatment) or (D) CD31. n = 9. Boxes indicate 25th to 75th percentile, whiskers indicate 5th to 95th percentile, and line indicates median. Statistical error analysis was performed with 2-tailed Wilcoxon's signed-rank test. APC = allophycocyanin; CXCR4 = C-X-C chemokine receptor type 4; EMS = electric muscle stimulation; FITC = fluorescein isothiocyanate; HSC = hematopoietic stem cell; JAM-A = junctional adhesion molecule A; PBMC = peripheral blood mononuclear cell.

The only reported side effects were muscle soreness, 1 to 2 days after the EMS treatment; of the treated patients, 5 reported muscle soreness with severity of symptoms as mild. No significant changes were found in glucose metabolism (as measured by HbA_{1c} and fasting glucose), renal function (as measured by creatinine), or any parameters in the complete blood count. However, diastolic blood pressure decreased significantly between baseline (before first EMS) and before the fourth EMS treatment ($P = 0.043$), as did the levels of adrenaline and noradrenaline metabolites metanephrine ($P = 0.006$) and normetanephrine ($P = 0.007$) in the plasma (Table I). Bivariate correlation analysis revealed an inverse correlation between participants' age and their glomerular filtration rate ($\rho = -0.58$, $P = 0.001$). The age of the participants correlated with the fasting glucose levels ($\rho = 0.394$, $P = 0.042$). Neither plasma antioxidative capacity (see Supplemental Figure 1A in the online version at http://dx.doi.org/10.1016/j.clinthera.2017.05.340)

