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Decreased levels of stem cell factor in subjects with incident coronary events

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Abstract

Vascular progenitor cell have been proposed to play an important role in vascular repair but their possible clinical importance in cardiovascular disease remains to be fully characterized. **Vascular endothelial growth factor A (VEGF-A), placental growth factor (PlGF) and stem cell factor (SCF) are three growth factors important in recruiting vascular progenitor cells. In the present study we investigated the association between the plasma levels of these growth factors and** incident coronary events (CE). Growth factors were measured by proximity extension assay in baseline plasma samples from 384 subjects with first incident CE (mean follow up time 14.0±4.3 years) and 409 event-free controls matched by gender and age as well as in homogenates from 201 endarterectomy specimens. Subjects in the lowest SCF tertile had a hazard ratio (HR) of 1.70 (95% confidence interval (CI) 1.14-2.54) compared with subjects in the highest SCF tertile, when controlling for known cardiovascular risk factors in a Cox regression model. Low SCF levels were also associated with more severe carotid disease, less fibrous atherosclerotic plaques and an increased incidence of heart failure. Expression of the SCF receptor c-kit could be demonstrated in the subendothelial layer and fibrous cap of human atherosclerotic plaques. Smokers and subjects with diabetes had decreased levels of SCF. To our knowledge, this is the first clinical study to support a key role for SCF and **progenitor** cells in vascular repair and we suggest that the SCF-c-kit pathway is a promising biomarker and therapeutic target in cardiovascular disease.

Most acute cardiovascular events are caused by rupture of an atherosclerotic plaque.[1] This rupture is the consequence of an imbalance between inflammatory processes degrading the plaque extracellular matrix and cellular repair responses aimed at restoring tissue integrity.[2] Experimental studies have provided evidence that vascular **progenitor** cells have a role in maintaining vascular integrity.[3, 4] These **progenitor** cells can develop into differentiated endothelial (EC) and smooth muscle cells (SMC) and have been shown to originate both from the vasculature itself as well as from the bone marrow (BM).[5-8] The adventitia has been identified as a particularly important reservoir of both endothelial and SMC progenitor cells and several studies indicate that these cells play important roles in vascular repair responses.[5] Studies in *apo* $e^{-/-}$ mice also suggest that adventitial SMC progenitor cells can contribute to the formation of atherosclerotic lesions.[5, 9] The most studied type of vascular **progenitor** cells are the endothelial progenitor cells (EPC) that are generated in the BM and recruited to tissues in response to physiological and pathological stimuli including myocardial ischemia.[7] BM-derived EPC have been demonstrated to contribute in maintaining endothelial integrity in atherosclerotic *apo* $e^{-/-}$ mice.[10] Vascular endothelial growth factor A (VEGF-A) and placental growth factor (PlGF) are growth factors that primarily promote vascular repair by enhancing recruitment of EPC.[11, 12] They have important functions in neovascularization and have been shown to play a role in the healing process after a myocardial infarction.[13-15] Stem cell factor (SCF) stimulates hematopoietic stem cells from the BM but is also important in EPC mobilization and recruitment in response to ischemia.[16, 17] The receptor for SCF, c-kit, has been detected on SMC progenitor cells in the artery wall indicating that SCF can have a functional role the vessels.[18] Presence of **progenitor** cells has also been demonstrated in human arteries,[18-20] but the understanding of their clinical importance in cardiovascular disease (CVD) remains limited. To further explore the possibility that factors that simulate vascular **progenitor** cells play a role in CVD

we determined the baseline levels of VEGF-A, PlGF and SCF in a cohort of subjects participating in the Cardiovascular Cohort of the Malmo Diet and Cancer study. During a mean follow-up period of 14.0±4.3 years in that study, 384 subjects had first- incident coronary event (CE) and we matched these cases with 409 CVD-free controls by gender and age. Subjects that suffered from CE during follow-up had lower plasma levels of SCF, whereas no differences were found for VEGF-A and PlGF between cases and controls. We show that low levels of circulating SCF are associated with incident CE and heart failure as well as with more severe carotid disease. Our findings provide the first clinical evidence for a role of SCF in protection against CVD. We also show that smokers and subjects with diabetes have lower levels of SCF which may contribute to the increased risk for CVD in this disease.

Methods

Study population

The Malmö Diet and Cancer (MDC) study is a population-based, prospective epidemiological cohort of 28 449 persons enrolled between 1991 and 1996.[21] From this cohort, 6103 persons were randomly selected to participate in the MDC Cardiovascular Cohort (MDC-CC), which was designed to investigate the epidemiology of carotid artery disease. Of MDC-CC participants, fasting plasma samples were available in 5433 subjects of whom we excluded 143 subjects who had history of MI or stroke prior to the baseline examination. During a mean follow-up time of 14.0±4.3 years, 384 first-incident coronary events (CE) occurred. A CE was defined as a fatal or nonfatal myocardial infarction [i.e. International Classification of Diseases**,** 9th Revision (ICD-9) code 410)] or death attributable to underlying coronary heart disease (ICD-9 codes 410–414).We matched incident coronary cases with CVD-free control

subjects during the follow-up based on gender and age and also required that the follow-up time of the control was at least as long as that of the corresponding incident CE case, **but did not use further propensity scoring.** For heart failure only those with a hospital diagnosis at least one day before a possible myocardial infarction were included. Hypertension was defined as blood pressure ≥140/90 mm Hg or use of blood pressure-lowering medication. Diabetes mellitus was defined as fasting blood glucose >6.0 mmol/l, self-reported physician diagnosis, or use of anti-diabetic medications. Smoking was categorized as current smokers at baseline, previous smokers and never smoked. Blood pressure, body mass index (BMI), smoking status, white blood cells (WBC), HbA1c, fasting glucose and lipoprotein lipid levels were determined as previously described.[22] The study was approved by the Regional Ethical Review Board in Lund and was conducted in accordance with the Declaration of Helsinki. All participants gave written consent.

