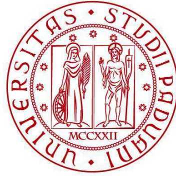

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BONE MARROW DYSFUNCTION IN DIABETES

Direttore della Scuola : Ch.mo Prof. Gaetano Thiene

Coordinatore d'indirizzo: Ch.mo Prof. Roberto Vettor

Supervisore :Ch.mo Prof. Angelo Avogaro

Dottorando: Gian Paolo Fadini

INDEX

Pag 2 Abstract

Pag 4 Riassunto

Pag 7 Introduction

Pag 9 PART 1. Diabetes impairs stem cell and proangiogenic cell mobilization in humans

Pag 19 PART 2. Diabetes impairs mobilization of stem cells for the treatment of cardiovascular disease. A meta-regression analysis

Pag 28 PART 3. Stem cell compartmentalization in diabetes and high cardiovascular risk reveals the role of DPP-4 in diabetic stem cell mobilopathy

Pag 47 Conclusions

Pag 49 References

Pag 62 Tables

Pag 72 Figures

Pag 92 Acknowledgements

ABSTRACT

Background. Diabetes mellitus (DM) increases cardiovascular disease (CVD) and this is attributed, at least in part, to shortage of vascular regenerative cells derived from the bone marrow (BM). Indeed, the BM harbours several subsets of progenitor cells for endothelial, smooth muscle cells and cardiomyocytes, which derive from a common CD34+ ancestor. Recent data from experimental models of type 1 and type 2 diabetes highlight BM pathologies that include microangiopathy, neuropathy, altered gene expression and niche dysfunction.

Aims. The set of experiments herein described aim to portray the alterations of BM function in clinical and experimental diabetes.

Methods. The approaches are diversified and include: 1) A prospective trial of direct BM stimulation with human recombinant granulocyte colony stimulating factor (G-CSF) in diabetic and non diabetic patients; 2) A meta-regression analysis of trials using G-CSF to stimulate cardiovascular repair in diabetic and non diabetic patients; 3) A study of stem/progenitor cell compartmentalization in the BM and peripheral blood (PB), in relation to diabetes; 4) An animal study to dissect the role of DPP-4 dysregulation in the impaired stem/progenitor cell mobilization induced by diabetes.

Results. Part 1: in response to G-CSF, levels of CD34+ cells and other progenitor cell phenotypes increased in non DM subjects. DM patients had significantly impaired mobilization of CD34+, CD133+, CD34+CD133+ hematopoietic stem cells and CD133+KDR+ endothelial progenitors, independently of potential confounders. The in vivo angiogenic capacity of circulating mononuclear cells increased after hrG-CSF in non DM controls, but not in DM patients. DM was associated with inability to upregulate CD26/DPP-4 on CD34+ cells, which is required for the mobilizing effect of G-CSF.

Part 2: for the meta-regression analysis 227 articles were screened, 96 were retrieved for evaluation and 24 retained for the analysis of the primary end-point. There was a strong negative correlation between prevalence of diabetes and achieved CD34+ cell levels after G-CSF stimulation ($r=-0.68$; $p<0.0001$), while there was no correlation with other traditional risk factors. A multiple regression analysis showed that the correlation between diabetes and mobilization was independent. In 13 articles reporting pre- and post-G-CSF cell counts, the increase in CD34+ cells was also negatively correlated with prevalence of diabetes ($r=-0.82$; $p<0.0001$).

Part 3. PB and BM CD34+ cell counts were directly correlated, and that most circulating CD34+ cells were viable, non-proliferating and derived from the BM. Then, PB and BM CD34+ cell levels were analyzed in a 2-compartment model in 72 patients with or without cardiovascular disease. Self organizing maps showed that disturbed compartmentalization of CD34+ cells was associated with aging and cardiovascular risk factors, especially diabetes. High activity of DPP-4, a regulator of the mobilizing chemokine SDF-1α, was associated with altered stem cell compartmentalization. The role of DPP-4 in the BM mobilization response of diabetic rats was then assessed. Diabetes differentially affected DPP-4 activity in PB and BM and impaired stem/progenitor cell mobilization after ischemia or G-CSF administration. DPP-4 activity in the BM was required for the mobilizing effect of G-CSF, while in PB it blunted ischemia-induced mobilization. Indeed, DPP-4 deficiency restored ischemia (but not G-CSF) -induced stem cell mobilization and improved vascular recovery in diabetic animals.

Conclusion. Evidences from multiple clinical and experimental approaches indicate that diabetes impairs the mobilization of stem/progenitor cells from the BM to PB. This primary BM defect is related to a maladaptive and tissue-specific DPP-4 dysregulation.

RIASSUNTO

Presupposti. Il diabete mellito (DM) aumenta il rischio cardiovascolare e ciò viene attribuito almeno in parte alla riduzione delle cellule vasculo-rigenerative di origine midollare. Infatti il midollo osseo contiene precursori per cellule endoteliali, muscolari lisce e cardiomiociti, che derivano da un progenitore CD34+. Dati recenti ottenuti da modelli sperimentali di diabete tipo 1 e tipo 2 indicano l'esistenza di difetti midollari che includono microangiopatia, neuropatia, alterazione dell'espressione genica e disfunzione della nicchia staminale.

Obiettivi. Questo set di esperimenti ha avuto l'obiettivo di descrivere in dettaglio le alterazioni della funzione midollare nel diabete clinico e sperimentale.

Metodi. Gli approcci metodologici sono diversificati e comprendono: 1) un trial di stimolazione midollare diretta con G-CSF ricombinante umano in pazienti con e senza diabete; 2) un'analisi di meta-regressione dei trials in cui il G-CSF è stato somministrato per indurre rigenerazione cardiovascolare in pazienti con e senza diabete; 3) lo studio della compartimentalizzazione delle cellule staminali/progenitrici nel midollo e nel sangue periferico, in relazione al diabete; 4) un modello animale per la definizione del ruolo di DPP-4 nel difetto di mobilizzazione midollare associato al diabete.

Risultati. Parte 1: in risposta al G-CSF, le cellule CD34+ circolanti aumentavano significativamente nel paziente non diabetico, ma non nel diabetico, che mostrava anche una difettosa mobilizzazione di cellule ematopoietiche CD133+ e CD34+CD133+, nonché di cellule progenitrici endoteliali CD133+KDR+, indipendentemente dai possibili fattori confondenti. La capacità angiogenica in vivo delle cellule mononucleate aumentava significativamente dopo G-CSF nei soggetti diabetici ma non nei non diabetici, rispetto al basale. Il diabete risultava associato ad una incapacità di upregolare DPP-4 sulle cellule CD34+ in risposta al G-CSF.

Parte 2: per la meta-regressione sono stati individuati 227 articoli, recuperati 96 e trattenuti 24 per l'analisi primaria. È stata identificata una forte correlazione negativa tra prevalenza del diabete all'interno di ogni trial e livello delle cellule CD34+ raggiunte dopo mobilizzazione con G-CSF ($r=-0.68$; $p<0.0001$). Una analisi di regressione multipla ha confermato che il risultato era indipendente da possibili fattori confondenti. In 13 articoli contenenti dati sui livelli di cellule CD34+ pre- e post-G-CSF, la correlazione negativa tra prevalenza del diabete e mobilizzazione appariva ancora più stretta ($r=-0.82$; $p<0.0001$).

Parte 3: i livelli delle cellule CD34+ nel midollo e nel sangue periferico risultano essere direttamente correlati e la maggior parte delle cellule CD34+ erano di origine midollare, non proliferanti e non apoptotiche. Lo studio della compartimentalizzazione delle cellule CD34+ in 72 pazienti con e senza malattia cardiovascolare mediante l'uso delle mappe auto-organizzanti ha permesso di rilevare alterazioni della mobilizzazione in presenza di diabete ed elevato rischio cardiovascolare. Inoltre, un'elevata attività plasmatica di DPP-4 si associava ad alterata compartimentalizzazione delle cellule CD34+. In ratti diabetici rispetto ai controlli, l'attività di DPP-4 risultava significativamente aumentata nel sangue periferico e ridotta nel midollo osseo. Lo studio di ratti geneticamente deficienti dell'enzima DPP-4 ha permesso di stabilire che l'alterazione tessuto-specifica di DPP-4 nel diabete è responsabile del difetto di mobilizzazione post-G-CSF e post-ischemia. La delezione di DPP-4 ripristinava la mobilizzazione post-ischemica di cellule staminali ematopoietiche e progenitrici endoteliali e favoriva il recupero del tessuto ischemico nel diabete.

Conclusioni. Diversi tipi di evidenze sperimentali indicano chiaramente che il diabete induce un difetto nella mobilizzazione delle cellule staminali/progenitrici midollari. Questo difetto primitivo del midollo osseo nel diabete è correlato ad una disregolazione tessuto-specifica dell'attività dell'enzima DPP-4.

INTRODUCTION

Diabetes mellitus (DM) increases cardiovascular disease (CVD) and this is attributed, at least in part, to shortage of vascular regenerative cells derived from the bone marrow (BM) (1). Indeed, DM is associated with reduced levels of several circulating progenitor cell phenotypes (2; 3). DM prevents post-ischemic progenitor cell mobilization in rats, which translates into impaired vascular recovery after ischemia (4). Recent data from experimental models of type 1 and type 2 diabetes highlight BM pathologies that include microangiopathy (5), neuropathy (6), altered gene expression (7) and niche dysfunction (8). These changes may account for an impaired mobilizing capacity in DM versus controls animals (9). The bone marrow (BM) harbours regenerative cells involved in the homeostasis of the cardiovascular system, including endothelial (EPCs), smooth muscle and cardiomyocytes progenitors (10; 11). These progenitor cell lineages derive from the common immature CD34+ stem cell population within the BM (11). In steady-state conditions, CD34+ cells circulate at a very low frequency (<0.01% of white blood cells) in peripheral blood, but can be mobilized from the BM by different stimuli, either physiologic or pathologic (12; 13). Vascular and myocardial injury, through the release of chemokines and growth factors, potently stimulates the BM to mobilize CD34+ cells into the bloodstream, as a reparative attempt (13). Preclinical studies indicate that CD34+ cells and EPCs repair the damaged vasculature, promote angiogenesis and favour recovery of the infarcted myocardium (11). Bone marrow (BM) derived stem and progenitor cells contribute to cardiovascular homeostasis through several mechanisms, including differentiation into vascular phenotypes and paracrine signals (14-17). The cross-talks between cardiovascular disease (CVD) and the BM system are manifold. Signals from ischemic tissues activate BM metabolism and function (18) and inform the BM for the need of vasculo-regenerative cells through soluble

mediators (e.g. VEGF and SDF-1α) produced locally (13). These cross-talks are disturbed in the presence of cardiovascular risk factors (12), which are associated with reduced and dysfunctional BM-derived circulating progenitor cells, a mechanism that contribute to development and progression of CVD (19-21). On this background, several clinical trials have been conducted during the last 10 years using autologous stem/progenitor cells to treat cardiovascular disease (CVD) (22). Cellular products were obtained by either BM aspiration or apheresis of peripheral blood mobilized with granulocyte-colony stimulation factor (G-CSF). Other trials were designed to test whether simply increasing circulating stem cell levels with G-CSF improved patients' outcomes (23). Data on BM function in human DM are scant, while there is no information on BM structure. In a retrospective case series of patients undergoing BM auto-transplantation, DM was statistically associated with poor mobilization in response to chemotherapy plus human recombinant G-CSF (hrG-CSF) (8). Moreover, in support of the existence of a BM defect in human DM, we have shown a reduction in BM CD34+ cells, compared with non diabetic subjects (24). The mechanism of action of the mobilizing factor G-CSF is complex and involves cleavage of SDF-1α through release of proteases, elastases and MMP9, suppression of osteoblastic function, and modulation of integrins (25). The mechanism whereby DM impairs stem cell mobilization may depend on altered local concentrations of the chemokine SDF-1α. Interestingly, SDF-1α is a natural substrate of the protease CD26/DPP-4, the activity of which is dysregulated in DM (26). The impaired stem cell mobilization in DM carries important implications for the care of patients in the hematology clinic. Furthermore, as the BM harbours a variety of regenerative non-hematopoietic progenitors, BM dysfunction may contribute to the onset of chronic diabetic complications (27). Unfortunately, exploration of BM structure and function in humans is limited by the intrinsic low availability of BM samples from non-hematologic patients.

PART 1

Diabetes impairs stem cell and proangiogenic cell mobilization in humans

AIM

To confirm the diabetic stem cell “mobilopathy” in humans, a pharmacologic test of BM reserve was herein devised in as a prospective trial of BM stimulation with a single subcutaneous injection of hrG-CSF in DM and in non-DM individuals.

RESEARCH DESIGN AND METHODS

Patients and treatment. The study was approved by the local ethical committee and is registered in ClinicalTrials.gov (NCT01102699). This was a prospective, parallel group study of direct BM stimulation with hrG-CSF in DM and non DM subjects. The primary end-point was change in circulating $CD34^+$ cells from baseline. Secondary end-points were changes in other progenitor cell phenotypes, proangiogenic capacity of peripheral blood mononuclear cells (PBMC), white blood cells and safety. The study was not designed and powered to detect baseline differences in progenitor cell levels. DM patients were recruited at the outpatient clinic of the University Hospital of Padova and healthy control subjects were volunteers from the local community. Both type 1 and type 2 DM patients were eligible because pre-clinical studies have shown similar BM alterations and progenitor cell reductions in both types of DM (5; 6). Exclusion

criteria were: age <25 or >65 years, any acute disease or infection, recent trauma, surgery or cardiovascular event, chronic immune or infectious diseases, current or past hematological disorders or malignancy, leukocytosis, leukopenia or thrombocytopenia, organ transplantation or immune suppression, advanced diabetic retinopathy, altered liver function, severe renal failure (eGFR<30 mL/min/m^2), anomalies in lymphocytes subpopulations, allergy to Filgrastim, bronchial asthma or other chronic lung disorders, impossibility to provide informed consent. For each patient, we collected anthropometric measures, data on concomitant risk factors, HbA1c, eventual diabetic complications and therapy.

After providing informed consent, patients were subjected to baseline examination and blood samples, including determination of the complete leukocyte counts and lymphocyte subpopulations, liver enzymes, renal function, plasma protein electrophoresis, erythrocyte sedimentation rate, C-reactive protein, prothrombin time, uric acid and standard urine exam. After verification of inclusion and exclusion criteria, at h8.30 am after an overnight fast, eligible patients were subjected to a baseline peripheral blood sampling for circulating progenitor cell quantification and collection of PBMC. Immediately after, they were injected subcutaneously with 5 µg/kg Filgrastim (Granulokine, Amgen). Twenty-four hours later, another peripheral blood sample was obtained to evaluate the effects of Filgrastim. Study subjects were invited to register and report any eventual side effect occurred after Filgrastim injection. Dosage of the drug was chosen as the minimum effective dose based on available pharmacodynamic data on Filgrastim, showing that 5 µg/kg is sufficient to increase absolute count of circulating $CD34^+$ cells in healthy controls (28).

FACS analysis. Circulating progenitor cells were quantified using flow cytometry as previously described in detail (29). Briefly, after red blood cell lysis, 150 µl of peripheral blood were stained with 10 µl of FITC-conjugated anti-human CD34

mAb (Becton Dickinson), 10 µl of PE-conjugated anti-human KDR mAb (R&D Systems) and 10 µl of APC-conjugated anti-CD133 mAb (Miltenyi Biotech). The frequency of peripheral blood cells positive for the above reagents was determined by a 2D side scatter-fluorescence dot plot analysis, after appropriate gating. We gated $CD34^+$ or $CD133^+$ peripheral blood cells in the mononuclear cell fraction and then examined the resulting population for the dual expression of KDR. At the intersection of the CD34 and CD133 gates we identified $CD34^+CD133^+$ cells, which were examined for KDR expression. In all patients, we also quantified the expression of CD26/DPP-4 on $CD34^+$ cells using a PE-labelled anti-CD26 mAb (BD). In separate analyses, CD45 co-staining was performed and showed that >90% of $CD34^+$ cells are CD45dim. For FACS analysis, $5x10^5$ cells were acquired and scored using a FACS Calibur (BD). Data were processed using the Macintosh CELLQuest software program (BD). The same trained operators, blind to the clinical status of the patients, performed the tests throughout the study. Absolute progenitor cell counts per unit of blood were derived by multiplying fractional data per white blood cell count. We have previously shown that reproducibility of $CD34^+$ cell quantification with this method is high (ICC 0.94, 95% C. I. 0.88-0.96; CV 6.3%) (29).

In vivo proangiogenic cell function. To gather information on the presence of functional circulating proangiogenic cells and how they are modulated by hrG-CSF in DM and non DM subjects, we used the in vivo Matrigel plug angiogenesis assay with patients' PBMC. Indeed, data suggest that diverse monocyte subsets, including monocytic EPCs and Tie2-expressing monocytes, have proangiogenic capacity (30; 31). Briefly, PBMC were isolated with Histopaque (Sigma-Aldrich). Cell count and viability were assayed with an automated BioRad TC20 cell counter. Then, 3×10^6 PBMC were resuspended in 500 µL phenol-free Matrigel (BD, cat no. 356237) and implanted subcutaneously into the dorsum of

immunodeficient RAG-2/gamma(c) double knock-out mice (in-house colony). The experiment was performed with pre- and post-hrG-CSF PBMC of n=5 non DM controls, n=5 T1D patients and n=5 T2D patients. To minimize variability, the same mouse received pre- and post-GCSF PBMC of the same subject. Plugs were explanted 10 days later for macroscopic inspection, histology (H&E staining), and determination of the hemoglobin/protein content ratio (Drabkin's solution and Bredford reagent respectively, Sigma-Aldrich), which is as a surrogate of perfusion. Hb/protein ratio was adjusted for the change in monocyte count after hrG-CSF to gather information on the proangiogenic capacity of circulating PBMC at each time point.

Statistical analysis. Data are expressed as mean±standard error (SE). Normal distribution of the variables of interest was verified with the Kolmogorov-Smirnov test. Comparisons between the diabetic and non diabetic group were performed using Student's t test for normal variables, Mann-Whitney' U test for non-normal variables and the chi-square test for categorical variables. As $CD34^+$ cell count is a normally distributed variable, the change in $CD34^+$ cell count from baseline to 24h after Filgrastim was assessed using paired Student's t test. We then calculated the mean±SE change of $CD34^+$ cells in the diabetic and non diabetic groups, which were compared using unpaired Student's t test. Linear associations were assayed using the Pearson's r correlation coefficient. To control for variables that were different between the 2 groups at $p \leq 0.10$ and may bias results, a multiple linear regression analysis was run with change in progenitor cell levels as the dependent variable. Non-normal dependent variables in secondary end-point analyses were log-transformed. Statistical significance was accepted at $p<0.05$ and SPSS version 17.0 was used.

