

Number of circulating pro-angiogenic cells, growth factor and anti-oxidative gene profiles might be altered in type 2 diabetes with and without diabetic foot syndrome

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Keywords

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ABSTRACT

Aims/Introduction: Type 2 diabetes is often complicated by diabetic foot syndrome (DFS). We analyzed the circulating stem cells, growth factor and anti-oxidant gene expression profiles in type 2 diabetes patients without or with different forms of DFS.

Materials and Methods: Healthy volunteers ($n = 13$) and type 2 diabetes patients: (i) without DFS ($n = 10$); or with (ii) Charcot osteoneuropathy ($n = 10$); (iii) non-infected ($n = 17$); (iv) infected ($n = 11$); and (v) healed ulceration were examined ($n = 12$). Peripheral blood endothelial progenitor cells (EPC), mesenchymal stem cells (MSC), hematopoietic stem cells (HSC) and very small embryonic-like (VSEL) cells were phenotyped using flow cytometry. Plasma cytokine concentrations and gene expressions in blood cells were measured by Luminex and quantitative real-time polymerase chain reaction assays, respectively.

Results: Patients with non-complicated type 2 diabetes showed reduced *HMOX1* expression, accompanied by *HMOX2* upregulation, and had less circulating EPC, MSC or HSC than healthy subjects. In contrast, VSEL cells were elevated in the type 2 diabetes group. However, subjects with DFS, even with healed ulceration, had fewer VSEL cells, more CD45-CD29⁺CD90⁺MSC, and upregulated *HMOX1* when compared with the type 2 diabetes group. Patients with Charcot osteopathy had lowered plasma fibroblast growth factor-2. Elevated plasma tumor necrosis factor- α and decreased catalase expression was found in all diabetic patients.

Conclusions: Patients with type 2 diabetes and different forms of DFS have an altered number of circulating stem cells. Type 2 diabetes might also be associated with a changed plasma growth factor and anti-oxidant gene expression profile. Altogether, these factors could contribute to the pathogenesis of different forms of DFS.

INTRODUCTION

Diabetic foot syndrome (DFS), which includes ulcerations, infections and Charcot osteoneuropathy, is a frequent cause of amputations in diabetic patients. The pathogenesis of DFS, in

addition to well established risk factors; that is, long diabetes duration and chronic hyperglycemia¹, could be associated with alterations in stem or progenitor cell mobilization², changes in growth factors^{3,4} and impairment in cellular anti-oxidant capacity^{5,6}; however, data supporting this hypothesis are scarce. Even less is known about how these factors might influence different forms of DFS, especially when the ulcer is further complicated with infection or when it is healed.

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The number of smooth muscle or endothelial progenitors in peripheral blood (PB) is altered in diabetic patients, which might result in reduced vascular repair capacity⁷. Of note, type 2 diabetes also impairs endothelial progenitor cell proliferation, migration and the ability to incorporate to the pre-existing blood vessels^{8,9}. Furthermore, other populations, such as mesenchymal stem cells (MSC) or hematopoietic stem cells (HSC), which can contribute to revascularization and wound healing^{10–13}, can be affected. Mesenchymal stem cells, which are precursors of connective tissue cells; that is, fibroblasts, osteoblasts and chondrocytes¹⁴, are of special interest in the Charcot foot that affects both bones and joints¹⁵. Additionally, the role of very small embryonic-like (VSEL) cells, claimed to be pluripotent and mobilized in stroke, severe burns and acute myocardial infarction¹⁶, has not yet been studied in the complications of type 2 diabetes.

We compared the circulating stem cell subpopulations, growth factor, and anti-oxidant gene expression profiles in type 2 diabetes patients with and without specific clinical forms of diabetic foot syndrome.

METHODS

Patients

We examined healthy volunteers (H; $n = 13$) and five groups of type 2 diabetes patients: (i) without DFS (type 2 diabetes, $n = 10$); (ii) with Charcot peripheral osteoneuropathy ($n = 10$); (iii) with non-infected ulceration (DFU; $n = 17$); (iv) with infected ulceration (DFU-I; $n = 11$); and (v) with healed ulceration (DFU-H; $n = 12$). The diagnosis of infection was based on clinical examination. We used the perfusion, extent or size, depth of tissue loss, infection, sensation or neuropathy (PEDIS) scale criteria¹⁷. The infected ulceration was diagnosed only in the case of sure and typical signs of infection, such as the presence of necrotic tissues, and/or purulent exudation and odor. All patients had at least moderate infection (grade 2 PEDIS). The non-infected ulceration was diagnosed in patients without any clinical signs of infection (grade 1 PEDIS). The characteristics of the patients are shown in Table S1. The research complied with the Declaration of Helsinki and was approved by the local ethics committee. Patients provided written informed consent for the study.