B-mode ultrasound

Analysis of common and bulb carotid intima–media thickness (IMT) was performed using an Acuson 128 CT system with a 7-MHz transducer as described previously. [23]

Growth factor analyses

SCF, VEGF-A and PlGF were analyzed by the Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD^{96x96} reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala. The Proseek technique is based on PEA and measures 92 biomarkers simultaneously in one μL of plasma. 92 oligonucleotide labeled antibody probe pairs were allowed to bind to their respective

targets present in the plasma sample. Addition of a DNA polymerase led to an extension and joining of the two oligonucleotides and formation of a PCR template. Universal primers were used to preamplify the 96 different DNA templates in parallel. Finally, the individual DNA sequences were detected and quantified using specific primers by microfluidic real-time quantitative PCR chip (96.96, Dynamic Array IFC, Fluidigm Biomark). The chip was run with a Biomark HD instrument.[24] The CV for intra-assay variation (within-run) and inter-assay variation (between-run) VEGF-A, SCF and PlGF were 7% and 10%, 5% and 9%, and 8% and 9%, respectively. Data analysis was performed by a preprocessing normalization procedure using Olink Wizard for GenEx (Multid Analyses, Sweden). All data are presented as arbitrary units (AU). Calibrator curves to transform AU into pg/ml of the biomarker are accessible at www.olink.com/. The analytical measuring range for VEGF-A, SCF and PlGF are 0.24-15630 pg/ml, 0.12-7810 pg/ml and 1.91-31250 pg/ml, respectively.

Analysis of endarterectomy specimens

Human endarterectomy specimens were obtained from subjects participating in the Carotid Plaque Imaging Project (CPIP). The present study included 201 carotid plaques were collected. The mean age of the patients was 69.6±8.1 years and 68.7% of the subjects were males. One-hundred-seven plaques were associated with symptoms (TIA, stroke or amaurosis fugax) and had a degree of stenosis >70%, as assessed by ultrasound. Ninety-four plaques were considered asymptomatic and had a degree of stenosis >80%. Plaques were snap-frozen in liquid nitrogen immediately after surgical removal. One mm fragments, from the most stenotic region, were taken for histology and the remaining tissue was homogenized prepared as previously described.[25] SCF levels **were** analyzed by the Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD^{96x96} reagents kit as described above. For

elastin analysis the Fastin Elastin assay was used (Biocolor, Carrickfergus, Northern Ireland, UK). Plaque homogenate (30µl) were mixed with cooled Elastin Precipitating Reagent (1000µl), incubated and centrifuged (10000g, 10 min). Supernatants were discarded, Fastin Dye Reagent and 90% saturated ammonium sulfate (1000 µl+100µl) were added. Samples were mixed, centrifuged (12000g, 10 min), supernatants were discarded and Fastin Dissociation Reagent (1000µl) was added. Samples were read on a Tecan Elisa plate reader (492 nm). For determination of collagen we used the Sircol soluble Collagen assay (Biocolor, Carrickfergus, Northern Ireland, UK). Samples (5µl) were added to 995 µl of Sircol Dye reagent, mixed, incubated (30 min) and then centrifuged (10000g, 10 min). This assay detects acid-soluble and pepsin-soluble collagens types I to V. The supernatants were discarded and alkali reagent (1000µl) was added. Samples were read (absorbance 540nm) on a Tecan Elisa plate reader.

Immunohistochemistry

Immunohistochemistry for c-kit **and CD45 were** performed on carotid plaques derived from patients (n=10) who underwent carotid endarterectomy. After surgical removal, the carotid plaques were snap-frozen in liquid nitrogen and 1-mm tick fragments were cut from the most stenotic region of the plaque. Sections (8 µm thick) were fixed in acetone, permeabilized with 0.5% Triton-X, and incubated in phosphate-buffered saline (PBS) containing 3% H_2O_2 to neutralize endogenous peroxidase activity. After pre-incubation in PBS containing 10% goat serum, sections were incubated in c-kit antibody (sc-5535, Santa Cruz Biotechnology), **CD45 antibody (ab10558, Abcam)** or rabbit IgG isotype control antibody (ab172730, Abcam) diluted in PBS/10% goat serum over-night **for c-kit and control IgG or 1 hour for CD45**. After washing, the sections were incubated in biotinylated goat anti-rabbit antibody (BA-

1000, Vector Laboratories) for 1 hr. Sections were washed and developed using DAB peroxidase substrate kit, according to manufacturer's instructions (ImmPACT DAB, Vector Laboratories). After color development slides were immediately washed under tap water. Counterstaining was achieved using Mayer's hematoxylin. Slides were mounted with VectaMount (Vector Laboratories) and observed under a light microscope. Specificity of immune staining was confirmed by the absence of staining in mouse IgG isotype control sections.