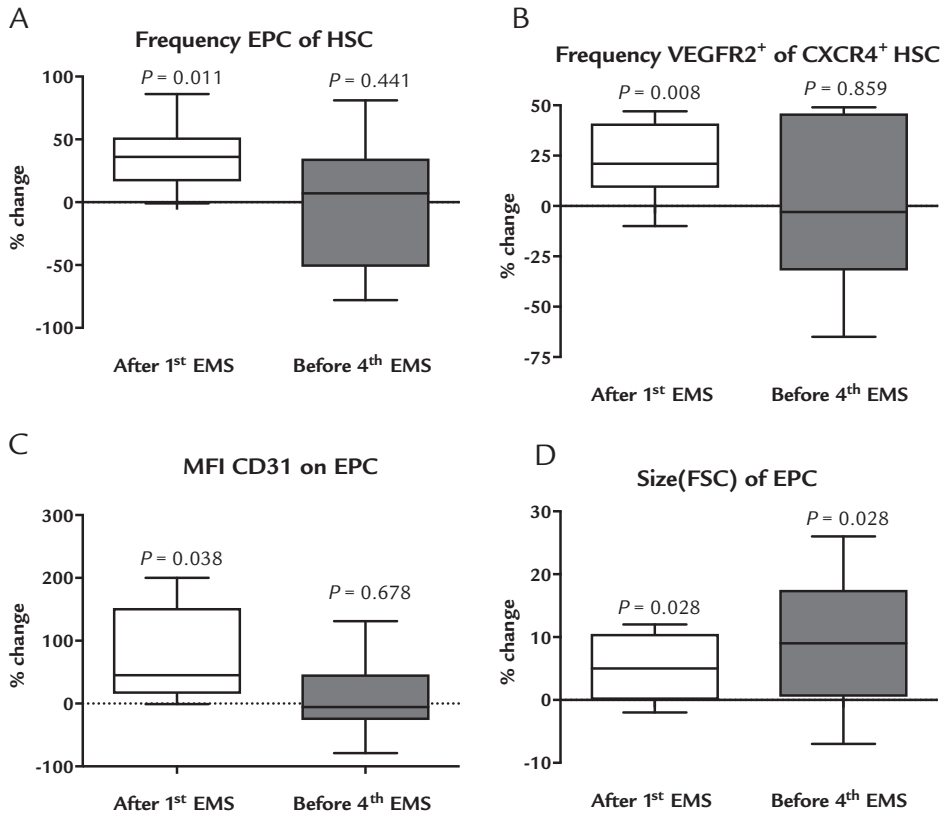

Figure 3. Functional markers of EPC activation were increased on HSCs in EMS-treated patients with diabetes. Percentages of change were calculated between samples taken immediately before and after the first EMS treatment (white boxes) or before the fourth EMS treatment (gray boxes). (A) Change in frequency of HSCs expressing VEGFR2 (ie, frequency of EPCs; 1 outlier excluded before the fourth treatment). (B) Change in frequency of VEGFR2$^+$ on CXCR4$^+$ HSCs. (C) Changes in mean fluorescence intensity (MFI) of CD31 on EPCs. (D) Change in size as measured by forward scatter (FSC) of EPCs. n = 9. Boxes indicate 25th to 75th percentile, whiskers indicate 5th to 95th percentile, and line indicates median. Statistical error analysis was performed with 2-tailed Wilcoxon's signed-rank test. CXCR4 = C-X-C chemokine receptor type 4; EMS = electric muscle stimulation; EPC = endothelial progenitor cell; HSC = hematopoietic stem cell; VEGFR2 = vascular endothelial growth factor receptor 2.

nor the concentration of methylglyoxal changed significantly over the study period (see Supplemental Figure 1B in the online version).

Before the first EMS treatment, an average of 0.55% (25th–75th percentile, 0.40%–0.64%) of PBMCs expressed CD34 on their surface in blood from patients with diabetes, a significantly lower frequency than what was found in 9 younger, healthy control volunteers ($P = 0.007$) (Figure 1A and B). Although no significant difference was found between circulating HSC frequencies at the beginning and at the end of the study (Figure 1B), the first EMS treatment caused a significant and transient decrease in the frequency of circulating HSCs both in patients with diabetes ($P = 0.001$) and in healthy control volunteers ($P = 0.015$) (Figure 1C). After the first EMS treatment, the HSC frequency was reduced to 0.45% (25th–75th percentile, 0.28%–0.45%) in the patients with diabetes. Mean fluorescence intensity (MFI) of CD34 on the remaining HSCs was significantly higher ($P = 0.015$) directly after the first EMS treatment (Figure 1D).

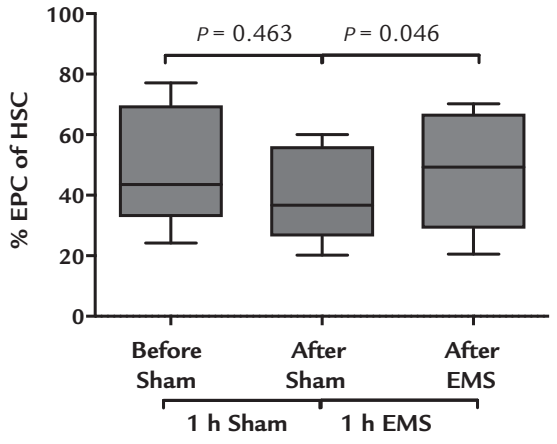

Figure 4. EMS treatment, but not sham treatment, caused an increased frequency of EPCs among the HSCs. Six of the healthy volunteers were recruited to receive a sham treatment according to standard protocol for EMS. Blood was drawn before sham, after sham, and after EMS treatments. Red blood cells were lysed, and leukocytes were immediately stained with anti-CD34 and anti-VEGFR2. Boxes indicate 25th to 75th percentile, whiskers indicate 5th to 95th percentile, and line indicates median. Statistical error analysis was performed with 2-tailed Wilcoxon's signed-rank test. EMS = electric muscle stimulation; EPC = endothelial progenitor cell; HSC = hematopoietic stem cell; VEGFR2 = vascular endothelial growth factor receptor 2.