Blood Sample Collection

Blood samples were collected in three 5.0-mL ethylenediaminetetraacetic acid tubes (Sarstedt, Numbrecht, Germany) at the Clinic of Metabolic Diseases at 08.00–09.00 h after overnight fasting, and processed within 1 h. Plasma levels of glucose, total cholesterol, blood urea nitrogen, total bilirubin and aspartate aminotransferase (GOT/AST) were measured with SpotChem Panel I Multi-parameter Strips, using a SpotChem EZ SP-4430 (Arkray, Amstelveen, the Netherlands) analyzer. Plasma was transferred to the 1.5-mL tubes and frozen after the centrifugation for 10 min at 670 g. Total nucleated cells (TNCs) were obtained from the blood samples after the double ammonium

chloride red blood cell lysis (0.15 mol/L NH_4Cl , 10 mmol/L KHCO_3 , 0.1 mmol/L ethylenediaminetetraacetic acid) and resuspended in autoMACS Running Buffer (Miltenyi Biotec, Auburn, CA, USA) supplemented with 2% of foetal bovine serum (Lonza, Basel, Switzerland).

Analysis of Cell Subpopulations

Progenitor and stem cells in peripheral blood were evaluated using flow cytometry. The following subpopulations were analyzed based on their surface markers: (i) EPC: $\text{CD45}^{\text{dim}}\text{CD31}^+\text{CD133}^+$ and $\text{CD45}^{\text{dim}}\text{CD31}^+\text{CD34}^+\text{KDR}^+$; (ii) MSC: $\text{CD45}^-\text{CD105}^+\text{STRO1}^+$ and $\text{CD45}^-\text{CD29}^+\text{CD90}^+$; and (iii) HSC: $\text{Lin}^-\text{CD45}^+\text{CD133}^+$ and $\text{Lin}^-\text{CD45}^+\text{CD34}^+$. Additionally, we evaluated the $\text{Lin}^-\text{CD45}^-\text{CD133}^+$ and $\text{Lin}^-\text{CD45}^-\text{CD34}^+$ cells, described as VSEL. Peripheral blood TNC were stained using fluorescently conjugated antibodies: $\text{CD45}^{\text{dim}}\text{CD31}^+\text{CD133}^+$ cells were stained with anti-CD45-FITC (clone HI30; BD Biosciences, San Diego, CA, USA), anti-CD31-PE (clone WM59; Biolegend, San Diego, CA, USA), anti-CD133/AC133-APC (clone AC133; Miltenyi Biotec), $\text{CD45}^{\text{dim}}\text{CD31}^+\text{CD34}^+\text{KDR}^+$ with anti-CD45-FITC (clone HI30; BD Biosciences), anti-CD31-PE (clone WM59; Biolegend), anti-CD34-PE-Cy5 (clone 581; BD Pharmingen, San Diego, CA, USA), anti-KDR(CD309)-APC (clone Avas12; Biolegend), $\text{Lin}^-\text{CD45}^+\text{CD133}^+$ and $\text{Lin}^-\text{CD45}^-\text{CD133}^+$ anti-CD45-PE (clone HI30; BD Pharmingen), anti-CD133/AC133-APC (clone AC133; Miltenyi Biotec), $\text{Lin}^-\text{CD45}^+\text{CD34}^+$ and $\text{Lin}^-\text{CD45}^-\text{CD34}^+$ with anti-CD45-PE (clone HI30; BD Pharmingen), anti-CD34-APC (clone 581; BD Pharmingen) and a cocktail of antibodies for hematopoietic lineage markers (Lin) anti-CD2-FITC (clone RPA2.10), anti-CD3-FITC (clone UCHT1), anti-CD14-FITC (clone M5E2), anti-CD16-FITC (clone 3G8), anti-CD19-FITC (clone HIB19), anti-CD24-FITC (clone ML5), anti-CD56-FITC (clone NCAM16.2), anti-CD66b-FITC (clone G10F5), anti-CD235a-FITC (clone GA-R2[HIR2]), all from BD Pharmingen; $\text{CD45}^-\text{CD105}^+\text{STRO1}^+$ cells were stained with anti-CD45-FITC (clone HI30; BD Biosciences), anti-CD105-PE (clone 43A3; Biolegend), anti-STRO1-APC (clone STRO-1; Biolegend), $\text{CD45}^-\text{CD29}^+\text{CD90}^+$ with anti-CD45-FITC (clone HI30; BD Biosciences), anti-CD29-PE-Cy5 (clone MAR4; BD Pharmingen) and anti-CD90-PE (clone 5E10; Biolegend). Cells were stained for 30 min at 4°C, then washed with phosphate-buffered saline, collected on BD LSR II flow cytometer (Becton Dickinson, San Diego, CA, USA) and analyzed with BD FACS Diva software (Becton Dickinson). Number of cells per 1 mL of peripheral blood (PB) was calculated based on the total leukocyte count (WBC, 10^3 cells per 1 mL of PB) and the percentage of each population within the collected events. Gating strategies for flow cytometry are shown on Figures S1–S3.