Statistical analysis

Measures of skewness and kurtosis were used to test for normality. Differences between means of normally distributed continuous variables were assessed with independent sample *t* tests and differences in proportions between subjects with and without coronary events were assessed using the chi-squared test. Differences between means of non-normally distributed continuous variables were assessed using the non-parametric Mann–Whitney test. Pearson or Spearman's rank correlation coefficients were used to examine relationships among continuous variables. Differences in stem cell growth factors between different categories of smokers and non-smokers were analyzed by one-way ANOVA. The relation between SCF (in tertiles) and incidence of first CE during follow-up was assessed by Kaplan Meier survival curves and quantified by Log rank test. A Cox proportional hazards regression model was used to assess the hazard ratio (HR), and 95% confidence interval (CI) of first CE in relation to tertiles of SCF. The model included adjustment for age, sex, prevalent diabetes, systolic blood pressure, triglycerides, LDL/HDL ratio, WBC, smoking and use of statins or blood pressure-lowering medication. The proportionality of the hazards assumption was confirmed by visual inspection of log–negative log survival curves.

Results

We determined the baseline levels VEGF-A, PlGF and SCF in a cohort of subjects participating in the Cardiovascular Cohort of the Malmo Diet and Cancer study. During a mean follow-up period of 14.0±4.3 years in that study, 384 subjects had first- incident CE and we matched these cases with 409 CVD-free controls by gender and age. The clinical characteristics of the study cohort are shown in table 1.

Stem cell growth factors and incident CE

First, we focused on the relation between incident CE and levels of **the three** growth factors in plasma samples that had been obtained at baseline. Subjects who had a CE during follow up had significantly lower plasma levels of SCF at baseline than the controls, whereas no differences were found for VEGF-A and PlGF (figure 1 A-C). Kaplan-Meier plots demonstrated a linear relation between tertiles of SCF and event-free survival (log rank test $p<0.0001$, figure 1D). Subjects in the lowest SCF tertile had a hazard ratio (HR) of 1.70 (95% confidence interval (CI) 1.14-2.54) compared with subjects in the highest SCF tertile, when controlling for known cardiovascular risk factors in a Cox regression model. **Using a threshold value of 125 arbitrary units for SCF (approximately equivalent to 10 pg/mL) 61.2% of the 183 subjects below the cut off suffered from incident CE versus 44.6% in those with a SCF values above this cut off ($p<0.0001$ as determined by chi-square test). The positive predictive value (PPV) for this SCF threshold was 61.2% and the negative predictive value was 55.4%. For a comparison the PPV and NPV for an LDL cholesterol value of above 5.0 mmol/L (n=160) were 51.3 and 80.6%, respectively and for an HDL**

cholesterol value below 1.0 mmol/L (n=188) 59.9 and 56.0%, respectively. These findings suggest that subjects with high levels of SCF have a lower risk of developing acute CE.

SCF and atherosclerosis

To investigate the possible role of vascular **progenitor** cell growth factors in CVD further, we determined their association with carotid intima-media thickness (IMT), a well-established surrogate marker of atherosclerosis. We found an inverse association between SCF and the baseline IMT in the common carotid artery and the carotid bulb as measured by ultrasound (table 2). By contrast, plasma VEGF demonstrated a positive correlation with carotid bulb IMT, while no significant association were found for PlGF.

To determine if the SCF receptor c-kit is expressed on cells in human atherosclerotic plaques we stained carotid endarterectomy specimens from the Carotid Plaque Imaging Project (CPIP) for c-kit. Presence of c-kit expressing cells was found in 40% analyzed plaques (n=10) with a preferential location in the endothelial and immediate sub-endothelial layer but several cells in the fibrous cap were also c-kit positive (figure 2A-D). To explore possible associations between the plaque content of SCF and the stability of the plaque we determined SCF, collagen and elastin contents in 201 endarterectomy specimens. The entire plaque (with the exception of a 1-mm section from the most of stenotic part of the lesion which was used for histology) was homogenized and the content of elastin and collagen standardized as mg/g plaque wet weight. The plaque homogenate content of SCF was determined by PEA technology and also standardized against the plaque wet weight. The level of SCF in the plaques was found to demonstrate significant correlation with both the elastin ($r=0.38$, $p<0.000001$) and collagen ($r=0.21$, $p=0.002$) contents of the plaques (figure 2E-F). These findings show that cells expressing the receptor for SCF are present in some human

atherosclerotic plaques and provide evidence for an association SCF and less severe atherosclerotic disease.

SCF and heart failure

Vascular **progenitor** cells have also been implicated in the repair of cardiac tissue and have in some clinical studies been shown to inhibit loss of myocardial function in patients with acute myocardial infarction.[7] To explore possible associations between **SCF, VEGF-A, PlGF** and heart failure we identified subjects who developed heart failure without a prior or concomitant diagnosis of acute myocardial infarction during follow-up (n=32). Subjects with incident heart failure had significantly lower levels of SCF at baseline, whereas no differences were found for VEGF-A and PlGF (figure 3A-C). These findings imply that SCF may be of importance for maintaining cardiac function.