RESULTS

Characteristics of the study population. A total of 24 DM patients (10 T1D and 14 T2D) and 14 non DM controls have been enrolled and treated. Diabetic patients had a higher prevalence of hypertension and tended to be older than control subjects. T1D patients had a significantly longer disease duration and lower prevalence of cardiovascular disease, compared to T2D (Table 1).

Stem and progenitor cell mobilization. In non DM subjects, absolute $CD34^+$ cell level significantly increased 2.2-fold after hrG-CSF, while DM patients completely failed to mobilize $CD34^+$ cells (mean±SE change versus baseline: non DM 3475±800 cells/mL; DM 52±332 cells/mL; $p=5x10^{-5}$; Figure 1A). Results were similar when T1D or T2D patients, considered separately, were compared with age-matched non DM controls (Figure 1B-C). Clinical characteristics of the subgroups are shown in Table 1.

After adjusting for potential confounders that were different between the 2 groups at p≤0.10 (age, hypertension and cardiovascular disease), DM remained significantly associated with reduced $CD34^+$ cell increase after hrG-CSF (p=0.002).

In response to hrG-CSF, DM was associated with impaired mobilization of all the other progenitor cell phenotypes, such as $CD133^+$, $CD34^+CD133^+$, $CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells (Figure 2). Upon correction for potential confounders, DM remained significantly associated with defective mobilization of $CD133^+$ (p=0.015), $CD34^+CD133^+$ (p=0.011) and $CD133^+KDR^+$ (p=0.013) cells, while the association between defective $CD34^+KDR^+$ mobilization and diabetes was marginally significant (p=0.056) and blunted by age (p=0.024).

Percentage expression of CD26/DPP-4 on $CD34^+$ cells significantly increased after hrG-CSF in non DM controls (delta +14.1±3.9%), consistent with previous findings in vitro (32). In DM, CD26/DPP-4 expression was elevated at baseline

and tended to decline after hrG-CSF treatment (delta -8.8±5.5%, $p=0.013$ vs non DM. Figure 3).

In the DM groups, progenitor cell mobilization was not significantly correlated to HbA1c, disease duration, pattern of complications or treatment regimen.

After treatment with hrG-CSF, WBC, neutrophil and monocyte counts significantly increased in both groups and there were no differences between DM patients and non DM controls (Table 2 and Figure 5). This suggests that DM affects immature and not mature cell mobilization.

Proangiogenic cell function in vivo. Patients' mononuclear cells collected before and after hr-GSCF administration were embedded into Matrigel plugs and implanted in immunodeficient mice to assess the presence of proangiogenic cells. Baseline PBMC from non DM subjects showed higher neovascularisation capacity compared to DM patients, which was statistically significant versus T1D. Plugs with non DM PBMC before hrG-CSF (baseline) showed vascular invasion at gross inspection and the presence of vascular structures containing erythrocytes at histology. Plugs implanted with T1D PBMC at baseline showed almost no vascularization, lower hemoglobin content and no evidence of perfused vascular structures. Plugs containing baseline T2D PBMC showed non-significantly lower vascularisation capacity compared to non DM at baseline. After hrG-CSF, the proangiogenic capacity of patients' cells significantly increased in non DM, but not in DM groups, and post-hrG-CSF neovascularisation capacity was lower in T1D ($p=0.058$) and T2D ($p=0.045$) versus non DM (Figure 4). Matrigel plug neovascularisation capacity was significantly correlated with circulating $CD34^+$ ($r=0.47$; $p=0.003$), $CD34^+KDR^+$ ($r=0.38$; $p=0.024$), $CD133^+KDR^+$ ($r=0.46$; $p=0.005$) and $CD34^+CD133^+KDR^+$ ($r=0.41$; $p=0.014$) cell levels (Figure 6). These data suggest that hrG-CSF mobilizes functional proangiogenic cells in non DM subjects but not in DM patients.

Safety. Treatment with hrG-CSF was safe and uneventful: 5 subjects in the non DM group (35.7%) and 5 patients in the DM group (20.8%, p=0.53 DM vs. non DM) reported mild back pain 12 to 18 hours after Filgrastim injection, which resolved at 24h and required analgesic therapy with acetaminophen in 3 non DM cases.

DATA INTERPRETATION AND DISCUSSION

We show that DM is associated with impaired stem and progenitor cell mobilization after direct BM stimulation, independently of potential confounders. Remarkably, this was true for both hematopoietic stem cells and EPCs despite baseline cell levels were not reduced in this DM cohort. Therefore, the present prospective trial substantiates the existence of a BM defect in human DM and suggests that the BM mobilization failure precedes reduction of circulating progenitors. Both T1D and T2D patients showed almost complete unresponsiveness to stem/progenitor cell mobilization, suggesting that this complication is independent of DM etiology; however, the role of autoimmunity in determining BM response may be worth of investigation.

In mice, long-term DM causes BM microangiopathy and altered oxygen gradients (5) that, in addition to reduced expression of pro-survival genes (7), lead to a pauperization of the stem cell pool. Microvascular BM alterations in experimental DM include capillary rarefaction, increased permeability, endothelial cell apoptosis and dysfunction (5), features that resemble diabetic microangiopathy of other organs, such as the kidney and retina. These histopathological aspects suggest that the BM is a hitherto unrecognized site of diabetic complication and are likely responsible for the mobilization failure. Data on the amount of BM stem cells in diabetes are discordant, with some studies showing normal (4; 33) or

even increased (8) primitive Sca-1$^+$c-kit$^+$Lin$^-$ hematopoietic progenitors. Therefore, the low CD34$^+$ cell count in BM aspirates from type 2 DM patients that we have previously shown (24) might reflect true stem cell deficiency or reduced accessibility of the niches to aspiration, owing to the sticky property of the diabetic niche, which is more prone to stem cell retention than mobilization (8). Importantly, DM did not impair mobilization of mature leukocytes, which are more loosely retained by the BM stroma than stem cells.

Normally, G-CSF stimulates expression and activity of CD26/DPP-4 and other proteases, with subsequent degradation of the chemokine and retention signal SDF-1α (32; 34). Thus, stem/progenitor cells migrate to the peripheral circulation following SDF-1α gradients. Herein, we suggest a possible mechanism of stem cell unresponsiveness to G-CSF in DM, by showing a maladaptive CD26/DPP-4 response. Systemic CD26/DPP-4 activity is increased in DM (26), and our new data indicate that G-CSF fails to upregulate CD26/DPP-4 on BM-derived cells in DM, likely preventing modification of the SDF-1α gradient. Studies show that DM mice mobilize stem cells after treatment with the SDF-1α receptor CXCR4 antagonists AMD3100 (8; 33; 35) and NIBR1816 (36). Therefore, the vascular niche containing stem cells that can be readily mobilized upon disruption of the SDF-1α retention signal seems to be preserved, but responsiveness to CXCR4 antagonists should be confirmed in DM patients.

The existence of a BM mobilopathy in DM might be ascribed to both structural alterations affecting the stem cell niche (microangiopathy) and functional defects preventing the cells from being mobilized (e.g. the CD26/SDF-1α/CXCR4 axis). Additionally, DM alters the activity of endothelial nitric oxide synthase (eNOS) (37) which is pivotal for EPC mobilization (38) and, though G-CSF activity is mainly eNOS-independent (39), this may be another mechanism accounting for depressed mobilization in DM. Among strategies to reverse BM dysfunction, our data showing no correlation between HbA1c and mobilization suggest that

glucose control might not be effective, while experimental data indicate that boosting the anti-oxidative defence is a suitable strategy to prevent BM alterations (5). Though CD26/DPP-4 inhibition increases EPCs (40), whether this represents a therapeutic target to restore BM responsiveness in DM needs to be ascertained.

We also found that DM impairs neovascularisation by mononuclear cells mobilized by hrG-CSF, as shown by the in vivo Matrigel plug assay. Interestingly, T1D patients already had defective neovascularisation capacity at baseline. This is possibly related to the longer disease duration in T1D versus T2D patients (Table 1). Both T1D and T2D patients were unable to increase significantly their PBMC proangiogenic capacity after hr-GCSF, again suggesting that mobilization failure precedes reduction of proangiogenic cells. Although we did not identify the subpopulation of PBMC involved (30; 31), change in neovascularisation capacity was correlated with stem/progenitor mobilization. These important results indicate that the stem cell mobilization failure can be pathophysiologically linked to impaired tissue repair and development of cardiovascular diabetic complications, which are characterized by defective angiogenesis (41; 42).

These findings have clinical implications. Reduced progenitor cell levels are powerful predictors of future cardiovascular events (21), and replenishment of progenitor cells may lower cardiovascular risk. Although the pathogenesis of diabetic vascular complications is complex and possibly diversified in T1D and T2D, it is remarkable that alterations in BM-derived cells have been consistently reported for both T1D (43) and T2D patients (44). Understanding the causes of progenitor cell reduction and the role of BM can identify novel strategies to reverse this defect and prevent vascular disease. Moreover, the immunomodulatory activity of mobilized BM-derived progenitors may be important in T1D (36). In addition, clinicians should be aware of the likelihood of

mobilization failure in DM patients undergoing stem cell collection for BM autotransplantation or for angiogenic cell therapy (8; 9).

The study has limitations. First, replication in other, possibly larger, cohorts is needed and comparison between recent-onset and long-term T1D patients would allow a description of the natural history of BM dysfunction. Second, while the imbalances in clinical characteristics between groups were adjusted by multivariate analyses, residual confounding may be present. Finally, while a single-dose hrG-CSF was used in this study, DM patients might respond to a full 5-day course of hrG-CSF, but it would not be ethical to perform maximal BM stimulation for research purposes only. Several DM patients have been treated with high-dose hrG-CSF in cell therapy protocols (45), but a formal comparison of the mobilization effect between DM and non DM patients has never been performed. Nonetheless, our data represent a proof-of-concept of diabetic BM dysfunction in humans. As the BM emerges as a novel target organ in DM, intensive investigation to reverse this complication becomes compelling.

PART 2

Diabetes impairs mobilization of stem cells for the treatment of cardiovascular disease. A meta-regression analysis

AIM

In Part 1, we have shown that response to low dose hrG-CSF is impaired in diabetic compared to non diabetic subjects involved in a trial of direct BM stimulation to assess BM reserve. In this substudy, to test whether this holds true also in a setting that is more relevant to diabetic complications, data on trials of G-CSF administration in patients with CVD were collected. Given that studies do not normally report mobilization efficiency in DM versus non DM patients, a meta-regression analysis testing the correlation between CD34+ cell levels and prevalence of DM in the study cohort was performed.

MATERIALS AND METHODS

Objective. The study was conducted to detect a significant correlation between prevalence of diabetes in each trial and achieved absolute CD34+ cell count (cells/μL) after G-CSF stimulation, as an indicator of mobilization (primary end-point). A separate analysis was pre-specified for trials reporting CD34+ cell levels before and after G-CSF administration. Finally, we looked for studies eventually

reporting CD34+ cell levels separately for DM and non DM patients or individual patients data.

Literature search. We searched the English literature from 1997 to 2012 (last updated 30 Aug 2012) for articles describing clinical trials in which patients with CVD were subjected to G-CSF stimulation for BM CD34+ stem cell mobilization alone or followed by apheresis and cell therapy. The search terms were: ("trial" OR "patients" OR "patient") AND ("G-CSF" OR "GCSF" OR "granulocyte colony") AND ("CD34" OR "HSC" OR "stem cell" OR "stem cells") AND ("mobilization" OR "mobilized" OR "mobilize") AND ("vascular" OR "cardiovascular" OR "myocardial" OR "cardiac" OR "critical" OR "arterial" OR "coronary artery disease" OR "angina" OR "heart failure"). We also examined cross-references among different articles. The minimal criteria for inclusion of the study in the primary analysis were: 1) description of the study cohort with clear indication of the prevalence of diabetes; 2) clear reporting of G-CSF dosage and treatment duration; 3) indication of the absolute CD34+ cell count (in cells/μL of peripheral blood) achieved after the course of G-CSF administration.

Quality assessment. Quality of the trials included in this meta-analysis was assessed according to an itemized methodological quality graph, as suggested by the Cochrane Handbook for Systematic Reviews of Interventions (46). Each item was scored as low, uncertain, or high risk of bias. The items included in the checklist and visualized in the graph were selected based on their relevance to the meta-regression on CD34+ cell mobilization. Therefore, detection bias was not related to the primary outcome of the trial, but on integrity of CD34+ cell data. Attrition bias was not considered, as availability of CD34+ cell data was an inclusion criterion. Quality was scored by the 2 authors independently. Quality

assessment was meant to generate separate analyses limited to trials with low risk of bias.

Data retrieval. We collected the following data: mean ± SD age of the patients, percentage of male patients, prevalence of diabetes, hypertension, dyslipidemia, and smoking habit, underlying disease, baseline CD34+ cell count/μL (when available), achieved CD34+ cell count/μL after the course of G-CSF stimulation. Total dose of G-CSF (μg/kg) received by the patient was calculated as daily dose (μg/kg/day) multiplied by the duration of treatment in days. The standard course of G-CSF stimulation is 5 μg/kg b.i.d. for 5 days, for a total of 50 μg/kg. As the total dosage varied considerably among studies, in order to make the achieved CD34+ cell count comparable, we normalized CD34+ cells/μL to the standard 50 μg/kg dosage using a proportionality formula: adjusted CD34+ cells/μL = reported CD34+ cells/μL × 50 ÷ total μg/kg G-CSF administered. In case the CD34+ cell count/μL was not directly reported, this was calculated by multiplying % of CD34+ cells in peripheral blood for total white blood cell count/μL. When CD34+ cell count was reported only in figures and not in the text, data were extrapolated from figures, but these data were considered low quality and marked as such.

Statistical analysis. Data are expressed as mean ± SD or as percentages, where appropriate. For merging continuous normal data from different studies (e.g. age) in order to provide a global overview of patients characteristics, we calculated the weighted average and standard deviation. Heterogeneity among studies was calculated using the chi-square test. The correlations between prevalence of diabetes and achieved CD34+ cell count or increase in CD34+ cell count were checked using the meta-regression method described by Thomson and Higgins (47), with the random effect model. Pearson's r and Spearman's rho

correlation coefficients were also calculated. A multivariate analysis was run to verify whether the correlation between prevalence of diabetes and CD34+ cell count was independent from the prevalence of other risk factors and underlying disease. The PRISMA guidelines were used to compile and report the data (48). Statistical significant was accepted at $p<0.05$.

RESULTS

Search results. Based on search criteria, we initially retrieved 227 articles for screening. Of these, 96 were retained for further evaluation, while the others were discarded as non relevant. Twenty-four articles were finally included in the analysis of the primary end-point, with two trials reporting two groups with different G-CSF dosages, which were considered separately (Table 3). Of the 24 articles, 13 also reported the baseline CD34+ cell count, which allowed a calculation of the increase in CD34+ cells after G-CSF stimulation (Figure 7). In one trial only, crude data of individual patients were reported (49), allowing a calculation of the achieved CD34+ cell count in DM and non DM patients.

Data quality assessment. Data quality is represented by an itemized methodological quality graph (Figure 8). Most studies (70%) were randomized, controlled trials almost free from baseline imbalance, in which patients were randomly assigned to treatment with G-CSF or placebo. This suggests that the risk of selection bias was low. Quality of CD34+ cell data was high in 80% of cases: in 2 trials data were calculated from % of CD34+ cells and WBC counts (uncertain risk of bias); in 3 trials, CD34+ cell levels were derived from figures (high risk of bias). Blinding was relatively low, but this was unlikely to affect the effects of G-CSF on CD34+ cells.

Study population. Pooled characteristics of the study population are reported in table 4. On average, patients were about sixty year old, prevalently males, treated for ST-elevation myocardial infarction. In most trials, prevalence of DM was lower than that of other traditional risk factors, reflecting the typical characteristics of patients in cardiovascular trials. The total dose of G-CSF administered was close to the standard 50 µg/kg dose, over a period of about 6 days. However, there was considerable heterogeneity in patients characteristics and treatment protocol among studies. Importantly, the chi-square test for heterogeneity among studies was statistically significant ($p<0.0001$), indicating that the study differed in the degree of CD34+ cell mobilization.

Meta-regression analysis. The primary aim of the analysis was to find a correlation between prevalence of DM and achieved absolute CD34+ cell count after G-CSF administration. Simple correlation analysis revealed a strongly significant negative correlation ($r = -0.68$; $p < 0.0001$; $rho = -0.65$; $p < 0.0001$). Using the random effect model by Bayesan tau taking into account weights of the single trials, the negative correlation was as well highly significant ($p < 0.0001$; equation $y = a + bx$ where $a = 91.2 \pm 11.4$; $b = -1.4 \pm 0.3$. Figure 9A). The analysis of variance (calculated as the residual error sums of squares and estimated using the chi squared test) was not significant ($p=0.72$), indicating no residual heterogeneity among trials. Excluding trials in which the absolute CD34+ cell count / µL was calculated or derived from figures ($n=5$), the correlation did not change ($r = -0.69$; $p < 0.0001$; $rho = -0.62$; $p < 0.0001$). Similarly, limiting the meta-regression to randomized, controlled trials did not modify strength of the association ($r = -0.69$; $p < 0.0001$; $rho = -0.61$; $p = 0.008$). These results indicate that data quality did not affect the correlation between prevalence of DM and achieved CD34+ cell count.

Including in the meta-regression only trials of patients with heart disease, thus making the population more homogeneous, did not significantly modify the correlation (r = -0.62; p = 0.002; rho = 0.56; p = 0.005). Further limiting to trials on ST-elevation myocardial infarction slightly blunted the correlation strength (r = -0.51; p = 0.034; rho = -0.48; p = 0.049).

The secondary aim was to find a correlation between the increase in CD34+ cell count after G-CSF and prevalence of DM. To this end, only trials reporting pre- and post-G-CSF data were included. The simple correlation analysis revealed an even stronger negative association (r = -0.82; p < 0.0001; rho = -0.84; p < 0.0001), which was also highly significant using the random effect model meta-regression (p < 0.0001; equation $y = a + bx$ where a = 63.5±8.3; b = -1.0±0.3; Figure 9B), again with no residual heterogeneity (p=0.91). This result indicates that including only trials with high quality data made the correlation even stronger.