Analysis of Gene Expression

Total ribonucleic acid was isolated from the peripheral blood nucleated cells with phenol–chloroform extraction, and reverse

transcribed with the oligo(dT) primers and RevertAid reverse transcriptase (Fermentas; Thermo Fisher Scientific, Waltham, MA, USA). The expression of heme oxygenase-1 (*HMOX1*), heme oxygenase-2 (*HMOX2*), Cu/Zn superoxide dismutase (*SOD1*), catalase (*CAT*), nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1 (*NQO1*) in total nucleated cells (TNCs) was assessed with quantitative real-time polymerase chain reaction, which was carried out in the StepOnePlus system (Applied Biosystems, Foster City, CA, USA) with the specific primers (Table S2), 50 ng of complementary deoxyribonucleic acid and SYBR Green Quantitative RT-PCR kit (Sigma-Aldrich, St. Louis, MO, USA), under conditions summarized in the Table S3.

Analysis of Growth Factor Concentrations

Concentrations of stem cell factor (SCF), leukemia inhibitory factor (LIF), thrombopoietin (TPO), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), fms-like tyrosine kinase-3 ligand (Flt-3-L), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor- α (TNF- α), and stromal cell-derived factor-1 α and β (SDF-1 α + β) in plasma were evaluated using Milliplex FlexMap 3D (Millipore, Billerica, MA, USA) according to the vendor's protocol.

Analysis of Plasma Total Anti-oxidant Capacity

Plasma total anti-oxidant capacity was measured with the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS) assay according to the protocol by Erel¹⁸. Briefly, 5 μ L of plasma was mixed with 200 μ L of 0.4 mol/L acetate buffer, pH 5.8, 20 μ L of 10 mmol/L ABTS⁺ in 30 mmol/L acetate buffer pH 3.6 and incubated for 5 min at room temperature. Absorbance at $\lambda = 660$ nm was read using the plate reader. Trolox was used for the standard curve.

Statistical Analysis

Normality of data was checked with the D'Agostino–Pearson omnibus test. Statistical significance was assessed with the Mann–Whitney *U*-test and accepted at $P < 0.05$ level. All data obtained was correlated using Spearman's rank correlation.

RESULTS

Circulating Stem and Progenitor Cells

The number of EPC defined as CD45^{dim}CD31⁺CD133⁺ (Figure 1a) or CD45^{dim}CD31⁺CD34⁺KDR⁺ (Figure 1b) was decreased in type 2 diabetes patients in comparison with healthy controls, and in general was not further modified by diabetic foot syndrome forms. The exemption was infected diabetic foot ulcer, which led to an increase in the CD45^{dim}CD31⁺CD34⁺KDR⁺ subpopulation ($P = 0.01$ for DFU-I vs DFU). Both EPC subpopulations correlated negatively with glycated hemoglobin (HbA_{1c}) (CD45^{dim}CD31⁺CD133⁺ Spearman's $r = -0.29$, $P = 0.014$, CD45^{dim}CD31⁺CD34⁺KDR⁺ Spearman's $r = -0.47$, $P < 0.0001$), although only in the DFU-H group HbA_{1c} was lower than in the type 2 diabetes and

DFU groups ($P = 0.0393$ and $P = 0.0375$, respectively). CD45⁻CD29⁺CD90⁺ MSC were less numerous in type 2 diabetes and Charcot osteoneuropathy patients than in healthy volunteers (Figure 1c), whereas in groups with infected or healed ulcers their numbers increased to the control values. However, a similar pattern without an increase in the DFU-I group was observed for MSC defined as CD45⁻CD105⁺STRO1⁺ (Figure 1d). Patients with type 2 diabetes either with or without DFS had also less Lin⁻CD45⁺CD133⁺ HSC (Figure 1e). Differences for Lin⁻CD45⁺CD34⁺ HSC were similar, but less pronounced (Figure 1f).

The number of Lin⁻CD45⁻CD34⁺ VSEL cells was increased in type 2 diabetes and Charcot osteoneuropathy patients ($P < 0.01$ and $P < 0.05$ vs the healthy group, respectively; Figure 1g) and correlated positively with HbA_{1c} (Spearman's $r = 0.32$, $P = 0.007$). This rise was weaker in diabetic patients with foot ulceration, especially in the DFU-I and DFU-H groups. The Lin⁻CD45⁻CD133⁺ VSEL population was also less numerous in comparison with the non-complicated type 2 diabetes group in all groups with DFS (Figure 1h). Of note, populations of endothelial progenitor cells, mesenchymal stem cells and hematopoietic stem cells/progenitors in general correlated positively with each other. Interestingly, numbers of Lin⁻CD45⁻CD133⁺ and Lin⁻CD45⁻CD34⁺ VSEL cells also correlated positively with each other, and the number of Lin⁻CD45⁻CD34⁺ cells correlated negatively with CD45^{dim}CD31⁺CD34⁺KDR⁺ EPC and CD45⁻CD29⁺CD90⁺ MSC populations (Table S4).