SCF, smoking and diabetes

The plasma level of SCF in current smokers (n=198) was 19.6% lower than in those that had never smoked (n=281) and 13.9% lower than in previous smokers (n=293, $p<0.000001$ and $p<0.000005$, respectively; supplemental figure 1). Interestingly, also the difference in SCF between those that had never smoked and previous smokers was statistically significant. Current smokers had marginally higher levels of VEGF-A than those that had never smoked (supplemental figure 1). In the present cohort, subjects with diabetes at baseline (n=109) had significantly lower plasma levels of SCF, while VEGF-A and PlGF were increased (figure 4A-C) compared with those without diabetes. Those patients who developed diabetes during follow-up (n=130) also had significantly lower plasma levels of SCF at baseline than those

without diabetes, while there were no differences in VEGF-A and PlGF (figure 4D-F). The physical characteristics and metabolic factors that are generally associated with metabolic syndrome and diabetes including high waist/hip ratio, BMI, triglycerides, fasting glucose, HbA1c and low HDL correlated with low plasma levels of SCF, whereas opposite trends were observed for VEGF-A and PlGF (table 3). The association between SCF at baseline and metabolic factors was not only found in subjects with prevalent diabetes, but also in those that developed diabetes during follow-up and in non-diabetics (table 4). There was no significant difference SCF content between plaques from subjects with (n=69) or without (n=132) diabetes (11.0±4.6 versus 12.1±5.6 AU) in the CPIP cohort.

Discussion

Vascular **progenitor** cells have been suggested to play a role in vascular repair and protection against CVD.[3, 5-8] In the present prospective nested case–control study we analyzed the association between the plasma levels of three **vascular progenitor cell** growth factors and development of CVD. We show for the first time that low levels of one of these growth factors, SCF, are associated with an increased incidence of CVD. **Using a threshold value of 125 arbitrary units/mL (corresponding to approximately 10 pg/mL (www.olink.com)) SCF had a predictive value that was in the same range as that of established risk factors such as LDL and HDL cholesterol.** Moreover, a low plasma level of SCF is associated with more severe carotid disease as assessed by ultrasonography and a low plaque content of SCF is associated with less stable plaques as determined by the amount of the major connective tissue proteins in the plaques. Although these associations do not provide evidence for a functional role of SCF they are in line with a body of evidence from studies performed on

human tissues and in experimental animal models implicating **progenitor** cells in maintenance of vascular integrity by incorporation and/or activation at sites of injury.

The role of **stem cells and vascular progenitor cells** cells in vasculogenesis is well characterized[26] while the possible function of adult stem cells in vascular repair remains to be fully elucidated. Autopsy studies on subjects that had received sex-mismatched bone marrow transplantation demonstrated that around 10% of the SMC in coronary atherosclerotic lesions were derived from the donor bone marrow and not from the recipient.[19] Moreover, studies on sex-mismatched cardiac transplantation patients have also demonstrated that significant endothelial replacement by circulating progenitor cells occurs in transplanted vessels.[27] The role of circulating stem **and progenitor** cells in endothelial cell replacement and generation of intimal SMC has been further confirmed in a number of animal organ transplantation models.[28, 29] Studies performed by Wang and coworkers[30] have demonstrated the importance of the SCF/c-kit pathway for activation of BM-derived vascular progenitor cells. Using wire-induced femoral artery injury in mice reconstituted with wild-type BM cells expressing yellow fluorescent protein they showed that more than 60% of the SMC is neointima were derived from the BM in their experimental model. Transfer of BM cells from c-Kit deficient *W/Wv* and SCF-deficient Steel–Dickie mice resulted in an almost complete inhibition of neointima formation demonstrating that BM-derived stem cells activated through the SCF/c-kit pathway differentiate into vascular SMC contributing to vascular repair. There is also evidence for the existence of resident **progenitor** cells in the vascular wall.[8] These cells can differentiate into both endothelial and SMC and are most commonly found in the adventitial layer of the vessel wall. Studies in *apo* $e^{-/-}$ mice have shown that adventitial SMC progenitor cells contribute to the formation of atherosclerotic lesions.[5, 9] The role of SCF in activation of resident vascular **progenitor** cells remains to be fully understood but the fact that many of these cells express c-kit[18, 20] suggests that at

least some resident vascular stem cell populations respond to SCF. Torsney and coworkers found a two- to three-fold increase in resident **progenitor** cells in the adventitia of atherosclerotic vessels compared with normal controls.[18] In line with the present observations they also detected presence of c-kit positive cells in about 40 to 50% of all atherosclerotic lesions. **Furthermore, SCF is an important growth factor for mast cells and melanocytes. The role of mast cells in atherosclerosis and CVD is not fully explained but the effector functions of mast cells, for example matrix degradation, increased inflammation and apoptosis, will result in increased disease [31]. However, as we show that higher plasma levels of SCF protects from CE this indicates that the protective effects mediated by SCF on other progenitor cells is dominating in CVD.** Taken together these findings demonstrate that both BM-derived and resident vascular **progenitor** cells play important roles in vascular repair and that many of these cells express the SCF receptor c-kit.