It was hypothesized that EMS increased adhesion to the vessel endothelium of the more differentiated ($CD34^{low}$) HSCs, explaining both the reduction in circulating HSCs and the increased CD34 expression of the remaining HSCs. To investigate whether EMS caused an increase in molecules mediating binding to vessel walls, the expression of receptors for homing (CXCR4), adhesion (JAM-A), and adhesion and differentiation (CD31) on the surface of the HSCs was measured in 9 of the patients with diabetes (Figure 2). Before the first EMS treatment, a median of 15.2% of the HSCs expressed CXCR4 (25th–75th percentile, 6.6%–21.6%), 5.6% expressed JAM-A (25th–75th percentile, 1.6%–12.9%), and 4.6% expressed CD31 (25th–75th percentile, 0.8%–11.95%). Expression of CXCR4 and JAM-A were mostly overlapping (Figure 2A). The frequencies of HSCs expressing CXCR4 ($P = 0.028$), JAM-A ($P = 0.021$), or CD31 ($P = 0.021$) were significantly increased directly after EMS treatment (Figure 2B, C, and D). Furthermore, JAM-A and CD31 surface expressions on the HSC population, as measured by MFI, were significantly increased ($P = 0.008$ and $P = 0.011$, respectively) (see Supplemental Figures 2A and B in the online version). CD31 MFI specifically on the subpopulations of HSCs expressing CXCR4 or JAM-A was also increased (see Supplemental Figure 2C and D in the online version). VEGFR2, a marker of differentiation on HSCs, defining the cell as an EPC,[26,27] was also increased on the surface of HSCs after the first EMS treatment (Figure 3A). Before the first treatment a median of 7.5% of the $CD34^+$ HSCs expressed VEGFR2 (25th–75th percentile, 5.3%–13.3%), and after treatment the frequency of HSCs expressing VEGFR2 increased significantly ($P = 0.011$) to 8.5% (25th–75th percentile, 7.0%–22.8%). The frequency of $VEGFR2^+$ in the CXCR4-expressing HSC population, capable of homing to hypoxic tissue, was also increased by EMS ($P = 0.008$) (Figure 3B). MFI of CD31 was increased on the EPCs after the first EMS treatment ($P = 0.038$) (Figure 3C), as a measure of EPC differentiation. Although all other changes were observed only immediately after the first EMS treatment, an enlargement of the EPCs remained significant ($P = 0.028$) also before the fourth EMS treatment (Figure 3D).

To investigate if the increase of EPC frequency in the HSC population was specific to EMS, 6 of the healthy control volunteers recruited from previous experiments (Figure 2) underwent a sham treatment (a power calculation based on the results in Figure 3 indicated that 6 treated participants would provide a power of 80% for a significant increase of VEGFR2 on the surface of HSCs). For an hour the test participants sat still with electrodes without current, a sample was drawn, and thereafter they were immediately treated with EMS. A significant increased frequency of EPCs among the HSCs was observed after the EMS treatment ($P = 0.046$) but not after sham treatment ($P = 0.463$) (Figure 4).

DISCUSSION

In a previous observational study it was reported that EMS can decrease neuropathic symptoms in patients experiencing DN.[45] Although it is not possible to perform a blinded control study of the treatment because of the nature of the EMS, which would have been required to assess the previous observation, patients experiencing DN have continued to be treated with EMS. In this prospective study, which aimed to investigate the changes in different factors related to the improvement of the pain symptoms of DN, it was found that EMS also caused a reduction of HSCs in circulation. This reduction of HSCs in circulation was observed only immediately after treatment in both patients with diabetes and healthy control volunteers. Although direct evidence to indicate that HSCs are exiting from the circulation by attaching to the vessel wall is lacking, it was found that the molecules required for homing, adhesion, and differentiation are increased by EMS on HSCs of patients with diabetes. EMS caused an increase in the proportion of HSCs expressing VEGFR2, the marker for EPCs, both in patients with diabetes and in a small group of young, healthy control volunteers. This increase was observed specifically after EMS treatment and not after a sham treatment.

CD34 is a marker of progenitor activity, which is lost on differentiation.[47] It was hypothesized that the higher MFI of CD34 observed on the HSCs remaining in circulation after EMS may reflect that primarily more differentiated $CD34^{low}$ HSCs had left the circulation. The possibility that EMS causes a blockage of HSC release from bone marrow into circulation was also considered. However, the 1-hour interval between the before and after EMS would be too short a time span for a restriction of HSC release from bone marrow to be evident, assuming that the half-life of HSCs in circulation is about 14 hours.[48]