Next, we checked whether the presence of stem cell subpopulations allowed for detection of the *POU5F1* transcript, which encodes OCT4A – a key regulator of pluripotency. The results show that primers designed to avoid detection of known pseudogenes (Figure 2a) did not show any *POU5F1* expression in PB TNCs (Figure 2b), although amplified in the positive control (Figure 2c). Additionally, no signal was detected in human Lin⁻CD45⁻ cells sorted from the bone marrow (data not shown).

Expression of Anti-oxidant Genes

Peripheral blood nucleated cells in all groups of diabetic patients showed a downregulation of catalase (Figure 3a), which correlated negatively with the Lin⁻CD45⁻CD34⁺ population ($P = 0.024$, Spearman's $r = -0.30$) and HbA_{1c} ($P = 0.023$, Spearman's $r = -0.33$). A similar decrease in patients with non-complicated type 2 diabetes was found for *HMOX1* (Figure 3b), regulated in response to oxidative stress by Nrf2 transcription factor. Here, the inhibition of *HMOX1* was possibly not associated with Nrf2, as *NQO1* – another Nrf2 target – showed an opposite tendency (Figure S4A). The expression of *HMOX1* was augmented in all groups with foot disorders (Figure 3b), and correlated negatively with the Lin⁻CD45⁻CD34⁺ VSEL population ($P = 0.024$, Spearman's $r = -0.291$). Furthermore, patients with healed foot ulcerations had higher expression of *HMOX1* than those with non-infected wounds ($P < 0.05$). Interestingly,