The EPC is the most studied vascular **progenitor** cell clinically.[7] It was initially described as a BM-derived CD34+VEGFR-2+ monocytic cell that differentiated into endothelial cells in cell culture.[32] It was subsequently found that only a minor CD45-CD34+KDR+ fraction (also referred to as late outgrowth EPC) of these cells were true EPC.[33] Increased levels of CD34+KDR+ EPC have been associated with a reduced risk of cardiovascular events in two prospective studies.[34, 35] Briguori and coworkers also found an inverse relationship between the progression of coronary disease as assessed by angiography and low circulating levels of EPC.[36] Several intervention trials have used EPC to stimulate angiogenesis and to preserve myocardial function in post-MI patients but the results of these studies have been inconsistent.[37] VEGF and PlGF are the most important growth factors for EPC.[11] Although SCF is known to activate EPC[17] we found no association between the most potent EPC growth factors, VEGF-A and PlGF, and incident CE suggesting that the protective effects of SCF are less likely to be mediated by EPC. Interestingly, patients that developed

heart failure independent of a previous or concomitant CE during follow-up had significantly lower levels of SCF at baseline. This could suggest a role for resident c-kit+ cardiac progenitor cells in protection against heart failure. Recent experimental data suggest that the ability of these cells to differentiate into cardiomyocytes is low while they amply generate cardiac endothelial cells.[38]

It is well documented that medial SMC de-differentiate to a synthetic phenotype and migrate into the intima during early stages of atherosclerosis.[39, 40] These SMC contributes to plaque growth but serves an important stabilizing function by forming a fibrous cap covering the lipid deposits and necrotic core of the plaque.[1] In advanced lesions, the risk for rupture increases when SMC dies or fails to replicate. The source of new SMC in the fibrous cap of advanced lesions remains to be fully characterized but is likely to involve a combination of proliferation of media-derived synthetic SMC as well as resident and BM-derived stem cells. Interestingly, also mature SMCs can express c-kit in response to injury[41] and studies have shown SCF-c-kit pathway protects SMC from apoptotic cell death and stimulates neointimal SMC proliferation.[42] Thus, it cannot be excluded that the present observation of an association between high plasma SCF levels and lower incidence of CE reflects an effect of SCF on non-**progenitor** cells in the vasculature.

An additional novel finding that has come out of the present study is that both smoking and diabetes are associated with reduced levels of SCF in the circulation. Also previous smokers had significantly reduced levels of SCF as compared with those that never had smoked although the difference was markedly smaller than between current smokers and those that never had smoked. It is an interesting possibility that lower levels of SCF in the circulation may result in an impaired vascular repair capacity both in smoking and diabetes. In a recent study on plaques from subjects participating in the CPIP cohort we found evidence suggesting that atherosclerotic plaques in subjects with diabetes type II are more prone to rupture due to

impaired repair responses rather than to increased vascular inflammation.[43] In particular, plaques from subjects with diabetes had markedly reduced levels of collagen and elastin. However, although we found highly significant associations between SCF and the plaque content of both elastin and collagen in the present study there was no significant difference in the SCF content between plaques from subjects with or without diabetes. The mechanisms responsible for the reduced plasma levels of SCF in smoking and diabetes remain to be characterized and need to be addressed in future experimental studies. In subjects with prevalent diabetes the plasma SCF level correlated inversely with fasting glucose and HbA1c while a positive correlation was seen with HDL suggesting an association between SCF and hyperglycemia and dyslipidemia. Interestingly, the same associations were found also in subjects without diabetes adding further support to the notion that metabolic factors may influence the expression of SCF.

There are some limitations of the present study that need to be considered. Most importantly observational studies like the present one can identify associations but not causality. Thus, our study does not provide direct evidence for a role of SCF or vascular stem cells in CVD. **It is also a limitation that variations in the levels of SCF, VEGF-A and PlGF over time cannot be determined using the present study sample.** Moreover, the plasma samples analyzed had been stored at -80ºC for about 15 years and there is limited information regarding the stability of stem cell growth factors during long-term storage. However, it is unlikely that the differences in SCF levels between subjects with and without incident CE reported here could be explained by storage artifacts.

In conclusion, the present study add clinical support to previous experimental studies suggesting an important role of vascular stem cells and other c-kit expressing cells in the vasculature in protection against CVD. Our findings also show these processes may be

impaired in diabetes and by smoking. Activation of the SCF-c-kit pathway represents a potential novel target for prevention and treatment of CVD.

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Authorship

M.W. analyzed data, performed research and wrote the paper, S.R. analyzed data, K.H. performed research, E.B., H.B., GN.F., I.G. designed the research, B.H., A.S., performed research and designed research, J.N. performed research, designed research and wrote the paper.

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Conclict of interest statement

None of the authors has any conflict of interest to declare

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Figure 1. Plasma levels of SCF, VEGF-A and PlGF at baseline and event-free survival time of tertiles of SCF.

Plasma levels of SCF, VEGF-A and PlGF were determined at baseline in 384 subjects with incident CE (cases) and 409 matched controls by the PEA technique using the Proseek Multiplex CVD^{96x96} reagents kit (A-C). Kaplan-Meier survival curves for tertiles of SCF shows associations between tertiles of SCF in plasma at baseline and development of a CE (fatal or nonfatal) during follow up and were calculated by a Log Rank test (D). Number of patients in tertile 1, 2 and 3 were 152, 123 and 109 respectively. Box plots show median values and values within the 25th and 75th percentiles. Whiskers show values within the 5th and 95th percentile. Significance was calculated by using the t-test.