We then looked at correlations with prevalence of other risk factors, mean age or underlying disease and only found marginal direct correlations with prevalence of male gender (achieved CD34+ cell count: r = 0.36; p = 0.067; rho = 0.34; p = 0.088; increase in CD34+ cell count: r = 0.48; p = 0.067; rho = 0.45; p = 0.092), which were not significant in the meta-regression with the random effect model (p=0.07 and p=0.13 respectively).

Finally, a multivariable analysis showed that the association between prevalence of DM and achieved CD34+ cell count was independent of age, other risk factors, underlying disease, and sample size (Table 5).

DATA INTERPRETATION AND DISCUSSION

The present study shows that, in clinical trials in which patients with CVD were treated with G-CSF for stem cell mobilization, the prevalence of DM is a negative

determinant of the achieved CD34+ cell level. This observation suggests that DM impairs the mobilization response to G-CSF and has 2 possible implications.

First, DM patients undergoing angiogenic cell therapy with mobilized peripheral blood stem cells are likely to receive a lower cell dose compared to non DM patients. In one study analyzing the outcome of 111 patients implanted with G-CSF-mobilized peripheral blood stem cells for the treatment of critical limb ischemia, a low dose of CD34+ cells was associated with a significantly shorter amputation-free survival (50). Additionally, 2 meta-analyses of stem cell therapy for ischemic heart disease showed that cell dosage is a direct determinant of the improved outcome (51; 52).

Second, the inability of DM patients to adequately mobilize stem cells upon direct BM stimulation points to the existence of a BM defect. This is supported by experimental animal studies showing BM microvascular rarefaction, altered location of stem cells in the niches, and reduced expression of pro-survival genes (5-7), that turn into impaired stem cell mobilization (9). This is of paramount importance, as BM-derived stem cells prevent cardiovascular damage and a low circulating CD34+ cell level independently predicts future events (21). Furthermore, after myocardial infarction, the levels of CD34+ stem cells mobilized by endogenous G-CSF secretion is a determinant of left ventricular function at follow-up (53; 54). Therefore, impaired stem cell mobilization in DM may represent a pathophysiological link among distant end-organ complications (1; 27).

The meta-regression approach has intrinsic limitations. Although data dredging is considered the principal pitfall leading to false positive results, this is not likely to be a matter of concern in the present study, because the analysis was pre-specified and a very limited number of covariates were tested. Additionally, the analysis of residual heterogeneity was non-significant, indicating that prevalence of DM was the most important determinant of mean CD34+ cell mobilization.

Quality of data might also be a concern. However, limiting the meta-regression analysis to trials with high quality data (reporting pre- and post-G-CSF CD34+ cell levels) strengthened the negative association between prevalence of DM and CD34+ cell mobilization. Limiting the analysis to randomized, controlled trials also did not modify the association between prevalence of DM and mobilization.

Publication bias cannot be fully ruled out in this meta-analysis, but is unlikely to be a major confounder, because the meta-regression was performed against a process indicator (CD34+ cells), not against the mean treatment effect (outcome indicator). In addition, the relationship between prevalence of diabetes and CD34+ cell mobilization in a given trial is unlikely to affect publication.

Importantly, our analysis should be considered as an ecological association extrapolated to individual patients, because the prevalence of DM is a summary of patient characteristics rather than a specific attribute of the trial. In other terms, trials in which patients had a higher prevalence of DM showed lower CD34+ cell mobilization, rather than patients with DM necessarily being the actual patients with lower CD34+ cell mobilization. Unfortunately, this issue cannot be further investigated without individual patient data. In the unique trial reporting individual patient data, CD34+ cell mobilization in non DM patients was 2.8-fold higher than in DM patients, supporting validity of the extrapolation of this meta-regression to individual patients (49).

Finally, it should be noted that all patients included in this meta-regression analysis had severe CVD and the interaction between DM and CVD in affecting stem cell mobilization cannot at present be dissected, nor it is possible to generalize these findings to DM patients without CVD.

To sum up, this meta-regression study supports the existence of a mobilization failure in DM patients receiving G-CSF for the treatment of CVD. As mounting evidence indicate that DM affects the BM microenvironment and stem cells

involved in vascular repair, intensive investigation into this hitherto unrecognized diabetic complication is warranted.

PART 3

Stem cell compartmentalization in diabetes and high cardiovascular risk reveals the role of DPP-4 in diabetic stem cell mobilopathy

AIM

In this substudy, we aimed to gather information on the clinical phenotype associated with an altered compartmentalization of stem/progenitor cells in the BM and peripheral blood (PB), which would be indicative of the mobilization capacity. Starting from these phenotypes, we devised a clinical data-driven approach to test the association of candidate regulators of angiogenesis with progenitor cell mobilization, to be validated in animal models.

MATERIALS AND METHODS

Patients. All studies involving human subjects were approved by the local ethical committee and conducted in accordance to the Declaration of Helsinki as revised in 2000. Patients with heart disease and healthy controls were recruited at the Johann Wolfgang Goethe University Hospitals. Patients with ischemic or non-ischemic heart disease were enrolled in protocols of progenitor cell therapy, which also included quantification and functional evaluation of progenitor cells in the BM aspirates and PB. The ethics review board of Goethe University

(Frankfurt, Germany), approved the protocols, and the studies were registered on Clinicaltrials.gov (NCT00962364 and NCT00284713). All patients undergoing BM aspiration for myocardial cell therapy in the trials NCT00962364 and NCT00284713 were eligible pending they did not have acute myocardial infarction. After giving informed consent, before undergoing cell therapy, patients were subjected to BM aspiration under sedation or general anesthesia. A small volume (about 1 mL) of the BM aspirate was collected for progenitor cell quantification. At the same time, a PB sample was drawn from a peripheral vein. Young healthy volunteers from the staff or the local community were subjected to the same procedure after providing informed consent for BM aspiration and collection of a venous PB sample.

For all subjects, we collected the following data: age, sex, prevalence of cardiovascular risk factors, total cholesterol, HDL cholesterol and triglycerides concentrations, fasting plasma glucose and medications. LDL cholesterol was calculated with the Friedwald formula. Patients were deemed to have diabetes mellitus if they were previously diagnosed with diabetes and/or were taking anti-diabetic medications, or if they had a fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) on two different occasions, or a random plasma glucose ≥200 mg/dL (11.1 mmol/L) associated with symptoms of hyperglycemia, or if they had a HbA1c ≥6.5%, measured by HPLC. None of the patients were taking medications active on the incretin system. Obesity was defined as a body mass index (weight in kilograms divided by height in meters^2) ≥30 kg/m^2. Patients were deemed as smokers if they habitually smoked one or more cigarettes per day. Hypertension was defined in the presence of a history of hypertension for which the patients were taking medications, and/or in the presence of blood pressure above 140/90 mm Hg. Family history for CVD was defined as a history of CVD in first degree relatives, excluding cases occurred at an advanced age. We calculated the

number of CV risk factors taking into account smoking habit, family history, diabetes, obesity, hypertension and dyslipidemia.

Measurement of plasma regulators of angiogenesis. PB plasma was obtained by centrifugation of venous blood samples. The plasma concentration of a panel of angiogenesis-regulating mediators was determined using multiplex suspension arrays (Bio-plex, Biorad), that permit the simultaneous automated analysis of several different biomolecules in a single microplate well. We used the angiogenesis array, which is designed to measure at the same time the following analytes: Angiopoietin-2, Follistatin, G-CSF, HGF, IL-8, Leptin, PDGF-BB, PECAM and VEGF. In parallel, we also used a custom multiplex array for the simultaneous automatic measure of IL18, SCF and SDF-1α.

Determination of DPP-4 activity. To measure plasma DPP-4 activity, we used the DPP-4 drug discovery Kit (Enzo Life Sciences, Farmingdale, NY, USA) with the Gly-Pro-para-nitroaniline (pNA) chromogenic substrate, according to the manufacturer's instructions. We have previously shown that this assay is able to detect significant differences in DPP-4 activity in diabetic versus non diabetic patients, and that the non-specific activity due to other DPP is less than 10% (55). Coefficient of variation of plasma DPP-4 activity, measured in replicate samples collected from the same patient 24 h apart, was <2%, indicating a high degree of reproducibility.

Flow cytometry. Human progenitor cells. The level of circulating progenitor cell phenotype in humans was determined by a protocol standardized at our laboratory, using multi-color flow cytometry. Briefly, after red blood cell lysis, blood cells were stained with FITC conjugated anti-human CD34 (BD Biosciences), APC-conjugated anti-human CD133 (Miltenyi Biotec) and PE-

conjugated anti-human KDR (VEGFR2, R&D Systems) monoclonal antibodies. After washing cells were analyzed using a FACS Calibur instrument (BD Biosciences) and the following gating strategy. In the SSC versus FSC morphological plot, we gated mononuclear cells and then examined this population for the expression of the relevant surface antigens. We first identified CD34+ cells, which were examined for the dual expression of CD133 and KDR. In the CD34+CD133+ cell gate, we also analyzed the expression of KDR to quantify triple positive cells. A total of 500.000 to 1.000.000 events were acquired for each analysis and the level of progenitor cells was expressed as number of positive events per 1.000.000 total events. In separate analyses, we stained CD34+ and CD133+ cells with a PE-conjugated anti-CD45 mAb to show that most (>90%) of CD34+ and CD133+ cells fall within the CD45dim region. A unique definition of EPCs is lacking (12); for this study, we defined CD34+, CD133+ and CD34+CD133+ cells as generic circulating progenitor cells (CPCs) and CD34+KDR+, CD133+KDR+ and CD34+CD133+KDR+ cells as EPCs.

Cell cycle analysis. To determine the proliferation rate of circulating human CD34+ cells, we performed a cell cycle analysis from fresh whole blood cells for anonymous healthy blood donors. Briefly, after red blood cell lysis, cells were stained with a FITC-conjugated anti-CD34 (BD Biosciences) mAb, fixed and permeabilized and stained with propidium iodide (PI). DNA staining, represented by PI content, was examined in the morphological gate of mononuclear cells and in the immunological gate of mononuclear CD34+ cells. The peak of 2n diploid (G0/G1) cells was identified in the large population of resting circulating mononuclear cells. A gate of PI intensity corresponding to the G2/M phase was also considered. We calculated the percentages of G2-M cells (proliferation rate) in all mononuclear cells and in gated CD34+ cells.

Analysis of apoptosis. The apoptotic rate of circulating CD34+ cells was analyzed in blood samples from healthy blood donors. Briefly, after red blood cell lysis,

cells were stained with a PE-conjugated anti-human CD34 mAb (BD Biosciences), and a FITC-conjugated Annexin V antibody (BD Biosciences), which recognizes early apoptotic cells that externalize phosphatidilserine. CD34+ cell apoptosis was assessed as the percentage expression of Annexin V on CD34+ cells. Dead cells were excluded from the analysis by PI staining.

Rat progenitor cells. A unique definition of rat CPCs and EPCs is lacking. According to previous studies (4; 56), we defined CPCs as Sca-1+c-Kit+ cells and EPCs as Sca-1+CD31+ cells. Similarly to human CPC and EPC phenotypes, CPCs are mainly hematopoietic stem/progenitor cells, while EPCs should be considered to be committed toward the endothelial lineage, because of the expression of the endothelial marker. Briefly, after red blood cell lysis, cells were stained with PE-conjugated anti-Sca-1 (Cederlane) and FITC-conjugated anti-c-Kit (CD117, BD Biosciences) or FITC conjugated anti-CD31 (BD Bisociences). After washing, cells were acquired and scored with a FACS Calibur instrument (BD Biosciences). In the SSC versus FSC morphological plot, we gated mononuclear cells and then examined this population for the expression of Sca-1 and c-kit or Sca-1 and CD31, relative to the respective negative controls. A total of 500.000 to 1.000.000 events were acquired for each analysis and the level of progenitor cells was expressed as number of positive events per 1.000.000 total events.

Fluorescence in situ hybridization. Peripheral blood samples were obtained form 3 women who received a bone marrow transplantation from a male donor more than 12 months earlier. We chose this criterion to assure complete hematopoietic reconstitution. This approach allowed to determine the donor or recipient origin of circulating CD34+ cells by analyzing that Y-chromosome signal. For this purpose, CD34+ cells were freshly sorted and spotted onto glass slides. A human Y chromosome-painting FITC-conjugate probe (Star*FISH,

Cambio, Cambridge, UK) was used. Slides were fixed in formaldehyde in PBS for 5 minutes and pass through three brief rinses in 50, 70 and 100 ethanol grading, after washes in 2X SSC. The DNA probe was applied to the sample and co-denatured at 85°C and then hybridized at 37°C. Washes were performed at 42°C in a solution of 2X SSC/0.3% Nonidet-P40 for 2 minutes. Nuclei were counterstained with TO-PRO 3. Probe was visualized by confocal microscope (TCS-SL Leica, Germany). In the positive and negative controls (represented by male and female cytospin from normal pheripheral blood), at least 200 nuclei were counted to test the efficiency of the probes. Efficiency of hybridization was 89%. The chimerism index was assessed by dividing the number of Y-chromosome-positive cells by the total number of cells of interest. Microphotographs were taken with a TCS-SL laser scanner confocal microscope.

Animal experiments. The animal protocol was approved by the Local and national institutional review boards. All experiments were conducted according to the “Principles of laboratory animal care” (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm). We used 8 week old Fisher 344 (F344) wild type (WT, Charles River Laboratories) and DPP-4 deficient (DPP4null or F344/duCrlCrlj, Charles River Laboratories, Japan) rats, weighting 200-250 g. F344/duCrlCrlj rats express Dpp4 mRNA but lack DPP-4 activity because of the translation of abnormal isoforms that fail to be processed to the biologically active mature glycosylated enzyme. We have previously shown that F344/duCrlCrlj rats >90% reduction in measured plasma DPP-4 activity compared to wild type F344 rats (1.0±0.1 U/l vs. 14.9±1.1 U/l, p<0.001) (55). F344/duCrlCrlj DPP4null rats have a normal phenotype compared to wild type F344 rats.

Induction of diabetes. Diabetes mellitus was induced in F344 WT and DPP4null rats by intraperitoneal injection of streptozotocin (STZ) 20 mg/kg/day in pH 4.5

citrate buffer. Capillary glucose was monitored after 3, 7, 14, and 28 days using a point-of-care glucometer (Freestyle, Abbott Diagnostics). Control (non diabetic) rats were injected with an equal volume of citrate buffer. Only animals with stably elevated (>250 mg/dL, >13.9 mmol/L) capillary glucose were considered as diabetic. We also determined the level of glycated haemoglobin (HbA1c) using a monoclonal antibody-based method (A1c now, Bayer). Animals were housed in controlled conditions to avoid complications attributable to polyuria and de-hydration that may derived from sustained hyperglycemia. Experiments were performed 5-7 weeks after induction of diabetes. Weigh, non fasting plasma glucose and HbA1c of the 4 groups of animals are reported in table 10.

<u>Hind limb ischemia</u>. Hind limb ischemia was induced in F344 WT and DPP4null diabetic and non diabetic rats (n≥5 per group) by femoral artery and vein excision. Briefly, under general gaseous isofluorane anesthesia (Forane, Abbott), a cranio-caudal 2-cm skin incision was made at the level of the right inguinal ligament. After removal of excess fat and accessing the vessels and nerve, the nerve was gently dissected from the vessels and preserved. Then, the femoral artery and vein were dissected from the surrounding tissues and fascia until the knee, cauterized with care to spare all non vascular structures and excised. All visible collaterals were also cauterized and excised. The skin was then closed with a silk suture and animals were allowed to recover from anesthesia and analgesic therapy was administered. Blood samples for enumeration of rat progenitor cells were obtained immediately before induction of ischemia and at days 3 and 7 after ischemia. At day 7, rats were euthanized by intracardiac injection of a cocktail of embutramide, mebenzonium iodide, tetracaine (Tanax, Intervet Italy Srl) and the solues muscles were collected from both ischemic and non ischemic hind limbs for histologic analysis. Before sacrifice, mice were injected systemically with pimonidazole (HydroxyProbe), which forms adducts at thiol groups in proteins at oxygen tension < 10 mm Hg, to stain hypoxic areas.

G-CSF stimulation. For direct bone marrow mobilization of progenitor cells in F344 WT and DPP4null diabetic and non diabetic rats (n≥3 per group), we used human recombinant G-CSF (hrG-CSF, Filgrastim, Amgen) at the dose of 60 microg/kg/day subcutaneously for 4 consecutive days. Blood samples were collected at baseline and after the last dose of G-CSF for progenitor cell count.

Histology. Seven-micrometer thick cryosections of ischemic and non-ischemic soleus muscles from F344 WT and DPP4null diabetic and non diabetic rats were stained with fluoresceing-labelled BSI-Lectin (Molecular Probes) to visualize the vascular network. Muscle fibers were visualized using phase contrast microscopy and images were overlapped with fluorescence microscopy detection of Lectin. We quantified the number of capillaries per area of section to derive a measure of capillary density in 10 randomly selected microscopic fields in 10 sections per muscle. The ischemic/non ischemic ratio of capillary density was then computed for each animal.

To visualize hypoxic areas, muscle sections were stained with a primary monoclonal IgG1 antibody reagent (Hydroxyprobe) that recognizes pimonidazole adducts in hypoxic cells. Immunohistochemical stained area were quantified using ImageJ in randomly selected field.

Apoptosis was assessed in 7 µm-thick criosections using the Apoptag Red Plus In situ Apoptosis Detection Kit (Millipore) according to the manufacture's instruction. Nuclei were counterstained in blue with Hoechst 33258 (Sigma-Aldrich, Schnelldorf, Germany) and the basement membrane was stained with a anti-laminin antibody (Sigma-Aldrich) and a FITC conjugated secondary antibody.

Statistical analysis. Data are expressed as mean ± standard error, or as percentage, where appropriate. Normal distribution of study variables was checked using the Kolmogorov-Smirnov test. Comparison between 2 or more

groups was performed using Student's t test or ANOVA (for normal variables) and with Mann-Whitney's U test and Kruskall-Wallis test (for non-normal variables), respectively. Linear correlations were analyzed using the Pearson's r (for normal variables) or the Spearman's rho (for non-normal data) correlation coefficients.