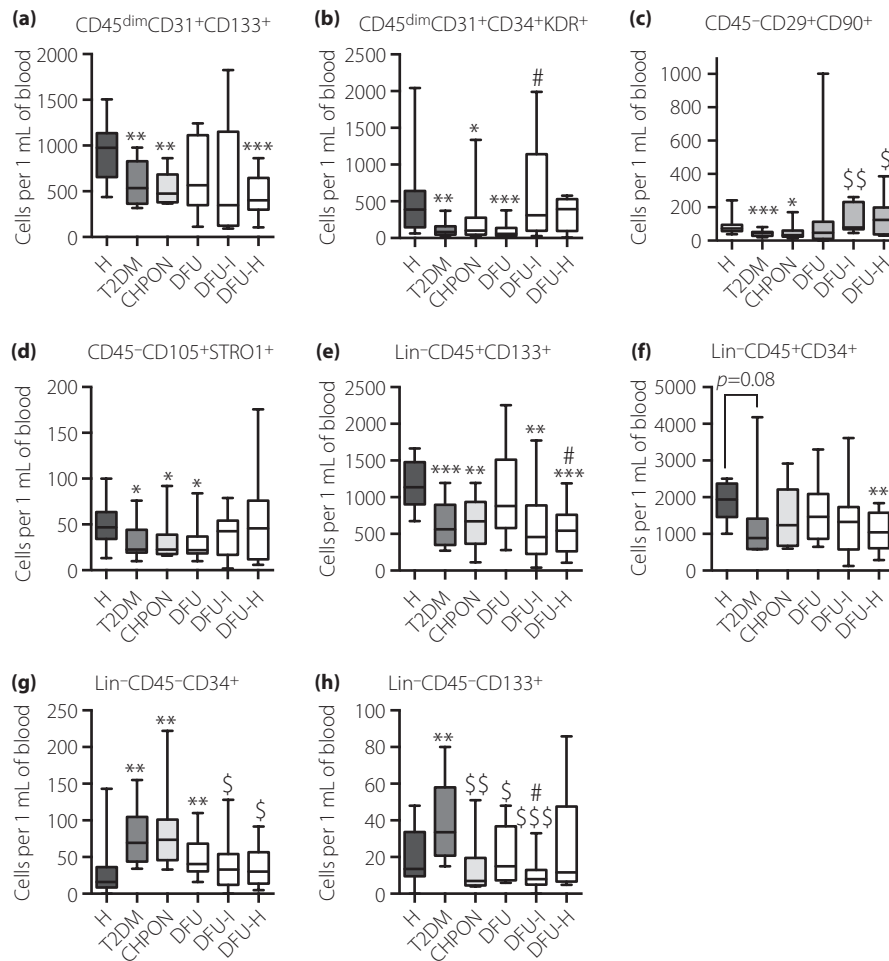


Figure 1 | Numbers of circulating stem and progenitor cells in the peripheral blood of patients. (a) $CD45^{dim}CD31^{+}CD133^{+}$ endothelial progenitor cells (EPC); (b) $CD45^{dim}CD31^{+}CD34^{+}KDR^{+}$ EPC; (c) $CD45^{-}CD29^{+}CD90^{+}$ mesenchymal stem cells (MSC); (d) $CD45^{-}CD105^{+}STRO-1$ MSC; (e) $Lin^{-}CD45^{+}CD133^{+}$ hematopoietic stem cells (HSC); (f) $Lin^{-}CD45^{+}CD34^{+}$ HSC; (g) $Lin^{-}CD45^{-}CD34^{+}$ very small embryonic-like (VSEL) cells; (h) $Lin^{-}CD45^{-}CD133^{+}$ VSEL cells. Flow cytometry phenotyping. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs healthy volunteers (H); \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ vs type 2 diabetes group (T2DM); # $P < 0.05$ vs type 2 diabetes patients with non-infected ulceration (DFU). Results are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles. CHPON, Charcot peripheral osteoneuropathy; DFU-H, type 2 diabetes patients with healed ulceration; DFU-I, type 2 diabetes patients with infected ulceration.

the expression of *HMOX2*, which is considered a constitutive gene, was higher in type 2 diabetes, Charcot osteoneuropathy and diabetic foot ulcer groups than in healthy subjects (Figure 3c), what might be considered as a kind of compensatory mechanism. Expression of *SOD1* was higher in patients with the non-complicated type 2 diabetes than in healthy controls (Figure 3d), and correlated positively with both VSEL populations ($Lin^{-}CD45^{-}CD133^{+}$ $P = 0.057$, Spearman's $r = 0.24$; $Lin^{-}CD45^{-}CD34^{+}$ $P = 0.006$, Spearman's $r = 0.34$), *NQO1* ($P = 0.037$, Spearman's $r = 0.28$), *HMOX2* ($P < 0.0001$, Spearman's $r = 0.72$), HbA_{1c} ($P = 0.036$, Spearman's $r = 0.28$), but negatively with $CD45^{dim}CD31^{+}CD34^{+}KDR^{+}$ EPCs ($P = 0.024$, Spearman's $r = -0.26$) and expression of *HMOX1* ($P = 0.002$, Spearman's $r = -0.40$). Finally, using the ABTS assay we checked the plasma total anti-oxidant capacity. Surprisingly, the

anti-oxidant capacity was higher in plasma of non-complicated type 2 diabetes or DFU and DFU-H groups than in healthy controls ($P < 0.01$, $P < 0.05$, $P < 0.05$, respectively; Figure S4B). Furthermore, plasma anti-oxidant capacity correlated positively with the *HMOX2* (Spearman's $r = 0.017$, $P = 0.33$) and *SOD1* (Spearman's $r = 0.28$, $P = 0.045$) expression. The expression of anti-oxidant genes, *CAT*, *SOD1* or *HMOX1*, was not affected by the metformin therapy (data not shown).

Concentration of cytokines

TNF- α was elevated in all diabetic patients, regardless of the presence of DFS (Figure 4a). Type 2 diabetes patients also had a trend towards the lowered concentration of EGF (Figure 4b), the effect was less pronounced in groups with DFS. Of note, the level of plasma EGF correlated positively with the number