Figure 2. Immunohistochemical detection of c-kit in human atherosclerotic plaquse.

c-kit immunopositive cells are present in atherosclerotic intima (A). Higher magnification of cells staining positive for c-kit (B). IgG isotype control tissue section is devoid of c-kit positive cells (C). Higher magnification of IgG isotype control staining (D). For all images (A-D); c-kit = brown, Mayer's hematoxylin counterstain = blue. Scale bars: 20 μm. Correlations between level of SCF in plaques from the CPIP cohort and the elastin (E) and collagen (F) contents of the plaques.

Figure 3. Plasma levels of SCF in smokers and SCF, VEGF-A and PlGF in prevalent and incident diabetes.

SCF was determined in plasma at baseline in 198 current smokers (A) and in 293 previous smokers and (B) and were compared with 281 subjects that never smoked. SCF, VEGF-A and PlGF were determined in plasma at baseline in 109 diabetics and 684 non-diabetic controls (C-E) and in 633 controls and 130 subjects who developed diabetes during follow up (F-H). Box plots show median values and values within the 25th and 75th percentiles. Whiskers show values within the 5th and 95th percentile. Significance was calculated by using the t-test. n.s., not significant.

Table 1. Baseline Clinical Characteristics of Study Cohort

	Controls (n=409)	Cases (n=384)
Age (y)[a]	61.6±5.3	61.4±5.4
Gender (%male)[b]	59.9	59.9
BMI[a]	25.4±3.8	26.1±4.0*
Current smoker (%)[b]	21.1	30.5***
Diabetes (%)[b]	8.3	20.2***
Hypertension (%)[b]	42.1	51.0*
Medication (%)		
Anti-diabetic[b]	1.2	7.3***
Lipid lowering[b]	3.9	7.0
Blood pressure lowering[b]	37.4	41.7
Laboratory parameters		
Triglycerides (mmol/L)[a]	1.2±0.7	1.4±1.0**
HDL (mmol/L)[a]	1.3±0.4***	1.2±0.4
LDL (mmol/L)[b]	4.1±1.0	4.4±1.0**
LDL/HDL ratio[a]	3.3±1.2	3.7±1.3***
Systolic BP (mm Hg)[a]	140.0±18.3	150.0±18.9***
Diastolic BP (mm Hg)[a]	86.0±9.2	90.0±9.4***
HbA1c (%)[a]	4.8±0.7	4.9±1.3***
Fasting venous blood glucose (mmol/L)[c]	5.2±1.6	5.8±2.3***
WBC (x 10^9 cells/L)[a]	5.7±1.6	6.4±1.8***

[a]) Mann-Whitney U test, [b]) χ^2 test for categorical data or [c]) t-test.

Diabetes is defined as history of diabetes, anti-diabetic medication or fasting glucose ≥6.1mmol/L. Hypertension is characterized as BP>159/94 mmHg or treatment for high blood pressure. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1c, glycosulated hemoglobin A1c; WBC, white blood cell counts; BP, blood pressure;

*p≤0.05, **p≤0.01, ***p≤0.001

Table 2. Correlations between IMT and SCF, VEGF-A and PlGF at baseline.

	Mean IMT CCA	Maximal IMT bulb
SCF	r=-0.08*	r=-0.131**
VEGF-A	r=-0.009 n.s.	r=0.087*
PlGF	r=0.011 n.s.	r=0.045 n.s.

IMT, inima media thickness; CCA, common carotid artery; n.s., non-significant.
*p≤0.05, **≤0.01

Table 3. Correlations between metabolic factors and SCF, VEGF-A and PlGF levels in plasma at baseline

	SCF	VEGF-A	PlGF
Fasting blood glucose (mmol/L)	r=-0.23**	r=0.09*	n.s.
HbA1c (%)	r=-0.17**	r=0.14**	n.s.
Triglycerides (mmol/L)	r=-0.22**	r=0.22**	r=0.16**
HDL (mmol/L)	r=0.28**	r=-0.14**	r=-0.18**
Waist hip ratio (index)	r=-0.19**	r=0.08*	r=0.14**
BMI	r=-0.17**	r=0.17**	r=0.11**

HbA1c, glycosulated hemoglobin A1c; HDL, high density lipoprotein; BMI, body mass index, n.s.; non-sognificant

*p≤0.05, **≤0.01

Spearman correlations

Table 4. Correlations between diabetes status and SCF levels in plasma at baseline

	Prevalent diabetes	Incident Diabetes	Non-diabetics
Fasting blood glucose (mmol/L)	r=-0.28**[a]	r=-0.21*[b]	r=-0.14**[a]
HbA1c (%)	r=-0.24**[a]	r=-0.21*[b]	r=-0.09*[b]
Triglycerides (mmol/L)	r=-0.112 n.s.	r=-0.22*[b]	r=-0.2**[a]
HDL (mmol/L)	r=0.310**[a]	r=0.33**[a]	r=0.24**[a]
Waist hip ratio (index)	r=-0.25**[a]	r=-0.25**[a]	r=-0.14**[a]
BMI	r=-0.21*[b]	r=-0.3**[a]	r=-0.12**[a]