Increases of VEGFR2 and CD31 on the HSCs remaining in circulation indicated that EMS treatment had rendered these cells more capable of differentiating into endothelial cells. Furthermore, the size of the EPC was increased, an independent indication of differentiation of the EPC.[49] The increased size of the EPC was the only significant change to the HSCs still observed before the fourth EMS treatment ($P = 0.028$). Induction of molecules essential for homing, adhesion, and differentiation on HSCs were found to be strictly transient in this study. This is not surprising, because up-regulation of these molecules directly or indirectly confer attachment to the vessel endothelium and additional differentiation,[35] causing extravasation of the cells. The increase in CXCR4 on the surface of HSCs of the patients with diabetes indicated that these cells became more capable of neovascularization in response to a hypoxia-induced SDF-1 gradient,[32] possibly counteracting the defects of HSCs from diabetic individuals.[10,12] In particular, $CXCR4^{+}$ HSCs exhibited an increased frequency of VEGFR2 expression. The observed up-regulation of JAM-A may also indicate that the HSCs of diabetic patients are more capable of binding to inflamed endothelium.[35]

The reduction of HSC frequency in circulation was transient, which is important, because a reduced frequency of HSCs has been associated with diabetes complications.[13] Although the baseline frequency of HSCs in the blood of patients with DN was significantly lower than that of the control participants ($P = 0.007$), the HSC frequency was no longer significantly different from baseline of control participants, when measured before the fourth EMS treatment ($P = 0.089$). Although the changes in expression of surface markers are indicators of changes in HSC biology, functional assays in vitro are required to determine to what extent EMS affects the ability of the HSC to differentiate into vascular tissue.

There was no evidence that the changes observed in neuropathy symptoms and HSC activation were directly related; there were no significant correlations between the clinical data and the changes observed in the HSC population. This lack of correlation may be due to the small number of participants analysed for HSC differentiation ($n = 9$) and that HSC activation may not be equally beneficial for all types of painful DN. The beneficial effect of the EMS treatment on symptoms of DN was not due to changes in plasma methylglyoxal or antioxidant capacity, both of which have been reported to be mediators of DN and pain.[50–53] Neither did EMS provide any short-term effect on glucose metabolism or renal function.

The scorings for neuropathic symptoms used in this study are subjective; however, the EMS treatment was also observed to decrease the diastolic blood pressure and the plasma concentrations of metanephrine and normetanephrine, metabolites of adrenaline and noradrenaline, respectively. A decrease in these stress parameters could be a direct consequence of the relief of pain and neuropathic symptoms. The reduced

plasma levels of normetanephrine could be the cause of the reduced diastolic blood pressure, which could also be a result of neovascularization; however, further studies would be required to validate this finding.

There are several possibilities for the mechanism of EMS-induced maturation in HSCs, such as the generation of a local ischemic/hypoxic environment through increased oxygen consumption in the contracting muscle. Hypoxic conditions were found to enhance the ability of stem cells to differentiate into EPCs in vitro.[54] However, HSCs of individuals with diabetes have an impaired ability to respond to hypoxia.[12] It is also unlikely that local hypoxia of the thigh would cause the effects observed on circulating HSCs, because hypoxia-inducible factor 1-α is rapidly degraded[32] as HSCs re-enter the normoxic circulation. It is also possible that EMS causes release of irisine, a myokine induced by exercise, which was recently reported to enhance migration and proliferation of EPCs in mice.[55]

A plausible mechanism would be that maturation of HSCs is voltage regulated. HSCs were found to express mRNA for voltage-gated potassium channels Kv1.3, Kv7.1,[56] and Kv2.1,[57] rendering these cells responsive to voltage-gated potassium efflux, which results in calcium influx.[58] Electrical pulsed currents activate homing of EPCs and other progenitor cells to the site of injury in vivo and thereby increase perfusion and drive regeneration of hypoxia-damaged tissue.[59] A mechanism for voltage-gated stem cell activation would allow for the specific activation of circulating cells, passing through the heart in a physiologic context, but not the dividing stem cell reservoir in bone marrow. Mesenchymal stem cells (MSCs) are also known to respond to electric stimuli, resulting in activation and support of nerve generation.[59,60] JAM-A was recently found to promote MSC homing to and wound-healing cytokine secretion in murine skin wounds.[61] Further studies are required to determine whether the regulation of JAM-A in response to EMS on MSCs is similar to that of HSCs.