Groups of patients were created according to their progenitor cell levels as below (termed "low") or above (termed "high") the median value in the peripheral blood and in the bone marrow. To render the separation of patients' groups more extreme, the 50° percentile cutoff was substituted with the 60° percentile cutoff as lower limit of the "high" groups and with the 40° percentile as the higher limit of the "low" groups. The resulting 4 groups were compared using ANOVA or Kruskall-Wallis test and then with appropriate test comparing couples of groups.

The distribution of clinical characteristics in the 4 groups was also analyzed using Self-Organizing-Maps (SOM) and statistically verified visualizations in the whole cohort and in patients belonging to the above mentioned groups defined by the median values or by the 40°/60° percentile. The SOM is an unsupervised pattern recognition method used for automated comparisons in a multivariate profile of clinical characteristics (57). In the map layout, patients with similar clinical characteristics are as close to each other, whereas those who have different characteristics are placed far apart on the map. After computing patients' positions, the map is colored according to the patients' characteristics. To obtain a p-value for each characteristic, a statistical test compares such color distribution with 10,000 random colorings obtained by permuting the data values. A multivariable analysis can also be performed by analyzing combined SOM layouts for different characteristics and was used to test the association of diabetes with progenitor cell compartmentalization group independently of other clinical features. The SOM analysis was performed with the online MeliKerion software and its web-based interface

(http://www.computationalmedicine.fi/software). Statistical significance was accepted at $p<0.05$ and SPSS ver. 16.0 was used.

RESULTS

Direct correlations between peripheral blood and bone marrow stem/progenitor cell levels. We quantified the levels of 6 stem/progenitor cell phenotypes in the PB and BM of 72 subjects, including 12 healthy controls and 60 patients with heart disease (Table 6). For each cell phenotype, there was a significant direct linear correlation between PB and BM levels, which was stronger for CD34+ cells ($r=0.43$, Figure 10A), CD133+CD34+ cells ($r=0.44$) and CD133+KDR+ cells ($r=0.50$) (Table 7). These direct correlations suggest that PB stem cell levels are informative of the BM status. The coefficient of variation was lower for both PB and BM CD34+ cells, the distribution of which was normal and less affected by extreme data. Therefore, we focused on CD34+ cells for further analyses.

Most circulating CD34+ cells are viable, non-proliferating and originated from the bone marrow. As also circulating mature endothelial cells may express CD34 (58), we analyzed the origin of CD34+ cells isolated from 3 female patients who received a male BM transplantation years before, by detecting the Y-chromosome signal using FISH. After adjusting for hybridization efficiency (89%) (59), we found that 92.5±3.1% CD34+ cells were Y+, indicative of a donor BM origin (Figure 10B). Therefore, the contamination by non BM-derived cells within the population of circulating CD34+ cells is <10%. In samples of healthy blood donors, we also show that the apoptotic rate of circulating CD34+ cells is on average 4.0±0.6% and most are resting (>95% in G0-G1 phase) (Figure 10C-D).

Therefore, variations in the number of PB CD34+ cells are mostly attributable to mobilization of BM CD34+ cells.

Distribution of PB and BM CD34+ cells. Despite the significant direct correlation between PB and BM CD34+ cell levels, data were largely dispersed around the regression line, suggesting that circulating CD34+ cell levels are associated with different mobilizing capacity. Thus, we divided patients according to their median values of PB and BM CD34+ cell levels. We defined as “healthy”, patients with high PB and high BM CD34+ cells, because most healthy subjects fell into this subgroup (8/12 of healthy subject, 67%). By converse, patients with low PB and low BM CD34+ cells were defined as “exhausted”. Patients with high PB and low BM CD34+ cell count were defined as “good mobilizers”, while patients with low PB and high BM CD34+ cells were defined as “poor mobilizers”. When we analyzed the clinical characteristics of these 4 groups, using conventional statistics, several cardiovascular risk factors were different, but a few reached significance (not shown). Therefore, as cases with values close to the medians of PB and BM cell counts were poorly informative, we excluded those falling within ±1 decile of the median value, to render groups more “extreme”. In other words, the median was substituted with the 40° and 60° percentiles as lower and higher cut-off, respectively (Figure11). The 40°/60° rule was a compromise to exclude borderline values without excess reduction of sample size.

Stem cell compartmentalization and the cardiovascular risk profile. The 4 groups identified by the 40°/60° percentile of PB and BM CD34+ cell counts were first analyzed for demographics, cardiovascular risk factors and therapies using conventional statistics. We found that subjects in the “healthy” group were (not significantly) younger, had a low number of cardiovascular risk factors (p=0.02),

and a low prevalence of diabetes, obesity and hypertension compared with patients in the “exhausted” group (post-ANOVA tests). The other groups, in which the compartmentalization of CD34+ cells was altered, were associated with higher prevalence of cardiovascular risk factors, and the “exhausted” group showed the worst risk profile. Among therapies, use of beta-blockers and insulin were higher in the group of “good mobilizers”, consistent with the previous findings that these drugs can increase circulating progenitor cell levels (4; 60) (Figure 11).

As stem cell compartmentalization is a bi-dimensional variable, we used self-organizing maps (SOM) to analyze the association between clinical characteristics of the patients and their belonging group in 2D planes. This visual and statistical approach shows co-localization of several high-risk features with the group of “exhausted” patients (age, diabetes, hypertension and obesity) or poor mobilizers (age, hypertension and obesity). Overlapping maps that represent groups with those that represent clinical features allows to describe the phenotype of a typical patient of each group. In compliance with its higher statistical power, SOM show significant and almost identical final results in the whole study cohort (Figure 12) and in patients of the 40°/60° percentile groups (Figure 17). A multivariable analysis run on SOM revealed that diabetes was associated with group distribution independently of age, obesity, hypertension and therapies. We also found that diabetes was a strong determinant of reduced PB CD34+ cells independently of BM CD34+ cells, suggesting that diabetes compromises CD34+ cell mobilization (Table 8). These data indicate that altered CD34+ cell compartmentalization is associated with diabetes and a high cardiovascular risk profile.

Compartmentalization was then repeated for other progenitor cell phenotypes (Table 9). Altered PB versus BM CD133+ and CD34+CD133+ cell compartmentalization was associated with a high cardiovascular risk profile,

similar to what obtained with CD34+ cell compartmentalization. However, the discriminative capacity of CD34+ cells was higher, as there were more clinical features differing among groups. These data confirm that CD34+ cell compartmentalization offers the best correlate of cardiovascular risk.

Stem cell compartmentalization and regulators of angiogenesis. We quantified the plasma concentrations of a panel of angiogenesis-regulating factors (Table 9). When patients were divided according to CD34+ cell compartmentalization, plasma VEGF concentrations and DPP-4 activity were lower in the “healthy” group, while other factors were not significantly different. SOM showed that high activity of the enzyme DPP-4 co-localizes with the “exhausted” phenotype (Figure 18).

The “healthy” group according to CD133+ and CD34+CD133+ cell compartmentalization had higher concentrations of the pro-angiogenic factors Angiopoietin-2 and PDGF-BB. Compartmentalization of CD34+CD133+KDR+ cells revealed high SDF-1α concentrations in the “healthy” and “good mobilizer” groups, consistent with the notion that circulating SDF-1α mobilizes EPCs (61). These data indicate that some mobilizing factors may explain progenitor cell compartmentalization in this cohort of patients. We also suggest that this clinical data-driven approach is a suitable platform to identify new strategies of progenitor cell mobilization.

We then aimed at validating one of these factors in diabetes, as a model of altered progenitor cell mobilization.

DPP-4 deficiency restores ischemia- but not G-CSF-induced mobilization in diabetes. DPP-4 has been shown to cleave pro-angiogenic factors, including SDF-1α (62). Consistently with this finding, in the whole cohort of subjects, we found a negative correlation between DPP-4 activity and plasma SDF-1α

concentrations (r=-0.27; p=0.017). Plasma DPP-4 activity was also directly correlated to the BM/PB ratio of CD34+ cells (r=0.25, p=0.028), supporting the hypothesis that DPP-4 regulates CD34+ cell mobilization. DPP-4 activity is increased in diabetic patients and its inhibition restores EPC levels in type 2 diabetes (40; 63). Taking into account that stem cell compartmentalization was associated with both diabetes and DPP-4 activity in our study, we hypothesized that DPP-4 may be a suitable target to restore progenitor cell mobilization in diabetes. We first analyzed DPP-4 activity in rats and found increased activity in PB and decreased activity in BM of diabetic compared to non diabetic rats (Figure 13). Then, we tested the bone marrow progenitor cell mobilization in wild type and DPP-4 deficient rats after either G-CSF stimulation or hind limb ischemia.

A 5-day course of G-CSF administration increased circulating Sca-1+c-Kit+ cells in non diabetic wild type rats, while this effect was markedly blunted in streptozotocin diabetic animals. Additionally, DPP-4 activity in the BM increased in non diabetic but not in diabetic animals (Figure 13). DPP-4 deficiency completely abolished Sca-1+c-Kit+ and Sca-1+CD31+ progenitor cell mobilization in diabetic and non-diabetic animals (Figure 14). These results confirm previous findings in non diabetic mice that DPP-4 is required for the mobilization effect of G-CSF (64) and indicate that the reduced DPP-4 activity in the BM contributes to unresponsiveness to G-CSF in diabetes.

In wild type non diabetic animals, hind limb ischemia induced a transient increase in Sca-1+c-Kit+ progenitor cells and Sca-1+CD31+ EPCs at day 3, which returned to baseline at day 7. This surge in progenitor cell levels after ischemia was completely abolished in wild type diabetic animals, which showed a paradoxical decrease in Sca-1+c-Kit+ and Sca-1+CD31+ cells at day 3 (Figure 15A). In DPP-4 deficient rats, diabetes did not impair post-ischemic increase in Sca-1+c-Kit+ progenitor cells, which was similar to non diabetic rats. Rather, the

increase of Sca-1+CD31+ EPCs at day 3 in diabetic DPP-4 deficient rats was even higher than that of non diabetic DPP-4 deficient rats. As a counterpart of progenitor cell mobilization, we analyzed microvascular density, blood flow, degree of ischemia and apoptosis in muscles at day 7. Recovery of capillary density was slightly impaired in wild type diabetic versus non diabetic rats, while it was not in DPP4null diabetic versus non diabetic rats (Figure 15B). The degree of tissue ischemia, as assessed by staining with the hypoxic probe pimonidazole, was higher in diabetic compared to non diabetic animals and was significantly lower in DPP-4null rats (Figure 15C). Therefore, DPP-4 deficiency abolished the detrimental effects of diabetes on development of ischemia and skeletal muscle microvascular recovery. We found no significant differences in superficial skin perfusion as assessed by Laser Doppler Imaging (Figure 19), suggesting that differences in blood flow recovery among groups occur earlier than day 7. Finally, the number of apoptotic myofibers, assessed by the ApopTag kit (in situ TUNEL labelling), was very low in all samples (average<2/field) and there was a non-significant trend toward reduced apoptosis in DPP-4null rats (Figure 20). Muscle DPP-4 activity was low (about 10% of that in PB) and unaffected by diabetes or ischemia (not shown), ruling out that local DPP-4 targets are responsible for the effects observed in DPP-4null rats.

DATA INTERPRETATION AND DISCUSSION

This study, by combining a clinical data-driven discovery approach with animal models, shows that altered mobilization of stem/progenitor cells is associated with a high cardiovascular risk profile and, in the setting of diabetes, is driven by a tissue-specific DPP-4 dysregulation.

Investigation into the mechanisms of reduced progenitor cell levels has led to several hypothesis, including excess apoptosis and senescence. In type 2

diabetes, we have previously shown that apoptosis is not responsible for the observed low level of circulating CD34+ cells (24). Rather, animal models suggest that BM alterations account for depressed circulating progenitor cells in diabetes (5; 6; 8). Unfortunately, the poor availability of BM samples limits our understanding of BM function in human diabetes. Herein, we analyzed BM and PB samples from patients undergoing cell therapy for heart disease and healthy volunteers. The direct correlation between BM and PB progenitor cell levels, although scattered, indicates that circulating progenitor cell levels are informative of the BM status. We also show that most circulating CD34+ cells are BM-derived, non-apoptotic and non-proliferating. As long as homing to the target tissues is not increased (65), these data indicate that variations in circulating CD34+ cells among patients are attributable to different capacity of BM mobilization. This allowed to categorize patients according to their BM and PB progenitor cell status into distinct mobilizer phenotypes. This original approach, although based on a snapshot of a dynamic system, led to the discovery that individuals with an altered distribution of CD34+ cells in the BM and PB display a strikingly high cardiovascular risk profile, characterized by aging, diabetes, obesity and hypertension. Using self-organizing maps, we visually and statistically analyzed cardiovascular risk factors in relation to the mobilizer phenotype: DM was strongly associated with exhaustion of PB and BM CD34+ cells independently of the distribution of other risk factors. While reduction of circulating progenitors is a consistent finding in animal and human DM, the effect of DM on the amount of BM progenitors is debated. Long-term diabetic mice appear to have reduced BM Sca-1+c-kit+ hematopoietic stem cells (5; 7), while short-term diabetic rats showed no reduction of BM Sca-1+c-kit+ (4) and short-term diabetic mice had normal (33) or even increased BM Sca-1+c-kit+ (8). While methodological issues can explain these discrepancies, we suggest that the low CD34+ cell count in BM aspirates of DM patients indicates a reduced

accessibility of stem cell niches to the aspiration manoeuvre, in compliance with the observation that the diabetic BM niche is sticky and more prone to stem cell retention than mobilization (8).

Among other clinical features, it appears that use of beta-blockers and insulin was associated with a good mobilizer phenotype. Experimental studies support a role for beta-blockers (60) and insulin therapy (4; 66) in improving progenitor cell mobilization, further indicating that the clinical phenotype associated with stem cell compartmentalization is biologically plausible.

Thanks to their instructing spatial networks, SOM provide complementary and incremental information over classic statistical group comparison, also indicating that the “good mobilizer” and, to a lesser extent, the “poor mobilizer” are less well defined phenotypes than the “healthy” and “exhausted”, as evidence from the uneven spatial distribution and a high p-value. Importantly, SOM allowed description of relevant clinical associations in the whole study cohort and is independent of the method of patients’ partitioning (the 40°/60° percentile rule).

We tested the plasma concentration of a selected range of regulators of angiogenesis to evaluate whether our stem cell compartmentalization analysis is a suitable benchmark for the discovery of stem cell mobilizing factors. Plausible associations were indeed found, such as between the CD34+CD133+KDR+ EPCs “healthy” and “good mobilizer” phenotypes and high concentrations of the mobilizing chemokine SDF-1α. An in-depth analysis of the plasma proteome could allow the identification of new mobilizing factors.

In addition, we found low activity of DPP-4 in the CD34+ “healthy” phenotype and high activity in patients with disturbed stem cell compartmentalization and mobilization. As peripheral DPP-4 activity is increased in DM (55), we performed pre-clinical experiments to validate causality of this association in the defective stem cell mobilization observed in DM. We employed 2 assays of stem/progenitor cell mobilization because the molecular mechanisms involved are different and

may be differently affected by DPP-4. First, we confirm that both stimuli fail to mobilize hematopoietic (Sca-1+c-Kit+) and endothelial (Sca-1+CD31+) progenitor cells in diabetic rats, lending further support to the diabetic bone marrow failure. There are important implications of these findings, as BM stem/progenitor cell mobilization after ischemia is a physiologic response, while responsiveness to pharmacologic stimulation could be used therapeutically (67). The phenotypes used to define rat and human progenitor cells differ because there is no rat homology of human CD34 and CD133. Moreover, Sca-1+c-Kit+ cells contain hematopoietic progenitor cells in mice and rats, while co-expression of the endothelial antigen CD31 on Sca-1+ cells is compatible with a EPC phenotype (12). Additionally, previous studies have found that these rat progenitor cell phenotypes are responsive to mobilizing stimuli (4; 56). Interestingly, DPP-4 deficiency restored ischemia-induced progenitor cell mobilization in diabetic rats, reduced the degree of ischemia and preserved from the detrimental effects of diabetes on microvascular recovery. These data suggest that DPP-4 deficiency improves the outcome of ischemic tissues, although the number of capillaries in genetically deficient mice might be influenced by the resulting phenotype and the link with progenitor cell mobilization is indirect.

We have previously demonstrated that impaired post-ischemic progenitor cell mobilization in diabetic rats is attributable to an altered activation of the hypoxia-sensing system HIF-1α leading to a blunted release of the chemokine SDF-1α, a circulating mobilizing stimulus (4). As SDF-1α is a natural substrate of DPP-4 and is increased in diabetic patients after treatment with a DPP-4 inhibitor (40), excess DPP-4 activity is therefore one mechanism for reduced of SDF-1α in diabetes. Thus, we hypothesize that restored peripheral SDF-1α concentrations in DPP4null rats preserved the ability to mobilize BM progenitors. The measure of rat intact versus cleaved SDF-1α is challenging because traditional sandwich ELISA poorly discriminate between the two forms. However, proof-of-concept

mass spectrometry analysis of mouse tissues confirmed that absence of DPP-4 reduces cleavage of native SDF-1α (62).

Differently from the post-ischemic assay, DPP-4 deficiency did not restore G-CSF induced HSC and EPC mobilization in diabetes, confirming data obtained in non-diabetic Dpp4-/- mice (64). The mobilizing effect of G-CSF relies on the establishment of a SDF-1α chemotactic gradient towards the vasculature, by reducing intra-marrow concentrations of SDF-1α, through DPP-4 induction and suppression of stromal cell activity. We now report that diabetes reduces BM DPP-4 activity, which was not stimulated by G-CSF, likely leading to high intra-marrow SDF-1α levels, a retention signal for stem cells. This is consistent with the previous observation of a failed SDF-1α switch in the BM in response to tissue injury in diabetes (33). Indeed, while DPP-4 deficiency restored the peripheral signal from the ischemic tissue to the BM, response to G-CSF remained deficient. Altogether these data indicate that tissue-specific DPP-4 dysregulation accounts for both defective post-ischemic and G-CSF induced mobilization (Figure 16). Recently, it has been demonstrated that DPP-4 negatively regulates CSF activity and stress hematopoiesis (68). Indeed, several CSF types, including native G-CSF, GM-CSF, erythropoietin, and IL-3 appear to be substrates of DPP-4 enzymatic activity (68). Cleavage of CSFs by DPP-4 reduces their biological activities and generates natural competitive inhibitors of receptor activation. DPP-4 inhibition or genetic deletion improved hematopoietic responses to stressing conditions, such as chemotherapy or radiation. Given that diabetic patients suffer from anemia and delayed recovery after myeloablation (8), DPP-4 inhibition holds clinical promise also in the setting of stressful conditions to which diabetic patients may be exposed. Mechanistically based on these data, as G-CSF induces DPP-4 and DPP-4 in turn cleaves G-CSF, this appears to be a negative feedback loop regulation of the balance between mobilization and engraftment of stem cells. Interestingly however, DPP-4 is not

able to cleave methionylated CSF such as hrG-CSF (Filgrastim), explaining why DPP-4 deficient rats are still not susceptible to G-CSF induced mobilization. A disruption of this mechanism in diabetes may have profound clinical implications for homeostasis of the hematopoietic system and its cross-talk with cardiovascular biology.