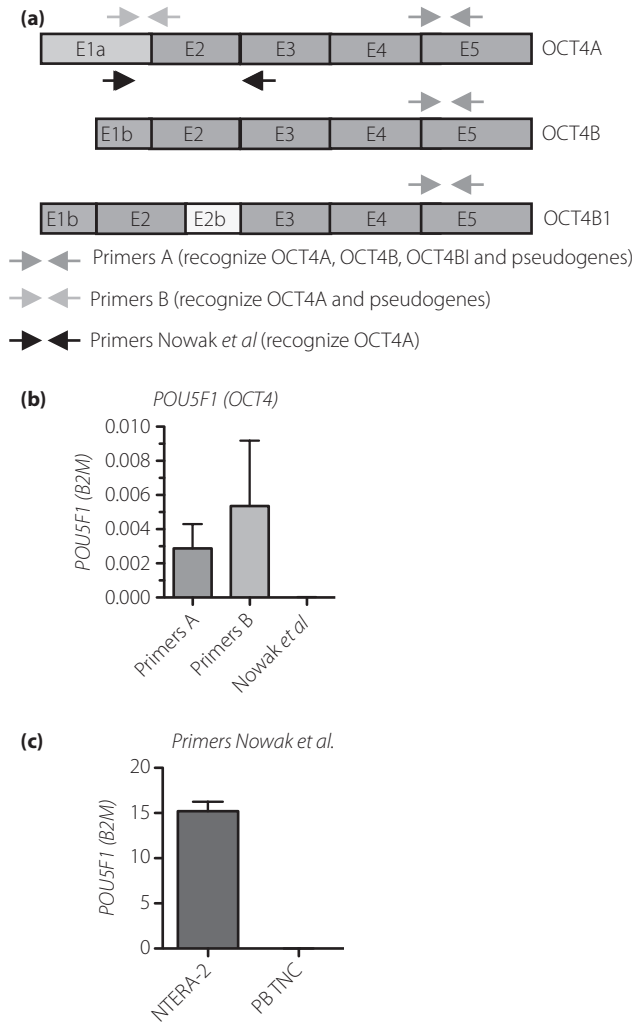
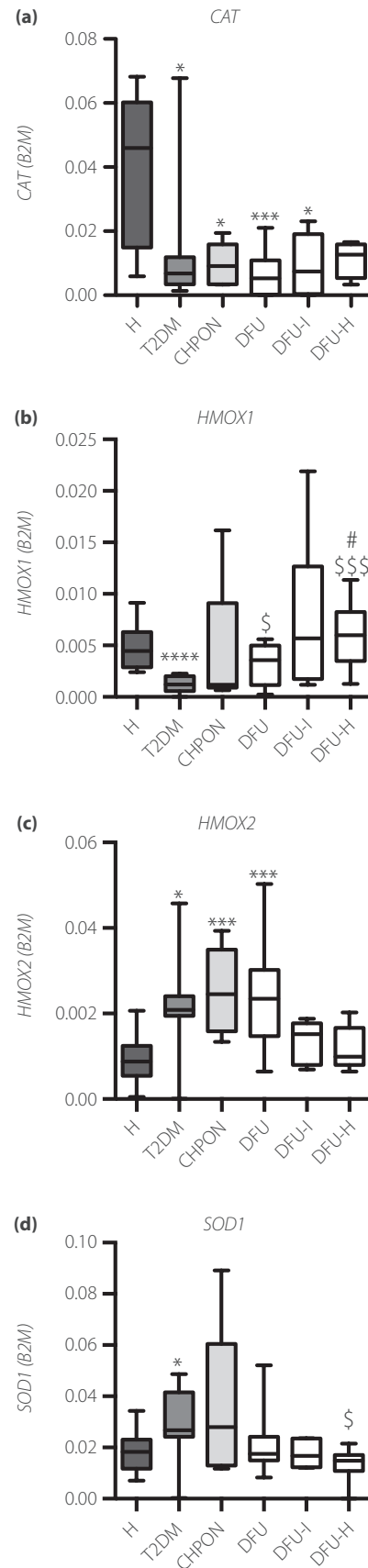


Figure 2 | Schematic localization of recognized sequences in the gene encoding OCT4. (a) E1a-E5 exons. (b) Analysis of *POU5F1* expression in peripheral blood total nucleated cells (PB TNC) of patients (mean \pm standard deviation, $n = 8$, randomly chosen patients). Primers A and B provide false-positive signals. (c) Primers by Nowak *et al.* produce no signal. Comparison of signal from NTERA-2 human embryonal carcinoma cell line (positive control) and PB TNC using primers by Nowak *et al.*

Figure 3 | Expression of anti-oxidant genes in circulating total nucleated cells and concentration of plasma cytokines in peripheral blood of patients. (a) Catalase. (b) *HMOX1*. (c) *HMOX2*. (d) *SOD1*. Expression of messenger ribonucleic acid was determined by quantitative real-time polymerase chain reaction. *B2M* served as a constitutive control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs healthy volunteers (H); \$ $P < 0.05$ vs type 2 diabetes group (T2DM); # $P < 0.05$ vs type 2 diabetes patients with non-infected ulceration (DFU). Results are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles. CHPON, Charcot peripheral osteoneuropathy; DFU-H, type 2 diabetes patients with healed ulceration; DFU-I, type 2 diabetes patients with infected ulceration.