To the best of our knowledge, this is the first study suggesting that EMS may be an efficient and non-invasive method for in vivo activation of HSCs in individuals with diabetes. However, because of the small size further experiments are required, including independent replications, to truly validate the potential for EMS to activate HSCs. Nevertheless, our finding indicates that the EMS activates the migratory capabilities of the steady state HSCs of healthy participants,[62] independently of vessel injuries and is sufficient for extravasation. The treatment is acting rapidly, well tolerated, and inexpensive. Although the improvement of neuropathy symptoms and the activation of HSCs may be purely coincidental, the ability to activate stem cells in vivo in patients with diabetes may be of value for other vascular complications of diabetes such as cardiovascular disease. If the EMS-mediated stem cell activation also applies to MSCs, this treatment may be beneficial for promoting healing of diabetic foot wounds, which is currently under investigation. EMS treatment, in combination with strategies for increasing numbers of circulating HSCs, may be a strategy to enhance the efficiency of stem cell therapies in individuals with diabetes or with cardiovascular complications.

EMS treatment has been reported to be an effective treatment to alleviate symptoms of DN. In this study we report that it increases the proportion of HSCs expressing VEGFR2 and other functional markers. We hypothesize that EMS treatment improves the function of HSCs, which promotes tissue regeneration and reduces symptoms of painful DN. EMS treatment may, as a complement to pharmacologic treatments, be a tool to improve the functionality of EPCs in patients at risk of cardiovascular disease.

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Availability of data and materials: The data sets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author contributions: A. Hidmark designed the flow cytometry analysis, performed the flow cytometry data analysis, made figures and wrote the article. I. Spanidis performed the EMS-treatments, collected clinical data, performed the flow cytometry analysis, performed the statistical analysis, made figures and contributed to writing the article. T. Fleming designed the study, performed the measurements for methylglyoxal, analysed the findings and contributed to writing the article. N. Volk performed the plasma measurements of antioxidant capacity. V. Eckstein performed and supervised flow cytometry analysis. J. Gröner participated in the study design, recruitment of study subjects. S. Kopf participated in the study design, recruitment of study subjects and data analysis. P. Nawroth participated in the study design, recruitment of study subjects, data interpretation and contributed with important intellectual content in the revision of the article. D. Oikonomou designed the study (i.e. wrote the application for the ethical approval), recruited study subjects, participated in data analysis and interpretation. All authors contributed with final approval of the version to be published.

CONFLICTS OF INTEREST

The authors declare that they have no financial interests in the outcome of this study. The manufacturer of the HiTop equipment was not informed of this study in advance. The study sponsors (see Funding section) had no influence over data collection, analysis, or the manuscript. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

SUPPLEMENTARY MATERIAL

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SUPPLEMENTARY MATERIAL

Determination of Antioxidant Capacity

Supplementary Figures S1 and S2.

Antioxidant capacity was based on the capacity of the plasma to inhibit the quenching of the fluorescence signal of fluorescein by 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH; Sigma-Aldrich Cat. 440914) as previously described [Ou, B., Hampsch-Woodill, M. & Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J. Agric. Food Chem. 49, 4619–26 (2001)]. A calibration curve was constructed using the vitamin E analogue, Trolox (Sigma-Aldrich Cat. 238813), and the results were normalized to total protein concentration, as measured by the Bradford assay.

Determination of Plasma Methylglyoxal

The concentration of methylglyoxal in the plasma was determined by liquid chromatography tandem mass spectroscopy following derivatization with 1,2-diaminobenezene, as described previously [Rabbani, N. & Thornalley, P. J. Measurement of methylglyoxal by stable isotopic dilution analysis LC-MS/MS with corroborative prediction in physiological samples. Nat. Protoc. 9, 1969–79 (2014).]