To sum up, we have conducted a reverse translational study that stems from the observation of the clinical phenotype in patients with disturbed progenitor cell mobilization and leads to the discovery of a central role for DPP-4 in the diabetic stem cell mobilopathy.

In this clinical data-driven discovery approach for regenerative medicine, we used a targeted strategy to screen soluble factors potentially involved in progenitor cell regulation, but future development into the human plasma proteome analysis may allow an unbiased approach.

The tissue specific regulation of DPP-4 in diabetes sheds light on the different mechanisms of ischemia- and GCSF-induced mobilization and has therapeutic implications based on clinically available DPP-4 inhibitors (63).

CONCLUSIONS

The 3 substudies presented here constitute a unique investigation into bone marrow function in clinical and experimental diabetes. Using very different methodological approaches, each substudy clearly shows that diabetes leads to an impairment in bone marrow stem/progenitor cell mobilization. This was apparent from a prospective clinical trial of direct bone marrow stimulation with human recombinant G-CSF in diabetic and control patients (Part 1), as well as from a meta-regression analysis of clinical trials using G-CSF to stimulate cardiovascular repair in patients with different forms of cardiovascular disease. Further, by analysing coupled bone marrow and peripheral blood samples of healthy and diseased patients in Part 3, an altered distribution of stem cells between these two compartments was noted in diabetic patients. In Part 1, initial mechanistic data were also provided, by showing a maladaptive DPP-4 response to G-CSF in diabetic patients. This was further confirmed in Part 3, where a correlation between DPP-4 activity and stem cell compartmentalization was noted. In the pre-clinical substudy of Part 3, a tissue specific dysregulation of DPP-4 in diabetic animals emerged as responsible for the defective G-CSF-induced and post-ischemic stem/progenitor cell mobilization. The clinically-relevant counterpart of these observations is that DPP-4 deletion restored ischemia-induced mobilization and improved tissue recovery after ischemic damage. Altogether, these data point to the existence of a primary bone marrow defect in diabetes, which has important implications for the development of

chronic complications and can be countered by modulation of DPP-4 activity. The translation of these findings into clinical practice may turn out to be life saving in diabetic patients.

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TABLES

Table 1. Characteristics of the study subjects in Part 1. In the left columns, all DM and non DM patients are compared. In the other columns, type 1 DM patients and type 2 DM patients are shown separately and compared with age-matched non DM controls. *$p<0.05$ for T1D versus T2D.

Characteristic	**All DM subjects**	**All non DM subjects**	**p**	**T1D**	**Age-matched controls**	**p**	**T2D**	**Age-matched controls**	**p**
Number	24	14	-	10	8	-	14	6	-
Age, yrs	48.8±2.5	40.1±3.8	0.06	37.1±2.6	29.4±2.3	0.06	57.1±1.6	54.5±2.7	0.39
Sex male, n (%)	20 (83.3)	11 (78.6)	0.73	8 (80.0)	6 (75.0)	0.81	12 (85.7)	5 (83.3)	0.89
Body mass index, kg/m^2	28.0±1.2	25.8±1.3	0.26	25.7±1.0	24.3±1.9	0.49	29.6±1.9	27.5±1.6	0.51
HbA1c, %	7.9±0.3	-	-	7.7±0.3	-	-	8.0±0.4	-	-
Plasma glucose, mg/dL	151.3±7.8	84.2±3.1	<0.01	148.2±6.3	80.9±3.6	<0.01	155.7±8.1	89.3±7.4	<0.01
Serum creatinine, µmol/L	78.2±6.5	81.3±3.4	0.65	77.4±5.4	78.2±3.8	0.88	84.3±4.5	83.7±2.9	0.89
Diabetes duration, yrs	16.1±2.3	-	-	21.2±3.1	-	-	12.5±2.9*	-	-
Hypertension, n (%)	15 (62.5)	2 (14.3)	0.01	2 (20.0)	0 (0.0)	0.56	13 (92.8)*	2 (33.3)	0.03
Complications									
Cardiovascular disease, n (%)	6 (25.0)	0 (0.0)	0.10	0 (0.0)	0 (0.0)	-	6 (42.9)*	0 (0.0)	0.07
Retinopathy, n (%)	8 (33.3)	-	-	5 (50.0)	-	-	3 (21.4)	-	-
Nephropathy, n (%)	2 (8.3)	-	-	1 (10.0)	-	-	1 (7.1)	-	-
Neuropathy, n (%)	6 (25.0)	-	-	3 (30.0)	-	-	3 (21.4)	-	-
Medications									
Insulin, n (%)	20 (83.3)	-	-	10 (100)	-	-	10 (77.0)	-	-
Oral anti-diabetic agents, n (%)	11 (43.8)	-	-	0 (0.0)	-	-	11 (78.6)*	-	-
ACE-inhibitors, n (%)	13 (54.2)	1 (7.1)	0.01	3 (30.0)	0 (0.0)	0.29	10 (77.0)	1 (16.7)	0.03
Other anti-hypertensives, n (%)	9 (37.5)	0 (0.0)	<0.01	2 (20.0)	0 (0.0)	0.56	7 (50.0)	0 (0.0)	0.05
Statins, n (%)	12 (50.0)	0 (0.0)	<0.01	2 (20.0)	0 (0.0)	0.56	10 (77.0)*	0 (0.0)	<0.01

Table 2. Hematological parameters and absolute progenitor cell counts in DM and non DM patients before and after administration of hrG-CSF in Part 1. *p<0.05 versus baseline.

Variable	Diabetic patients (n=24)		Non diabetic subjects (n=14)	
	Baseline	Post hrG-CSF	Baseline	Post hrG-CSF
White blood cells x 10^3 / μL	6.53±0.32	21.88±0.82*	6.05±0.34	23.54±1.14*
Neutrophils x 10^3 / μL	3.57±0.26	17.91±0.74*	3.29±0.22	19.94±0.97*
Lymphocytes x 10^3 / μL	2.20±0.10	2.60±0.14	2.02±0.12	2.34±0.20
Monocytes x 10^3 / μL	0.55±0.03	1.06±0.04*	0.52±0.05	1.00±0.04*
Eosinophils x 10^3 / μL	0.18±0.02	0.28±0.03	0.19±0.04	0.27±0.06
Basophils x 10^3 / μL	0.03±0.003	0.04±0.004	0.03±0.005	0.04±0.003
Red blood cells x 10^6 / μL	4.88±0.07	4.82±0.08	5.04±0.11	4.91±0.12
Platelets x 10^3 / μL	235.4±7.5	231.5±8.7	243.0±12.4	237.6±13.1
$CD34^+$ cells / mL	3236±269	3289±269	2837±351	6312±743*
$CD133^+$ cells / mL	1884±185	2128±212	937±184	2476±409*
$CD34^+CD133^+$ cells / mL	1204±179	1423±193	719±156	2152±374*
$CD34^+KDR^+$ cells / mL	253±34	223±38	201±46	515±145*
$CD133^+KDR^+$ cells / mL	287±55	274±40	249±84	754±266*
$CD34^+CD133^+KDR^+$ / mL	31±8	27±7	38±11	86±27

Table 3. Characteristics of the trials included in the meta-regression analysis in Part 2. Total G-CSF dose is reported in µg/kg. CD34+ cells refers to the achieved CD34+ cell count (cells / µL ± standard deviation) at completion of the G-CSF course. N, number of patients. HT, hypertension. DL, dyslipidemia. STEMI, ST-elevation myocardial infarction. CLI, critical limb ischemia. CHF, chronic heart failure. IHD, ischemic heart disease. IA, intractable angina. * Calculated. † Derived from figures. ‡ reports CD34+CD133+ cell count. N/A, not available.

Authors (ref)	G-CSF dose	Days	CD34+/µL	N	%DM	Age	%Male	%HT	%DL	%Smokers	Disease
Achilli et al. (69)	50.0	5.0	56.7 ± 61.1	29	20.7	61.0 ± 8.0	100.0	48.0	59.0	59.0	STEMI
Arai et al. (70)	35.0	10.0	4.7 ± 0.9	14	50.0	69.0 ± 2.0	50.0	N/A	N/A	N/A	CLI
Boyle et al. (71)	40.0	4.0	24.3 ± 9.0	5	40.0	65.4 ± N/A	80.0	100.0	100.0	0.0	IHD
Burt et al. (49)	45.0	4.5	20.2 ± 17.4	9	44.4	68.1 ± 19.3	55.6	77.8	55.6	77.8	CLI
Chih et al. (72)	22.5	5.0	33.0 ± 8.0†	18	22.2	62.0 ± 7.0	89.0	78.0	83.0	72.0	IHD
Ellis et al. (A) (73)	25.0	5.0	37.0 ± 30.0	6	16.7	53.0 ± 15.0	100.0	50.0	N/A	40.0	STEMI
Ellis et al. (B) (73)	50.0	5.0	29.0 ± 14.0	6	33.3	60.0 ± 11.0	83.0	33.0	N/A	50.0	STEMI
Engelmann et al. (74)	50.0	5.0	46.1 ± 32.9‡	23	21.7	60.0 ± 11.0	87.0	74.0	57.0	65.0	STEMI
Huttman et al. (75)	88.6	10.0	20.4 ± 22.4*	16	37.5	61.0 ± 10.0	100.0	56.3	81.3	62.5	CHF
Ince et al. (76)	60.0	6.0	66.0 ± 54.0	15	13.3	51.1 ± 8.2	93.3	40.0	66.7	80.0	STEMI
Ince et al. (77)	60.0	6.0	64.6 ± 37.1	25	8.0	50.5 ± 7.9	92.0	40.0	64.0	80.0	STEMI
Karimabad et al. (78)	50.0	5.0	24.3 ± 8.6	8	50.0	48.6 ± 8.7	87.5	12.5	12.5	62.5	STEMI
Kluethe et al. (79)	70.0	7.0	75.8 ± 44.6	14	21.4	57.7 ± 11.5	100.0	N/A	35.7	35.7	STEMI
Kovacic et al. (80)	50.0	5.0	100.0 ± 17.0†	20	15.0	62.4 ± 9.1	90.0	90.0	100.0	0.0	IA
Leone et al. (81)	50.0	5.0	50.3 ± 35.2	14	14.3	53.0 ± 11.0	93.0	57.0	64.0	71.0	STEMI
Losordo et al. (82)	25.0	5.0	28.7 ± 16.5	24	41.7	62.4 ± 25.0	79.2	77.8	94.4	72.2	IA
Ripa et al. (83)	60.0	6.0	55.1 ± 53.3	39	7.7	57.4 ± 8.6	72.0	33.0	N/A	56.0	STEMI
Ripa et al. (84)	60.0	6.0	47.1 ± 8.8	16	43.8	62.0 ± 9.0	87.5	44.0	100.0	13.0	IHD
Sprigg et al. (85)	50.0	5.0	37.7 ± 36.7	24	29.2	76.0 ± 9.0	50.0	75.0	33.0	N/A	Stroke
Stein et al. (86)	50.0	5.0	72.0 ± 20.0	56	10.7	59.4 ± 12.0	78.6	55.4	41.1	44.6	STEMI
Suzuki et al. (A) (87)	31.0	10.0	3.9 ± 2.1	11	54.5	63.0 ± 10.0	72.7	81.8	81.8	18.2	IA
Suzuki et al. (B) (87)	26.0	10.0	9.0 ± 4.7	12	25.0	65.0 ± 9.0	83.3	50.0	33.3	N/A	STEMI
Takano et al. (88)	12.5	5.0	15.0 ± 19.8	18	33.3	61.0 ± 8.0	77.8	44.4	33.3	50.0	STEMI
Valgimigli et al. (89)	20.0	4.0	33.0 ± 7.0†	10	20.0	62.0 ± 9.0	80.0	60.0	N/A	40.0	STEMI
Wang et al. (90)	30.0	6.0	22.9 ± 4.0*	13	46.2	63.0 ± 2.0	84.6	N/A	100.0	N/A	IHD
Wolfram et al. (91)	35.0	7.0	33.0 ± 22.8	8	37.5	69.0 ± 10.0	62.5	100.0	N/A	N/A	IA

Table 4. Pooled characteristics of the patients from trials included in the primary analysis of Part 2 substudy.

Characteristic	**Weighted average**
Number	453
Age, years (range)	60.7 ± 21.1 (50-76)
Male sex, % (range)	81.7 (50-100)
Diabetes, % (range)	24.7 (8-55)
Hypertension, % (range)	58.0 (13-100)
Dyslipidemia, % (range)	62.4 (13-100)
Smoking, % (range)	52.4 (18-80)
Type of disease	
ST-elevation myocardial infarction, n (%)	275 (60.7)
Chronic heart failure, n (%)	16 (3.5)
Ischemic heart disease, n (%)	52 (11.5)
Intractable angina, n (%)	63 (13.9)
Stroke, n (%)	24 (5.3)
Critical limb ischemia, n (%)	23 (5.1)
Total G-CSF dose, µg/kg (range)	46.6 ± 7.3 (12.5-88.6)
Treatment duration, days (range)	6.0 ± 1.9 (4-10)

Table 5. Multivariable analysis conducted in the n=16 trials reporting data on all the covariates (Part 2). The dependent variable was achieved CD34+ cell count.

Variable	**β-coefficient**	**P**
Diabetes mellitus	-1.128	0.028
Mean age	0.158	0.710
Male gender	-0.114	0.731
Hypertension	-0.163	0.726
Dyslipidemia	-0.460	0.384
Smoke	-0.113	0.671
Underlying disease	0.760	0.176
Sample size	0.050	0.867

Table 6. Characteristics of the study population in Part 3. *p<0.05.

Characteristic	**Healthy subjects (n=12)**	**Patients (n=60)**
Age (years)	31.3±2.0	58.3±1.8*
Male sex (%)	40.0	83.3*
Smoking habit (%)	33.3	25.9
Obesity (%)	0.0	41.6*
Family history for CV (%)	0.0	46.6*
Diabetes mellitus (%)	0.0	28.3*
Plasma glucose (mg/dL)	81.8±4.1	118.3±7.8*
Hypertension (%)	0.0	70.7*
Dyslipidemia (%)	0.0	70.2*
Total cholesterol	175.6±11.9	174.8±7.0
HDL cholesterol (mg/dL)	68.9±9.6	48.0±1.9*
Triglicerydes (mg/dL)	93.6±14.3	164.2±17.3
LDL cholesterol (mg/dL)	98.5±10.1	91.2±5.5
Medications		
Statin (%)	0.0	68.9*
ACE inhibitor /AR blocker (%)	0.0	74.1*
Beta-blockers (%)	0.0	77.6*
Other anti-hypertensives (%)	0.0	12.1
Insulin (%)	0.0	8.6
Oral anti-diabetic agents (%)	0.0	10.3

Table 7. Cross-correlation analysis of progenitor cell levels in Part 3. *p<0.05.

		Peripheral blood					
		CD34+	**CD133+**	**CD34+CD133+**	**CD34+KDR+**	**CD133+KDR+**	**CD34+CD133+KDR+**
Bone marrow	**CD34+**	0,43*	0,20	0,29*	0,27*	0,08	0,16
	CD133+	0,45*	0,31*	0,41*	0,20	0,01	0,16
	CD34+CD133+	0,44*	0,30*	0,44*	0,21	0,00	0,20
	CD34+KDR+	0,05	0,13	0,05	0,35*	0,43*	0,42*
	CD133+KDR+	0,06	0,17	0,13	0,30*	0,50*	0,46*
	CD34+CD133+KDR+	0,15	0,20	0,30*	0,19	0,28*	0,37*

Table 8. Multivariable linear regression analysis for identification of determinants of peripheral blood CD34+ cell count (dependent variable) in Part 3.

Variable	Beta coefficient	p value
Bone marrow CD34+ cells	0.392	0.002
Age	-0.110	0.450
Diabetes	-0.300	0.040
Hypertension	-0.044	0.754
Obesity	-0.032	0.813
Beta-blocker use	0.171	0.172
Insulin use	0.349	0.01

Table 9. Clinical characteristics and progenitor cell compartmentalization in Part 3. Group 1 "Healthy", group 2 "poor mobilizers" group 3 "good mobilizers", group 4, "exhausted".

Characteristic	CD34+	CD133+	CD34+CD133+	CD34+KDR+	CD133+KDR+	CD34+CD133+KDR+
Demographics						
Age	NS	Lower in group 1	NS	Higher in group 2	NS	NS
Sex	NS	NS	NS	NS	NS	NS
Risk factors						
No. risk factors	Lower in group 1	Lower in group 1	Lower in group 1	NS	NS	NS
Smoke	NS	NS	NS	NS	NS	NS
Family History	NS	Higher in group 4	Higher in group 4	NS	NS	Lower in group 4
Diabetes	Lower in group 1	NS	NS	NS	NS	NS
Hypertension	Lower in group 1	Lower in group 1	Lower in group 1	Lower in group 3	NS	NS
Dyslipidemia	NS	NS	NS	NS	NS	Lower in group 4
Obesity	Lower in group 1	NS	NS	NS	NS	NS
Medications						
Statins	NS	Lower in group 1 Higher in group 2	NS	NS	NS	NS
ACEi / ARBs	NS	NS	NS	NS	NS	NS
Beta-blockers	Lower in group 2 Higher in group 3	NS	NS	NS	NS	NS
Insulin	Higher in group 3	NS	NS	NS	NS	NS
Angiogenic factors						
Angiopoietin-2	NS	Higher in group 1	Higher in group 1	NS	NS	NS
Follistatin	NS	NS	NS	NS	NS	NS
G-CSF	NS	NS	NS	NS	NS	NS
IL-8	NS	NS	NS	NS	NS	NS
Leptin	NS	NS	NS	NS	NS	Higher in group 3
PDGF-BB	NS	Higher in group 1	Higher in group 1	NS	NS	NS
PECAM-1	NS	NS	NS	NS	NS	NS
VEGF	Lower in group 1	NS	NS	NS	NS	NS
SDF-1	NS	NS	NS	NS	NS	Higher in group 1 and 3
SCF	NS	NS	NS	NS	NS	NS
IL-18	NS	NS	NS	NS	NS	NS

Table 10. Body weight, plasma glucose and HbA1c of the four groups of animals (Part 3). *p<0.05 versus non diabetic of the same strain. There were no significant differences between non diabetic wild type and DPP4null mice and between diabetic wild type and DPP4null mice.