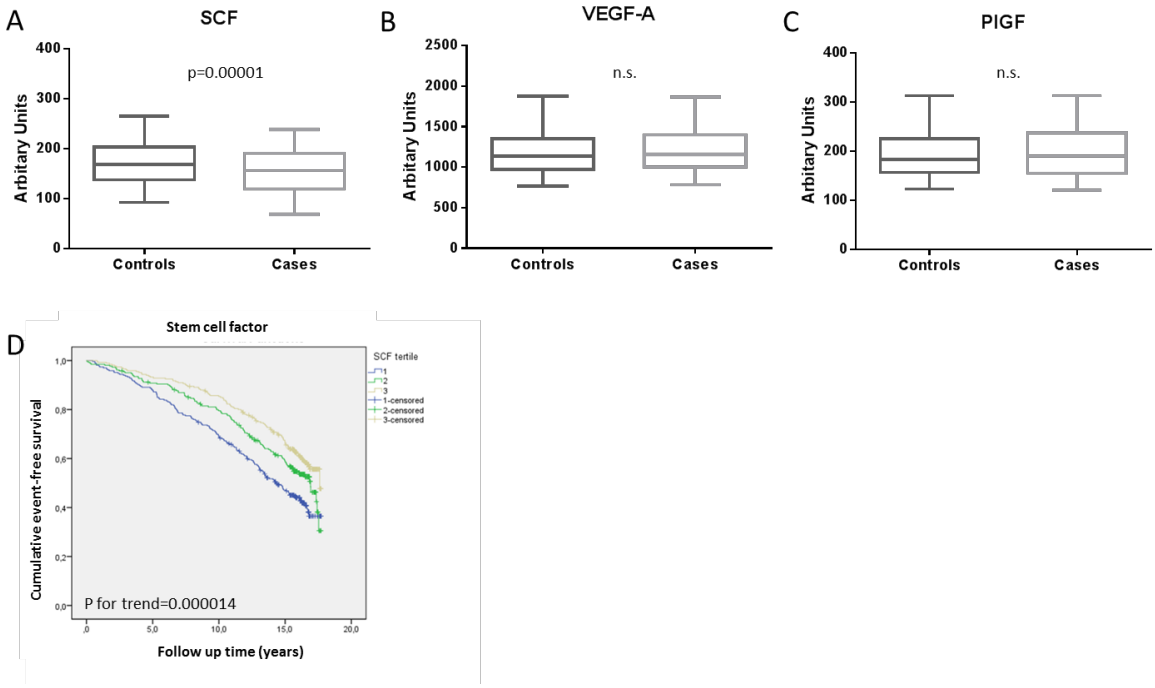
HbA1c, glycosulated hemoglobin A1c; HDL, high density lipoprotein; BMI, body mass index, n.s.; non-significant

*p≤0.05, **≤0.01

a) Spearman correlations to SCF in plasma

b) Pearson correlations to SCF in plasma

Figure 1


A
SCF
p=0.00001
Arbitary Units
400
300
200
100
0
Controls
Cases
B
VEGF-A
n.s.
Arbitary Units
2500
2000
1500
1000
500
0
Controls
Cases
C
PIGF
n.s.
Arbitary Units
400
300
200
100
0
Controls
Cases

D
Stem cell factor
SCF tertile
1
2
3
1-censored
2-censored
3-censored
Cumulative event-free survival
1,0
0,8
0,6
0,4
0,2
0,0
P for trend=0.000014
,0
5,0
10,0
15,0
20,0
Follow up time (years)