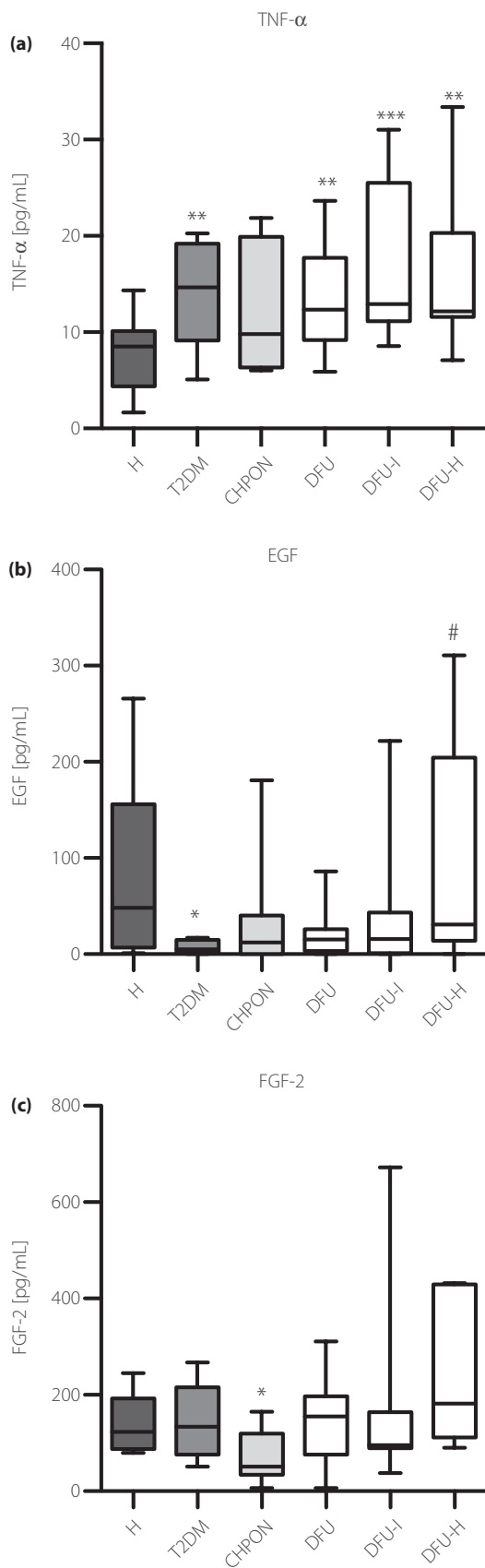


Figure 4 | Plasma levels of (a) tumor necrosis factor- α (TNF- α); (b) epidermal growth factor (EGF); and (c) fibroblast growth factor-2 (FGF-2). The concentration of cytokines in plasma was measured using the Milliplex FlexMap 3D method. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs healthy volunteers (H); \$ $P < 0.05$ vs type 2 diabetes group (T2DM); # $P < 0.05$ vs type 2 diabetes patients with non-infected ulceration (DFU). Results are shown as box and whisker charts displaying minimum, maximum, median, upper and lower quartiles. CHPON, Charcot peripheral osteoneuropathy; DFU-H, type 2 diabetes patients with healed ulceration; DFU-I, type 2 diabetes patients with infected ulceration.

of circulating CD45⁻CD29⁺CD90⁺ MSC (Spearman's $r = 0.34$, $P = 0.003$). Interestingly, patients with Charcot osteoneuropathy were the only participants with a reduced level of FGF-2 (Figure 4c). Concentrations of G-CSF, Flt-3-L, SDF1 α , SDF1 β , LIF, TPO and SCF (Figure S4C–H) were similar in all groups, and did not correlate with the number of stem or progenitor cells.

DISCUSSION

The most consistent finding of the current study shows that all groups of diabetic patients, with and without DFS, had an elevated concentration of plasma TNF- α and decreased expression of catalase in peripheral blood nucleated cells. This could indicate a chronic inflammatory reaction and reduced cellular anti-oxidative capabilities. A low level of catalase in the blood cells of diabetic patients, accompanied by enhanced oxidative stress, has been already reported⁶. Of note, catalase expression is regulated by peroxisome proliferator-activated receptor- γ , the target for antidiabetic thiazolidinediones¹⁹. Furthermore, the inherited catalase deficiency has been reported to be a risk factor for the development of type 2 diabetes²⁰.

Type 2 diabetes patients without DFS had also less circulating EPC, MSC and HSC, when compared with healthy subjects. The decrease found in EPC confirms the previously published data^{2,7}, whereas HSC and MSC in diabetes have not been this thoroughly studied hitherto. Interestingly, both subpopulations of EPC correlated negatively with HbA_{1c}. We can suggest that the simultaneous decrease in circulating EPC, MSC and HSC might reflect the impaired stem cell niche function in type 2 diabetes²¹.

In contrast, Lin⁻CD45⁻CD34⁺ and, to a lesser extent, Lin⁻CD45⁻CD133⁺ populations, both described as VSEL pluripotent cells^{16,22}, were elevated in type 2 diabetes patients. The latter ones were less numerous in groups with DFS than in patients without these complications. One could speculate that VSEL cell mobilization is a defensive mechanism, less effective in patients with diabetic complications. In contrast, a recent study showed that such populations (Lin⁻CD45⁻CXCR4⁺CD34⁺ among them) do not express pluripotency markers, including *POU5F1*, and contain many aneuploid cells and products of defective cell divisions²³. Accordingly, although using meticu-

lously designed primers, we were unable to detect any *POU5F1* transcript in peripheral blood nucleated cells. Furthermore, in our recent study on the murine bone marrow-derived $FSC^{low-}Lin^{-}CD45^{-}Sca-1^{+}$ events, which fulfil the VSEL criteria, we showed that such cells can be contaminated with the nuclei expelled during the erythropoiesis. Murine VSEs are also highly enriched in terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling (TUNEL) with deoxyribonucleic acid fragmentation. Importantly, we were not able to show Oct-4A expression in either sorted VSEL populations or at the single-cell level²⁴.