	Wild type non diabetic	**DPP4null non diabetic**	**Wild type Diabetic**	**DPP4 null diabetic**
Weight (g)	348±17	338±14	229±14 *	236±7 *
Plasma glucose (mg/dL)	154±23	120±6	480±42 *	410±22 *
HbA1c (%)	4.3±0.1	4.5±0.1	12.1±0.7 *	11±1.1 *

Figure 1. CD34$^+$ cell mobilization after hrG-CSF. A) Absolute pre- and post-G-CSF CD34$^+$ cell count in all DM and all non DM controls (study primary end-point). *p<0.05 versus baseline. B-C) Absolute pre- and post-G-CSF CD34$^+$ cell count in type 1 DM compared to young controls (B) and in type 2 DM compared to age-matched controls (C). *p<0.05 versus baseline. D) Changes in the absolute levels of circulating CD34$^+$ cell counts. *p<0.05 versus DM.

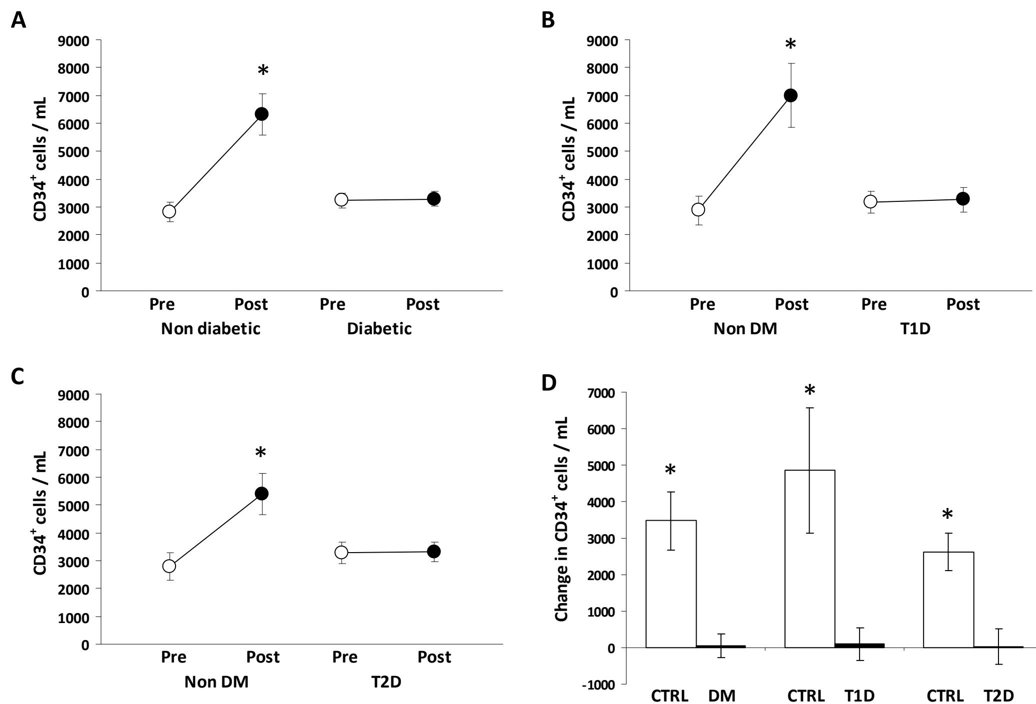


Figure 2. Mobilization of other progenitor cell phenotypes after hrG-CSF. A-E) Absolute pre- and post-G-CSF cell counts of circulating hematopoietic stem cells ($CD133^+$, $CD34^+CD133^+$) and endothelial progenitor cells ($CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$) in DM and non DM patients. *p<0.05 versus baseline. F) Changes in the absolute levels of stem/progenitor cell phenotypes in DM and non DM patients. *p<0.05 versus non DM controls.

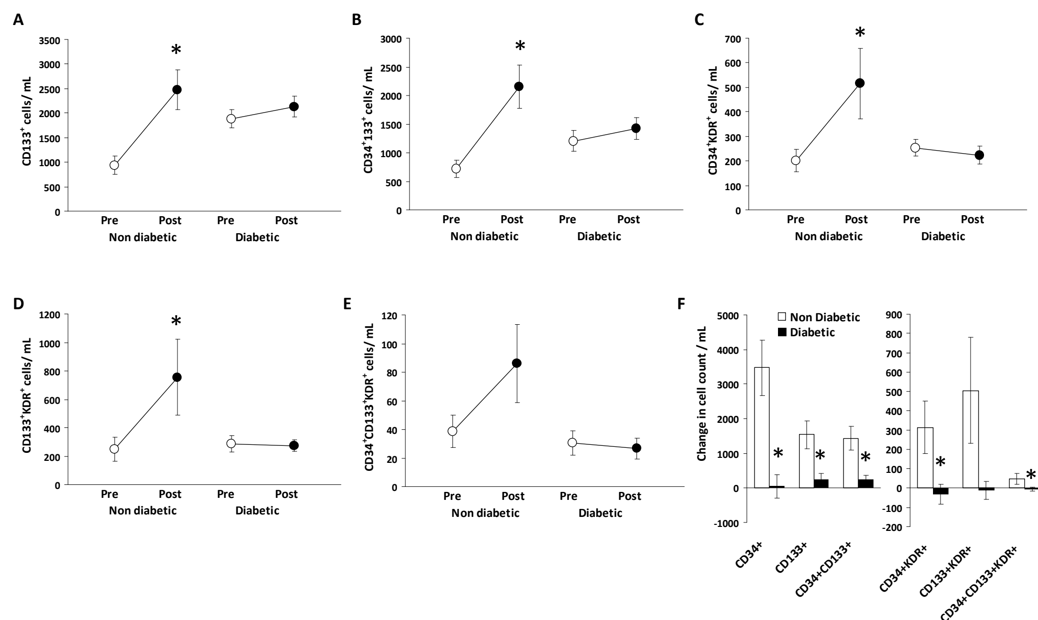


Figure 3. Effects of hrG-CSF on CD26/DPP-4 expression. Percentage CD26/DPP-4 expression on $CD34^+$ cells was significantly (*$p<0.05$) increased in non DM controls, while it was reduced in DM patients.

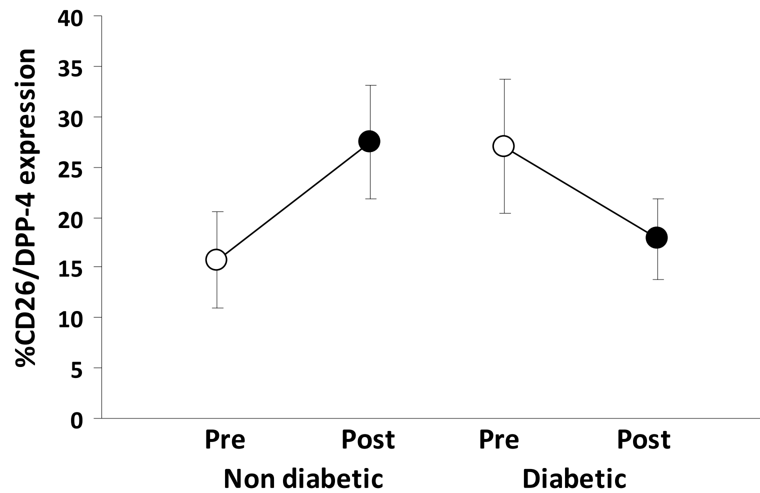


Figure 4. In vivo proangiogenic cell function. Patients' PBMC before collected pre- and post-GCSF were embedded into Matrigel plugs and implanted in immunodeficient mice. Representative gross appearance of the plugs (scale bar = 1 cm) and histological sections (scale bar 100 micron) are shown. In the bottom, quantification of hemoglobin content, a quantitative surrogate of perfusion, adjusted for mobilized monocytes, is shown. *p<0.05 versus pre-GCSF; † p<0.05 versus non DM.

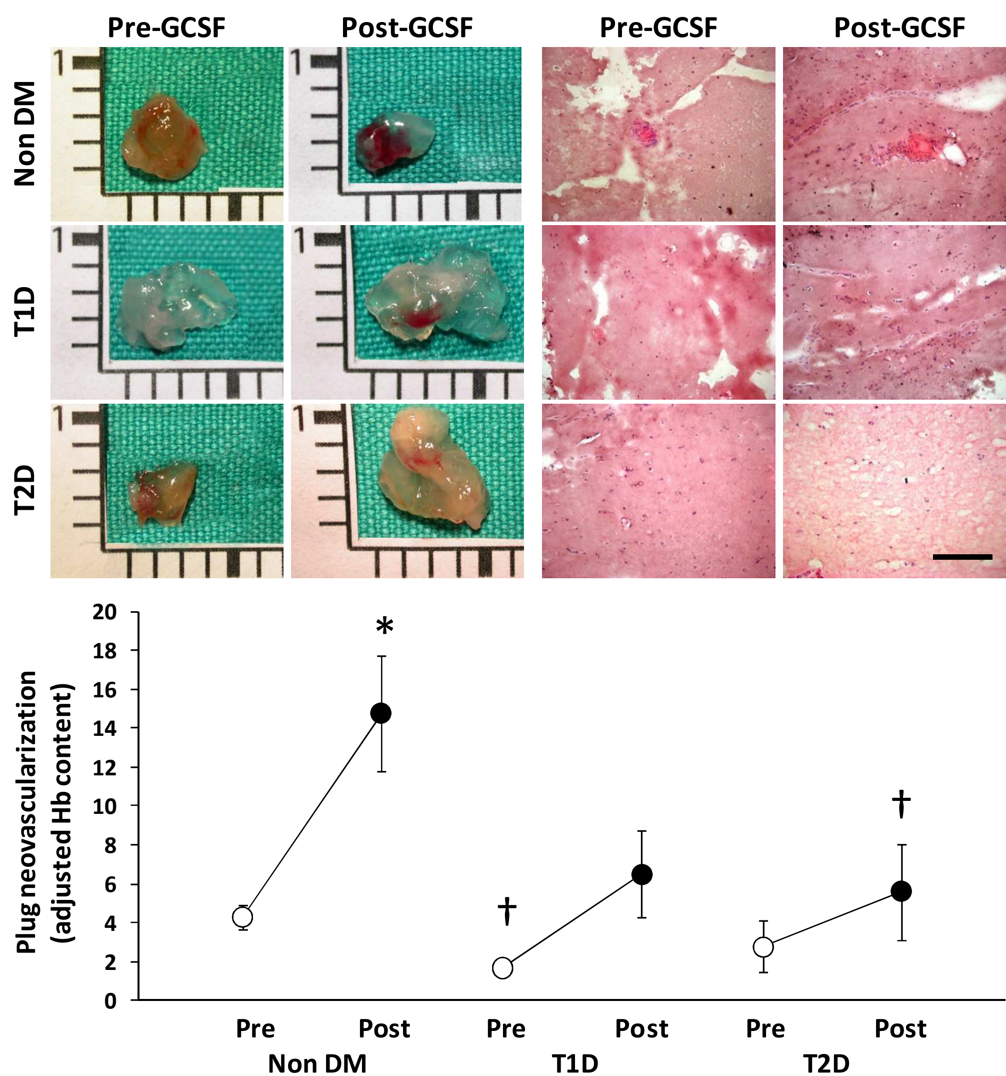


Figure 5. Pre- and post-G-CSF levels of white blood cells (WBC, A), neutrophils (B) and monocytes (C) count in DM and non DM patients. * p<0.05 versus pre-GCSF. D) Absolute changes in WBC, neurophils and monocytes: no significant differences were found between DM patients and non DM controls in the change in blood cell counts.

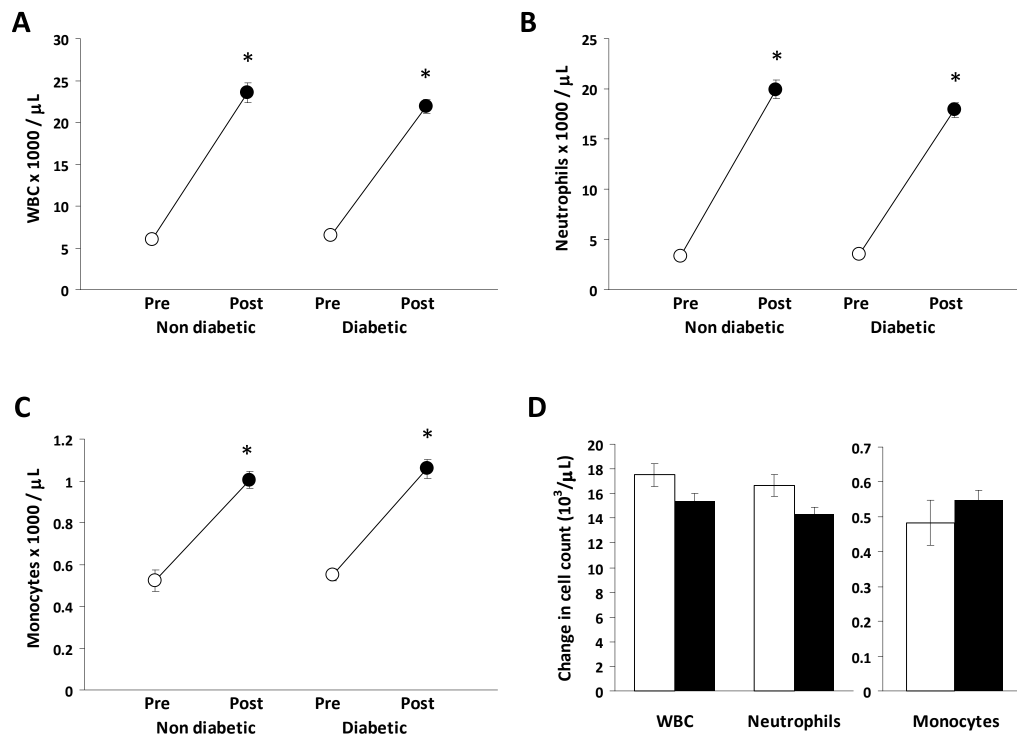


Figure 6. Significant linear correlations between Matrigel plug neovascularisation and progenitor cell levels. White circles indicate pre-GCSF; black circles indicate post-GCSF data.

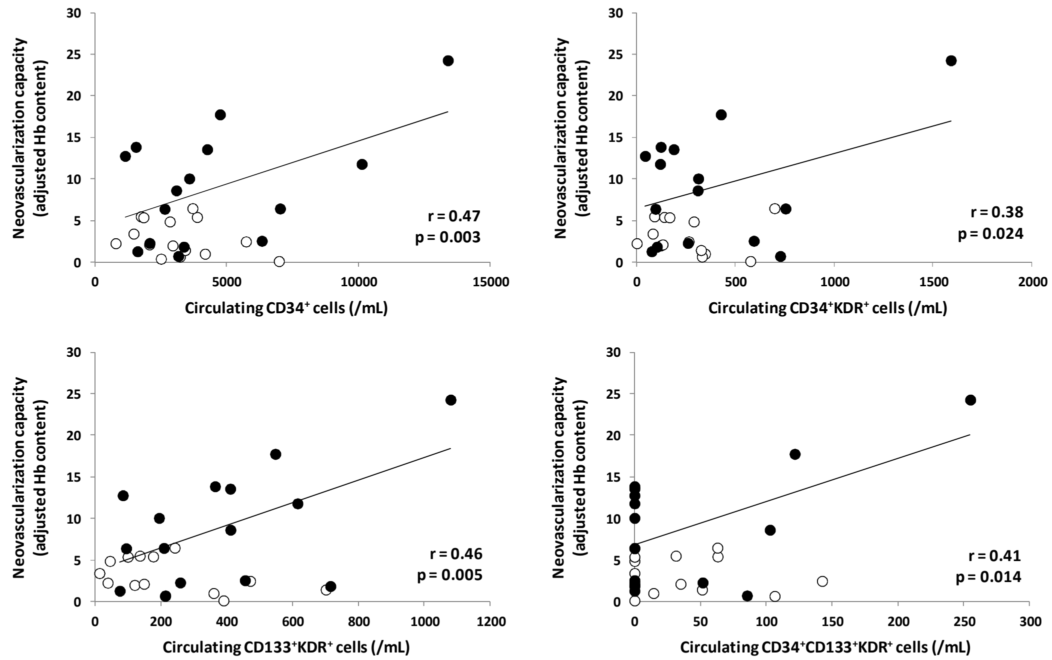


Figure 7. The PRISMA flow diagram illustrating the phases of data selection.

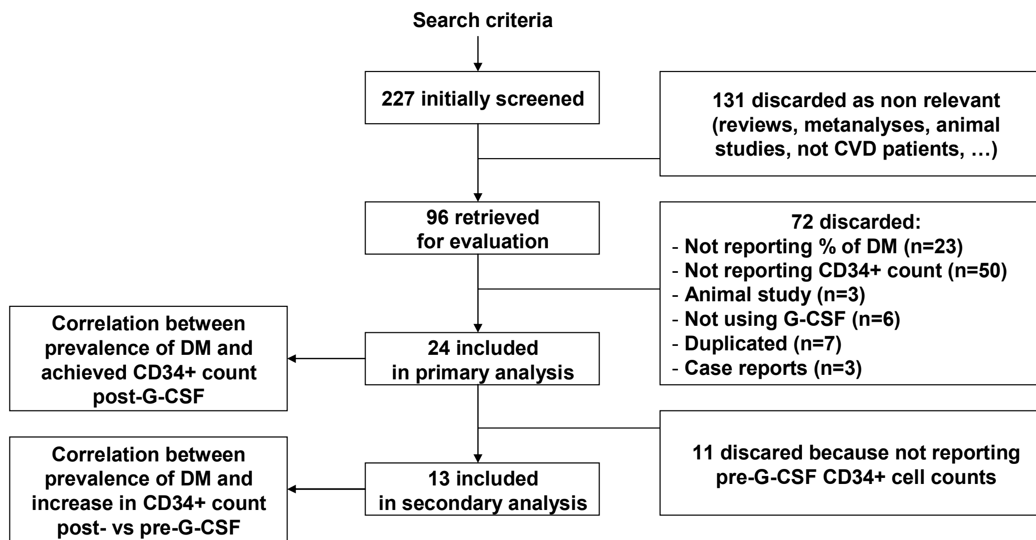


Figure 8. The itemized methodological quality graph. For each item, trials are scored as low risk of bias (complies with the item), uncertain risk of bias (not reported) or high risk of bias (does not comply with the item). The percentages of trials with low, uncertain and high risk of bias are plotted.