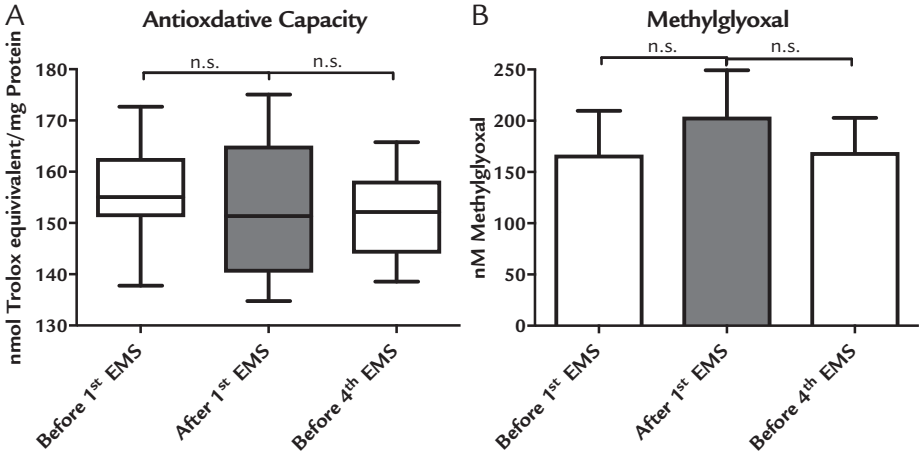

Figure S1. *EMS-treatment of diabetic patients did not significantly alter. plasma anti-oxidative capacity.* Plasma from diabetes patients immediately before and after the first EMS-treatment was used to measure antioxidant capacity (Boxes show 25-75 percentile, whiskers show 5-95 percentile, line indicates median) (n=9) (*a*), or methylglyoxal (bars show average, error bars show standard deviation) (n=10) (*b*). Measurement after treatment is depicted in grey bars. Statistical error analysis was performed with 2-tailed Wilcoxon's signed ranks test. ns=not significant.

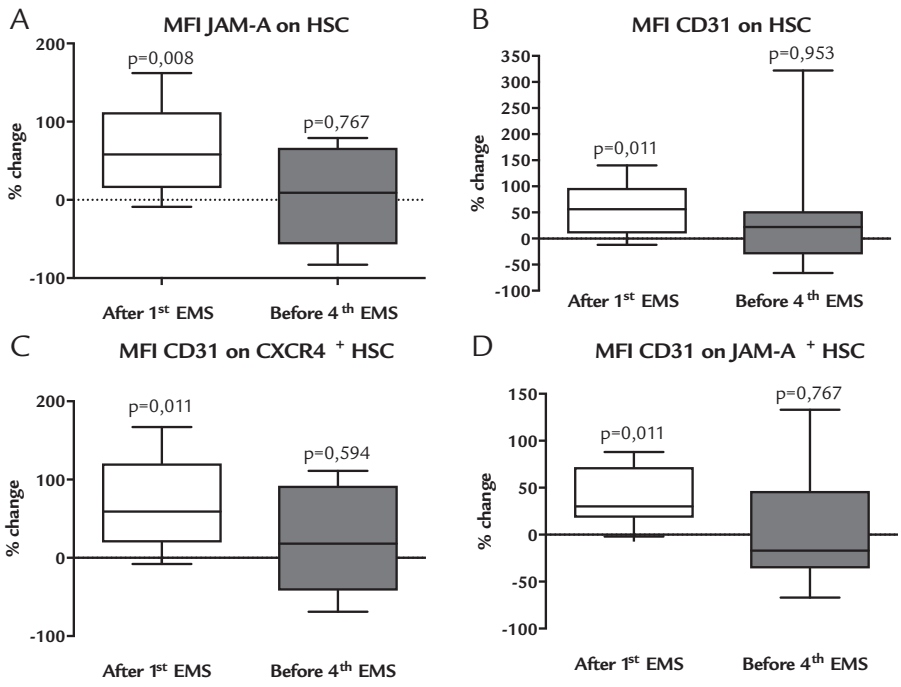

Figure S2. *Expression of endothelial adhesion molecule CD31 and JAMA increased in EMS-treated individuals with diabetes*. Changes of surface expression on HSC between samples taken immediately before and after 1st the first EMS (white boxes) or before 4th EMS (grey boxes): Changes in Mean Fluorescence Intensity (MFI) of surface expression of JAM-A (1 outlier excluded from before 4th treatment) (a) or CD31 (b). Change of MFI of CD31 on CXCR4^{+} HSC (c) or on JAM-A^{+} HSC (d). Per cent change is calculated between the baseline value (Before 1st EMS) and After 1st EMS or Before 4th EMS. Boxes show 25-75 percentile, whiskers show 5-95 percentile, line indicates median. Statistical error analysis was performed with 2-tailed Wilcoxon's signed ranks test.