The number of $Lin^{-}CD45^{-}CD34^{+}$ cells correlated positively with HbA_{1c} , and negatively with the expression of cytoprotective *HMOX1*. This might suggest that the elevation of these very rare populations results from hyperglycemia-induced tissue injury. Increases in the number of circulating $Lin^{-}CD45^{-}CD133^{+}$ and $Lin^{-}CD45^{-}CD34^{+}$ events have been previously shown in myocardial infarction^{25–27}, skin burns²⁸, stroke²⁹ and inflammatory bowel disease³⁰. However, the reported numbers of such events are highly variable, ranging from 0.035³⁰ to 0.33 per 1 μ L of blood²⁸, even in healthy controls. Finally, there is so far no proof for the active participation of human VSEs in the tissue regeneration or definitive evidence for their stemness. Altogether, we speculate that VSEs might be a heterogeneous population of events that are rather the markers of tissue injury.

Apart from changes in $TNF-\alpha$ and catalase levels, type 2 diabetes patients showed a trend towards decreased concentration of EGF, and reduced expression of *HMOX1*, compensated by an upregulation of *HMOX2*. In mice, a decrease in EGF or *HMOX1* enhances the risk of diabetic complications and impairs wound healing^{31,32}. In contrast, *HMOX1* was higher in DFU patients, which could indicate an increased oxidative stress or inflammatory reaction. Rapid upregulation of *HMOX1* in response to tissue injury is well-known³³. However, in the *db/db* diabetic mice, we showed that such induction was weaker and delayed as compared with that in healthy counterparts, and was not sufficient to maintain the proper vascularization and healing of the wounded tissue³³. Additional overexpression of *HMOX1* using adenoviral vector-mediated gene transfer³³ or injection of mesenchymal stem cells pretreated with EGF³⁴ significantly improves neovascularization and healing of wounded skin³³, or facilitates angiogenesis in ischemic limbs³⁴ in diabetic mice.

The parameters, which were different in patients with DFS, even with ulceration that had already healed, than in those without this complication included a lower number of VSEL cells, higher $CD45^{-}CD29^{+}CD90^{+}$ MSC, and augmented expression of *HMOX1*. Interestingly, intramuscular injection and topical administration of autologous MSC accelerated ulcer healing in the clinical study³⁵. Of note, the number of circulating EPC and HSPC was no different between the DFU group and healthy controls. Interestingly, in *db/db* diabetic mice, both

EPC and HSPC are normally released from the bone marrow, but poorly to the site of injury³⁶.

Infected ulcers were additionally associated with increased $CD45^{dim}CD31^{+}CD34^{+}KDR^{+}$ endothelial progenitors and $CD45^{-}CD29^{+}CD90^{+}$ mesenchymal stem cells, whereas numbers of hematopoietic stem and progenitor cells, as well as VSEs, remained low. Interestingly, the characteristic feature of patients with peripheral Charcot osteoneuropathy was a lowered level of plasma FGF-2, which might be related to the decreased number of endothelial progenitors preventing neuropathy in mice³⁷. FGF-2 has been found to be neuroprotective in the rat model of diabetic neuropathy³⁸, also when secreted by intramuscularly injected mesenchymal stem cells³⁹.

In summary, although the present study included a limited number of participants, the results show that patients with type 2 diabetes and different forms of DFS have an altered number of circulating stem cells. Type 2 diabetes might also be associated with a changed serum growth factor and anti-oxidant gene expression profile. Altogether, these factors can contribute to the pathogenesis of different DFS forms.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Characteristics of patients.

Table S2 | Sequence of primers used for the quantitative real-time polymerase chain reaction.

Table S3 | Conditions for the quantitative real-time polymerase chain reaction reactions.

Table S4 | Spearman's correlation of tested circulating cell populations.

Figure S1 | Gating strategy for CD45^{dim}CD31⁺CD133⁺ and CD45^{dim}CD31⁺CD34⁺KDR⁺ endothelial progenitor cells.

Figure S2 | Gating strategy for CD45⁻CD105⁺STRO-1⁺ and CD45⁻CD29⁺CD90⁺ mesenchymal stem/progenitor cells.

Figure S3 | Gating strategy for Lin⁻CD45⁺CD133⁺ and Lin⁻CD45⁺CD34⁺ hematopoietic stem and progenitor cells, and Lin⁻CD45⁻CD133⁺ and Lin⁻CD45⁻CD34⁺ enriched with very small embryonic-like cells [VSEL].

Figure S4 | Plasma total anti-oxidant capacity, expression of anti-oxidant genes in circulating total nucleated cells and concentration of plasma cytokines in peripheral blood of patients.