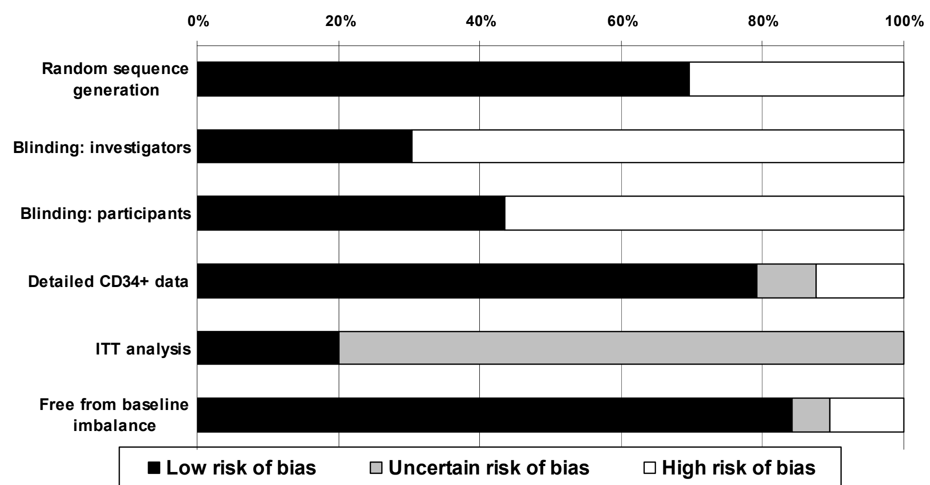


Figure 9. Plots illustrating the meta-regression between prevalence of diabetes (explanatory variable) and achieved (A) or increase (B) in absolute CD34+ cell count. Each circle represents a clinical trial included in the analysis. The area of the circles are proportional to weights of the studies. The regression line is shown with 95% confidence intervals (dashed lines).

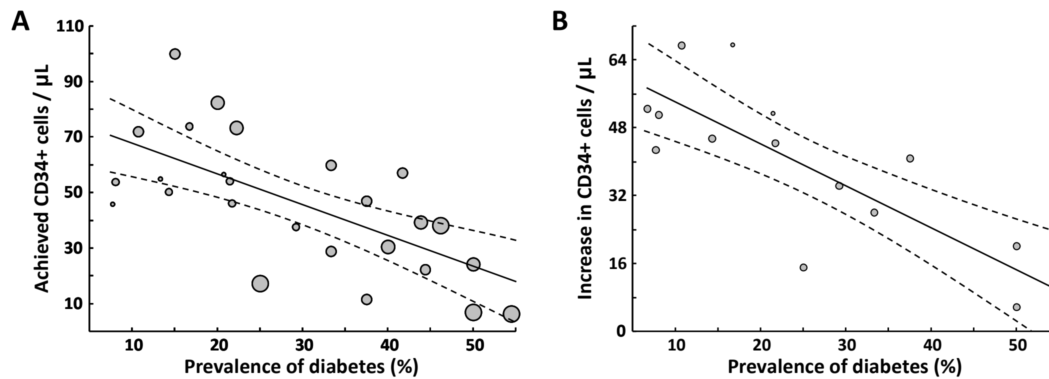
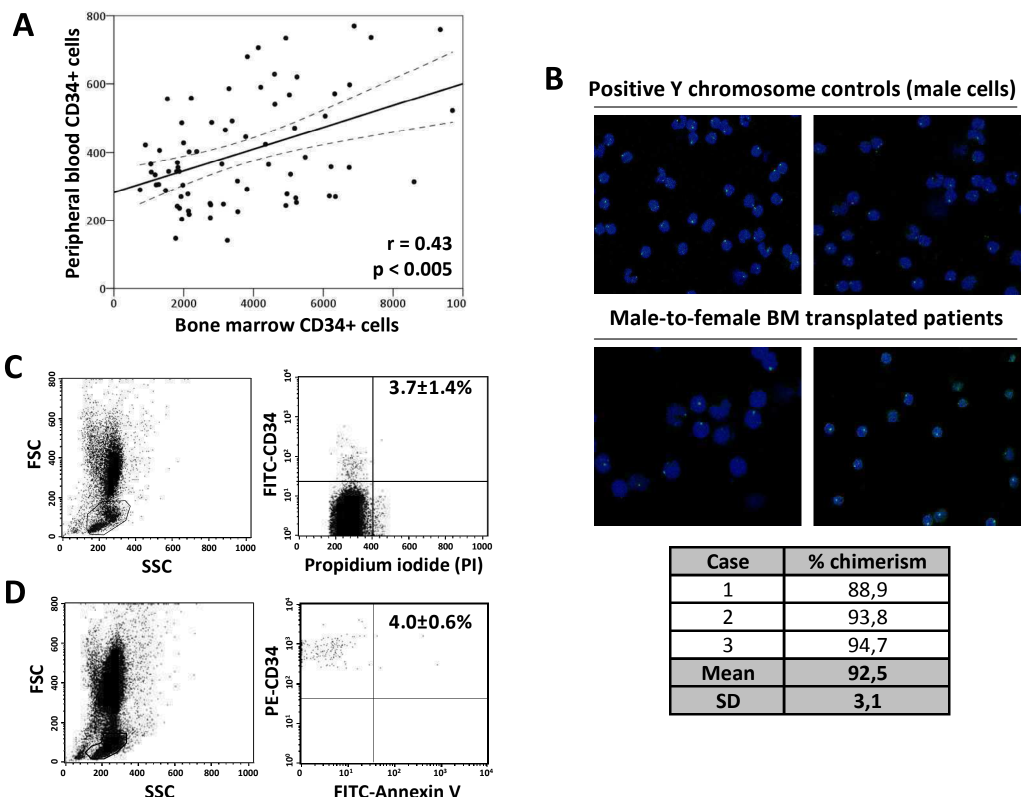
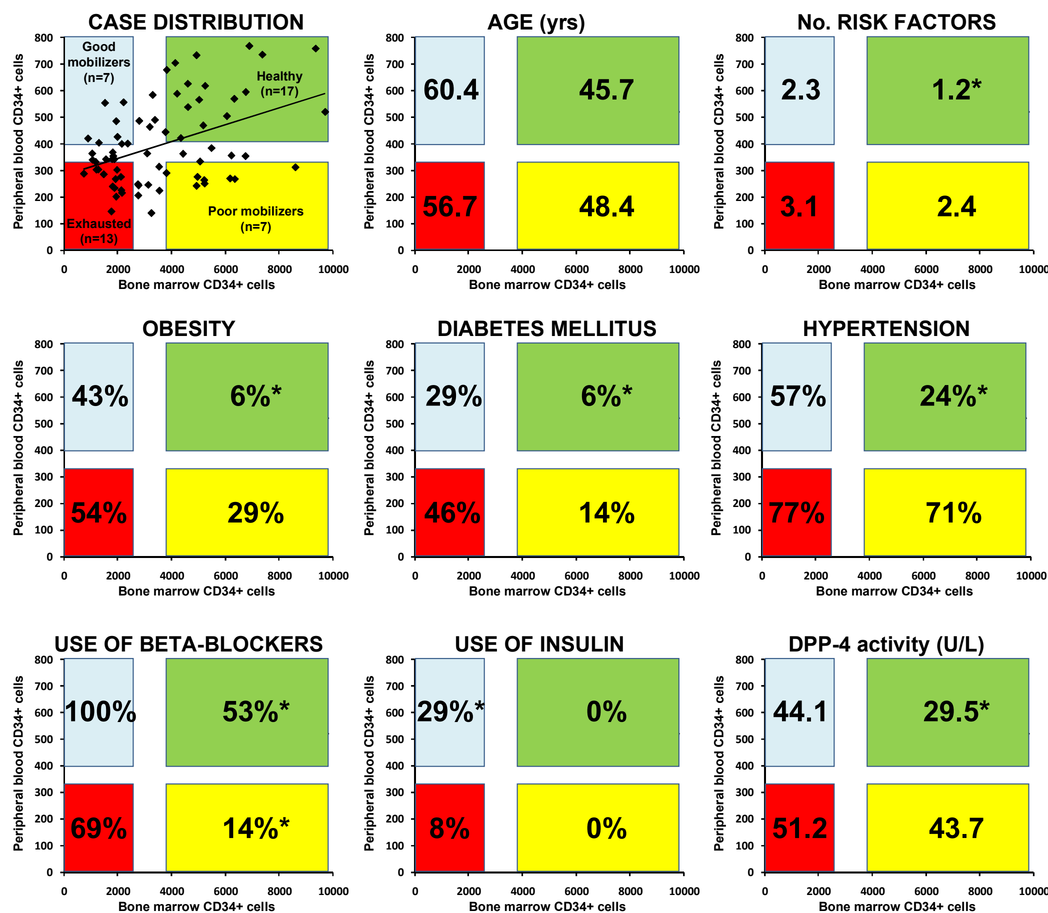


Figure 10. Most circulating CD34+ cells are viable, non-proliferating and bone marrow-derived. A) Correlation between bone marrow and peripheral blood CD34+ cell counts. Dashed lines indicate the 95% C.I. of the regression line. B) Fluorescence in situ hybridization (FISH) analysis of circulating CD34+ cell origin. The upper panels show positive controls of male cells (hybridization efficiency). The lower panels show Y-chromosome signal in sorted circulating CD34+ cells from females transplanted with a male bone marrow (zoom from 40x original magnification). The table summarizes the percentage of chimerism of 3 transplanted patients, after correcting for the Y-chromosome hybridization efficiency. C) Circulating CD34+ cells were analyzed for DNA content: the CD34 vs PI plot shows very few CD34+ cells above the PI threshold for the G2-M phase. The proliferating rate of 3 replicates is shown. D) Apoptosis of circulating CD34+ cells: the CD34 vs Annexin-V plot shows that very few CD34+ cells stained positive for the early apoptotic marker Annexin-V. The apoptotic rate of 6 replicates is shown.



Case	% chimerism
1	88,9
2	93,8
3	94,7
Mean	**92,5**
SD	**3,1**

Figure 11. Stem cell compartmentalization and the cardiovascular risk profile. Patients were divided into 4 groups according to their circulating and bone marrow CD34+ cell counts. Cut-offs for definition of groups were based on the 40° and 60° percentile of the distribution. Relevant clinical characteristics of the patients in each group are shown. *p<0.05 in post-hoc test versus the exhausted group after ANOVA or Kruskall-Wallis, as appropriate.



*** post-hoc test (ANOVA or Kruskal-Wallis p<0.05)**

Figure 12. Self-organizing maps showing clinical phenotypes associated with stem cell compartmentalization. A) Self-organizing maps colouring of clinical features in patients analyzed for stem cell compartmentalization. The colour scale indicates deviation from the population mean compared to random fluctuations that can be expected by chance. Numbers on selected hexagons indicate the prevalence (from 0 to 1) or the mean of each variable for that region. The p-values indicate the probability to observe equal colour distribution from random fluctuations. B) Topological localization of stem cell compartmentalization groups can be visually overlaid to maps in panel A to derive a representation of the clinical typical phenotype of each group. C) Each histogram of this figure represents a multivariate clinical profile in which bars can be interpreted as regression coefficients. The topological organization is the same as in the maps of panels A and B. Thus, bar plots illustrate the averaged profile for patients that reside on a given hexagon. The legends aids interpretation of the histograms: for instance, the upper-right region contains a typical patient with advanced age, diabetes, hypertension and obesity, belonging to the "exhausted" group. D) Typical phenotypes associated with stem cell compartmentalization groups are shown. Numbers in the hexagons represent sample size. The analysis was conducted in the whole cohort of 72 patient.

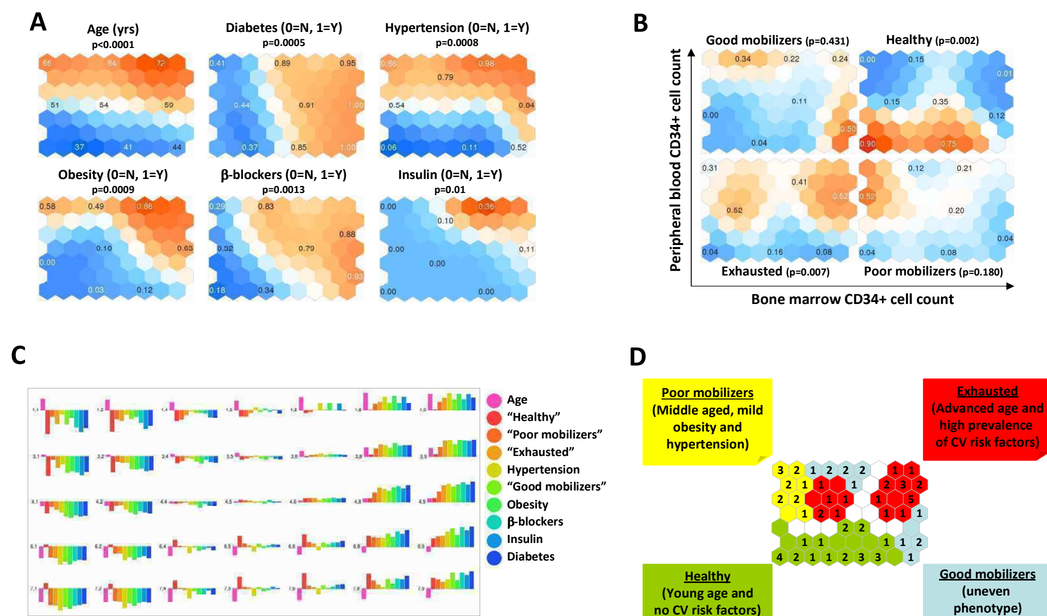


Figure 13. DPP-4 activity in diabetic and non diabetic animals. DPP-4 activity was measured in plasma and BM extracts of diabetic and non diabetic F344 rats. BM samples were also collected at the end of G-CSF stimulation. *p<0.05.

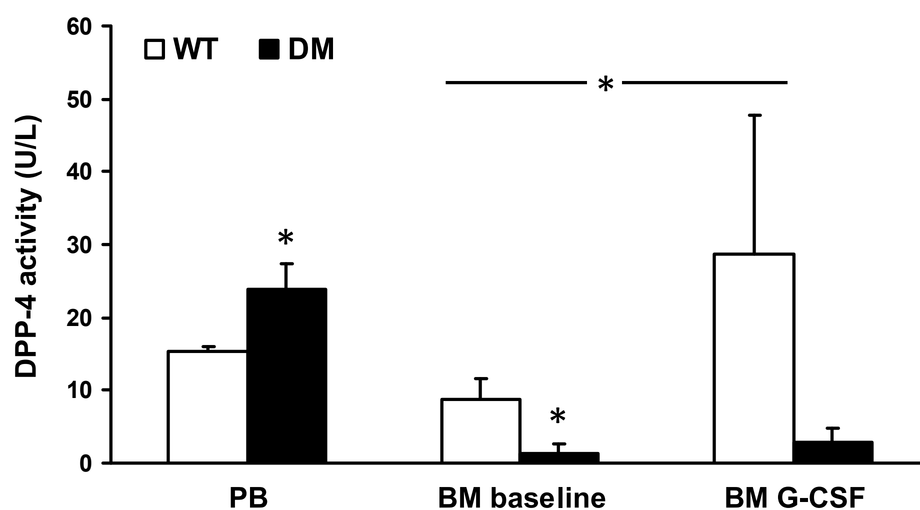


Figure 14. DPP-4 deficiency does not restore the defective G-CSF response in diabetic rats. The levels of circulating Sca-1+c-kit+ and Sca-1+CD31+ progenitor cells were determined by FACS at baseline, and after 5 day administration of G-CSF. A) The gating strategy used to enumerate Sca-1+c-kit+ and Sca-1+CD31+ cells. Data from a representative wild type (WT) non diabetic animal are shown. B) Effects of G-CSF on circulating Sca-1+c-kit+ and Sca-1+CD31+ cells in WT and DPP4null diabetic and non diabetic (control) rats. * $p<0.05$ post- vs. pre-G-CSF. Bars indicate SEM. Logarithmic scale.

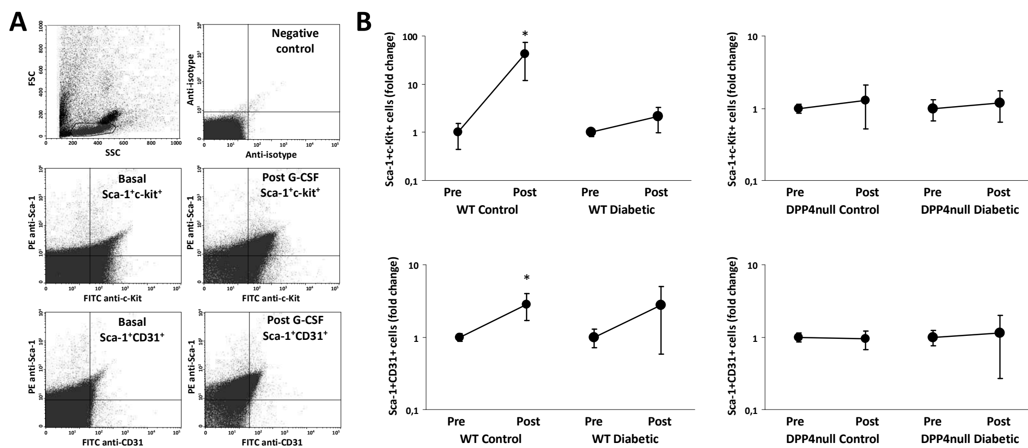


Figure 15. DPP-4 deficiency restores post-ischemic progenitor cell mobilization in diabetic rats. The levels of circulating Sca-1+c-kit+ and Sca-1+CD31+ progenitor cells were determined at baseline, day 3 and day 7 after hind limb ischemia. A) The gating strategy used to enumerate Sca-1+c-kit+ and Sca-1+CD31+ cells is shown on the left. Data from a representative wild type (WT) non diabetic animal are shown. Trends over time of circulating Sca-1+c-kit+ and Sca-1+CD31+ cells in WT and DPP4null diabetic and non diabetic (control) rats are shown on the right. * p<0.05 diabetic vs. controls. Bars indicate SEM. B) Capillary density in ischemic muscles at day 7 in wild type and DPP4null diabetic and non diabetic rats. Capillaries are stained in green with lectin (40x). C) Degree of ischemia assessed by staining with the hypoxia marker pimonidazole. Pimonidazole adducts formed in tissue areas with pO_2<10 mmHg were detected by immunohistochemistry *p<0.05 diabetic vs. non diabetic.

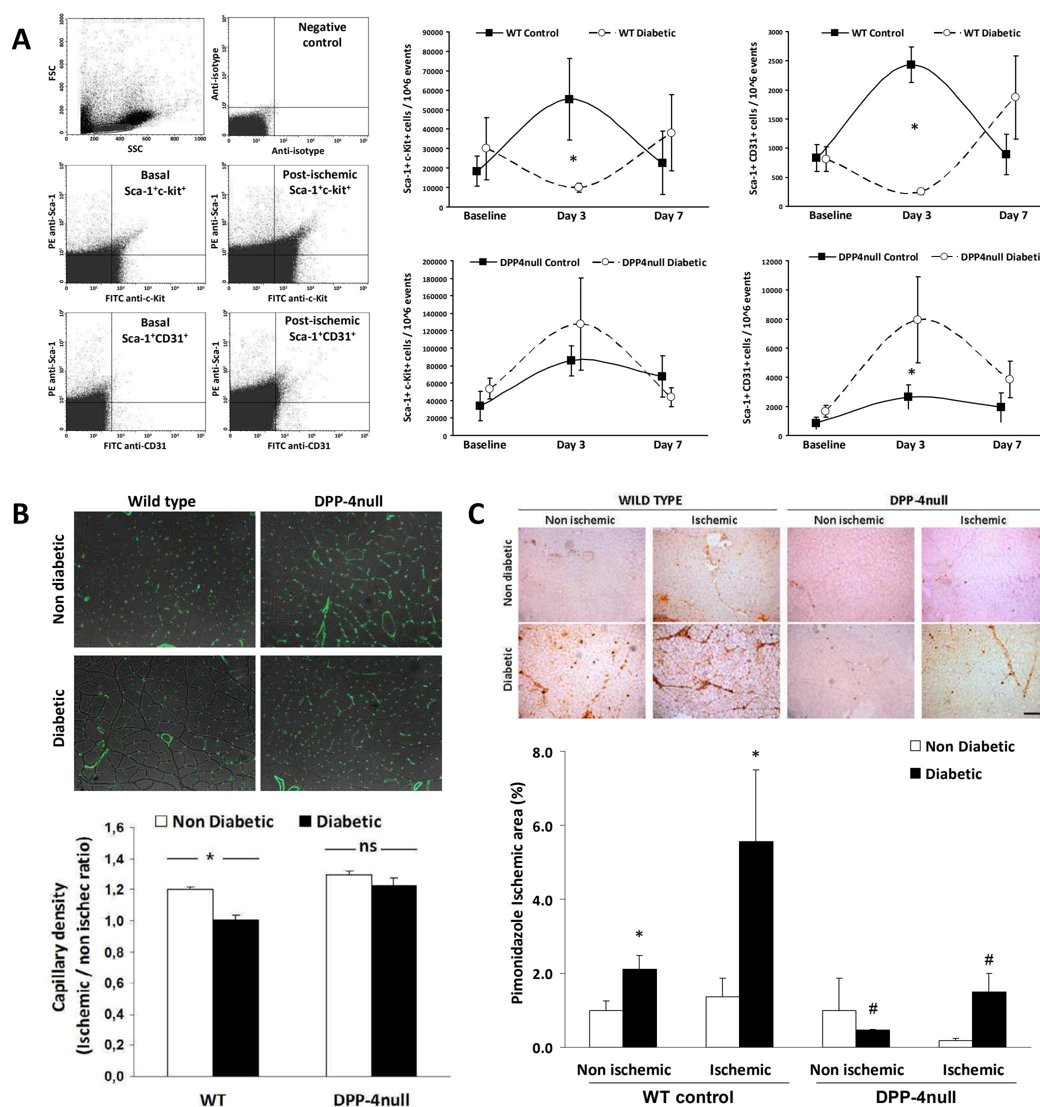


Figure 16. The role of DPP-4 in diabetic stem cell mobilopathy. Bone marrow stem cell mobilization is governed by SDF-1α gradients across the osteoblast/mesenchymal compartment and the vasculature within the niche. In the non diabetic condition, tissue ischemia upregulates circulating SDF-1α, which is partly cleaved by DPP-4. G-CSF activates bone marrow DPP-4 and reduces SDF-1α. As a result, both stimuli create a mobilizing gradient toward the vasculature. In diabetes, circulating DPP-4 activity in increased and ischemia-derived SDF-1α is reduced, thereby impairing post-ischemic mobilization. In addition, diabetes reduces DPP-4 activity in the bone marrow and prevents G-CSF from suppressing SDF-1α concentrations. Thus, tissue specific DPP-4 maladaptation in diabetes is responsible for both G-CSF and ischemia-induced impaired mobilization. DPP-4 deficiency in diabetes protects circulating SDF-1α from degradation and restores post-ischemic mobilization, but not G-CSF induced mobilization, which requires stimulation of DPP-4.

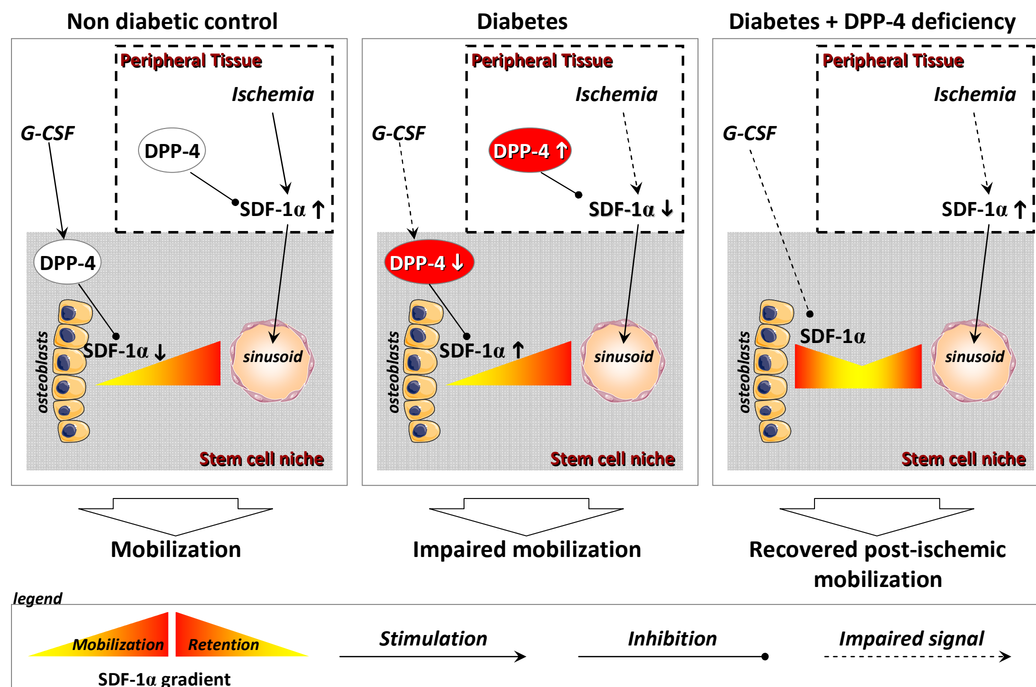


Figure 17. Self-organizing maps showing clinical phenotypes associated with stem cell compartmentalization. A) Self-organizing maps colouring of clinical features in patients analyzed for stem cell compartmentalization. The colour scale indicate the deviation from the population mean compared to random fluctuations that can be expected by chance. The numbers on selected hexagons indicate the prevalence (from 0 to 1) or the mean of each variable for that region. The p-values indicate the probability to observe equal colour distribution from random fluctuations. B) The topological localization of stem cell compartmentalization groups can be visually overlaid to maps in panel A to derive a representation of the clinical typical phenotype of each group. C) Each histogram of this figure represents a multivariate clinical profile in which bars can be interpreted as regression coefficients. The topological organization is the same as in the maps of panels A and B. Thus, bar plots illustrate the averaged profile for patients that reside on a given hexagon. The legends aids interpretation of the histograms: for instance, the bottom-right region contains a typical patient with advanced age, diabetes, hypertension and obesity, belonging to the “exhausted” group. D) The typical phenotypes associated with stem cell compartmentalization groups are shown. Numbers in the hexagons represent sample size. The analysis was conducted in the subgroup of patients defined by the 40°/60° percentile partitioning.

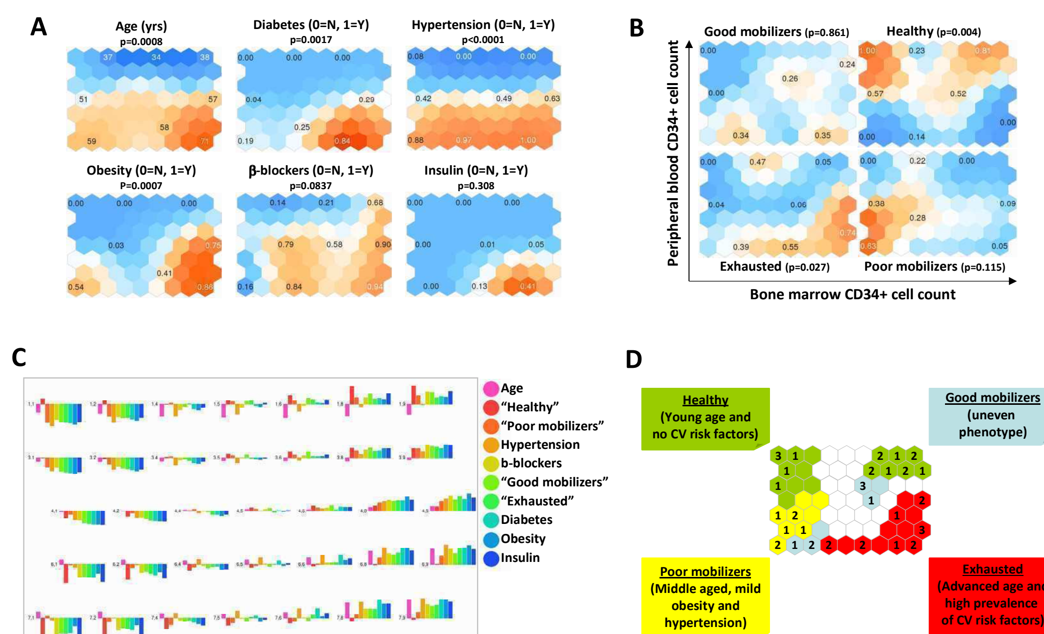


Figure 18. Self-organizing maps showing the co-localization of high DPP-4 activity with CD34+ cell compartmentalization. The right panel shows self-organizing maps colouring of the patients' CD34+ cell mobilizer phenotype. The left panel shows the corresponding SOM for DPP-4 activity, which clearly indicates co-localization of high DPP-4 activity with the exhausted phenotype and of low DPP-4 activity with the healthy phenotype.

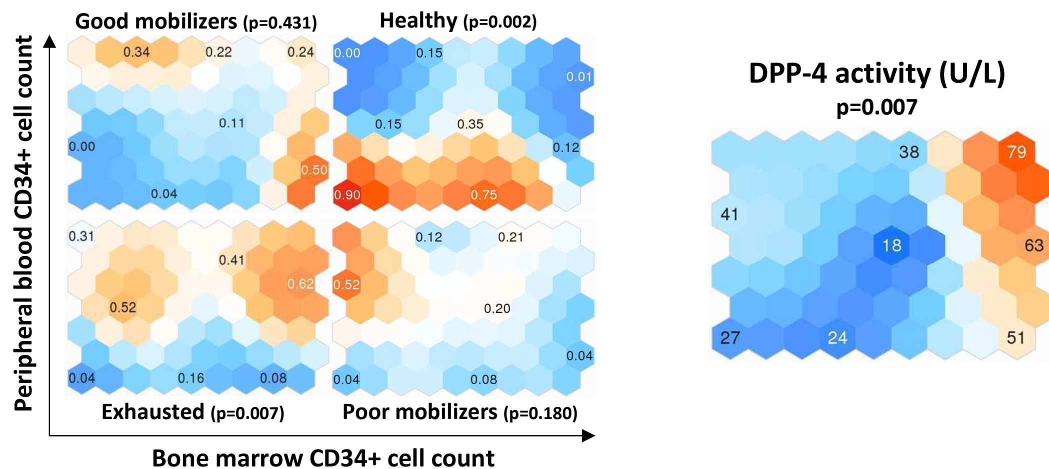


Figure 19. Assessment of skin blood flow after ischemia. Laser Doppler Imaging was used to determine superficial skin blood flow at day 7 after ischemia in diabetic and non diabetic, wild type and DPP-4null rats. Both limbs were monitored and the ischemic/non ischemic ratio is reported. There was no significant difference among groups in skin perfusion 7 days after femoral artery and vein excision, although diabetic rats tended to have a lower perfusion ratio.

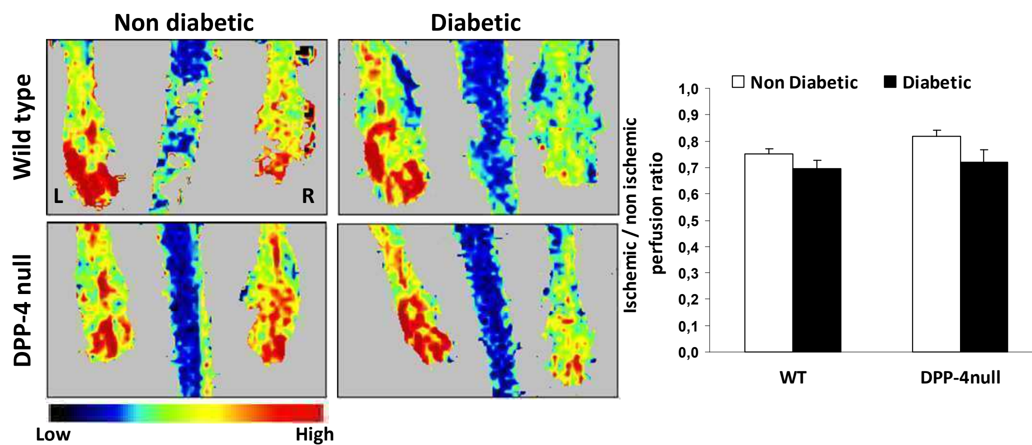
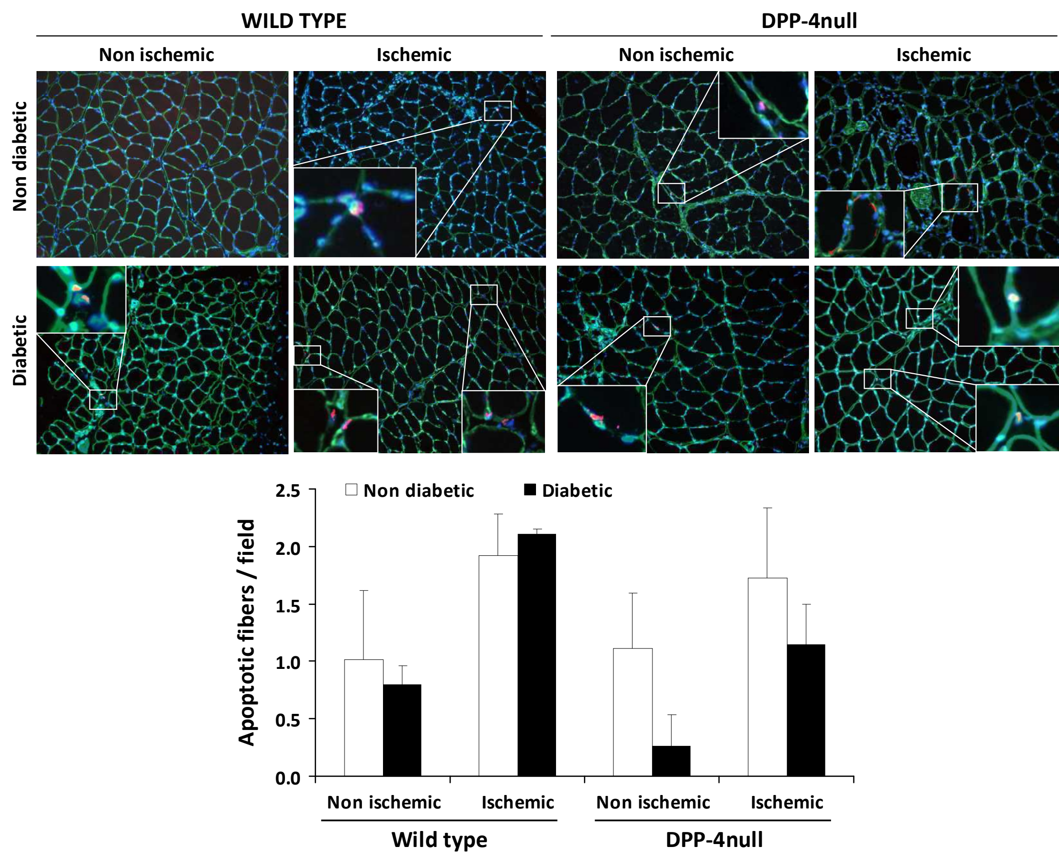


Figure 20. Apoptosis in muscle tissue. Apoptosis was evaluated with the in situ TUNEL method (ApopTag) in ischemic and non ischemic muscle sections of diabetic and non diabetic, wild type and DPP-4null rats at day 7 after induction of ischemia. Nuclei were stained in blue with Hoechst, the basement membrane was stained in green with laminin and apoptotic cells were stained in red.

The average number of apoptotic cells per section was very low and most apoptotic cells were located in the interstitium (see inserts). There was a non significant trend toward reduced apoptotic cell number in DPP-4null rats.



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