Figure 2

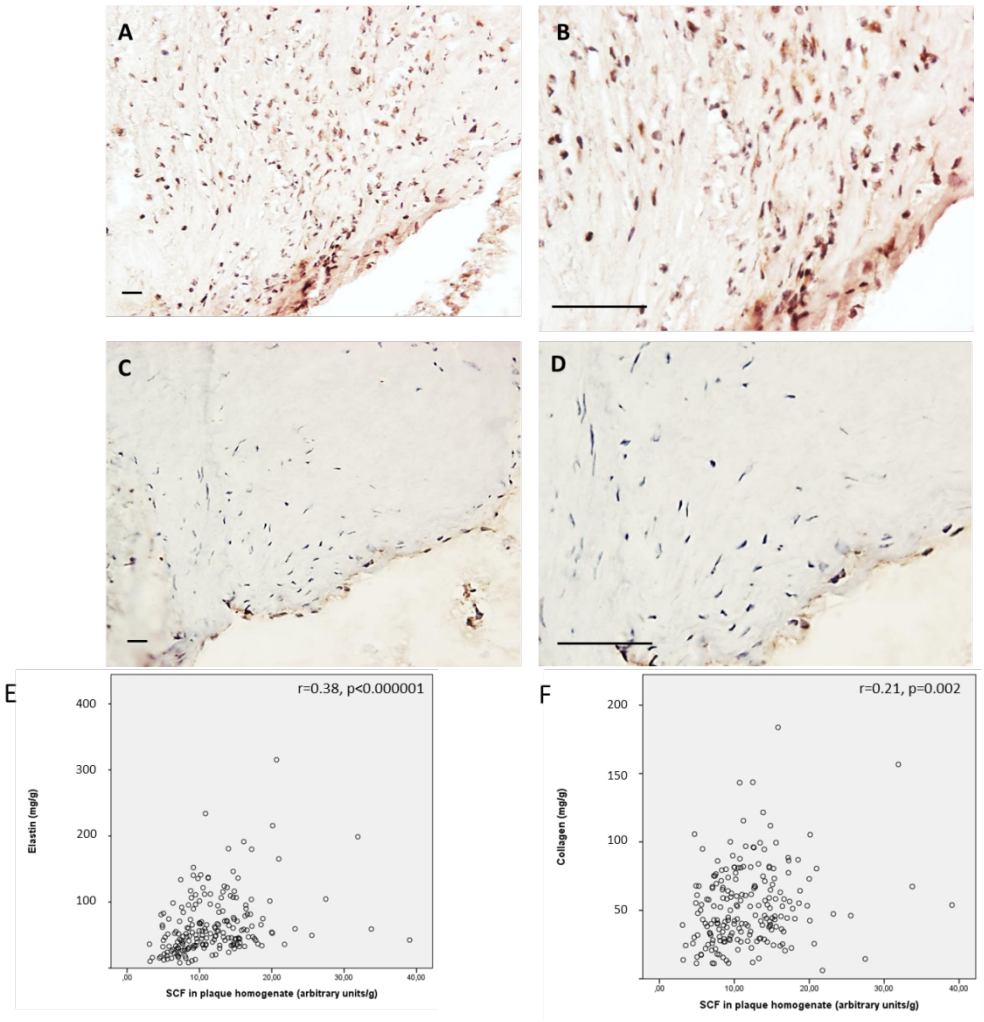
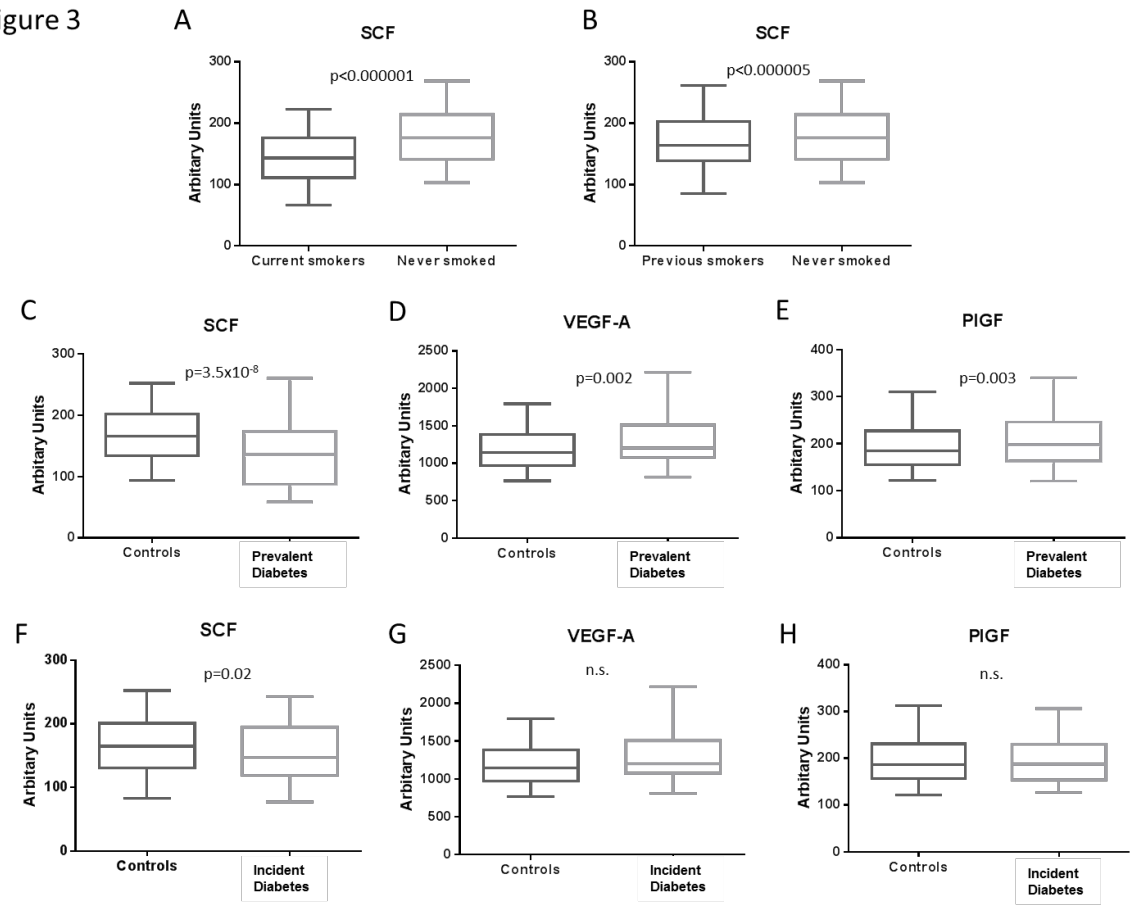
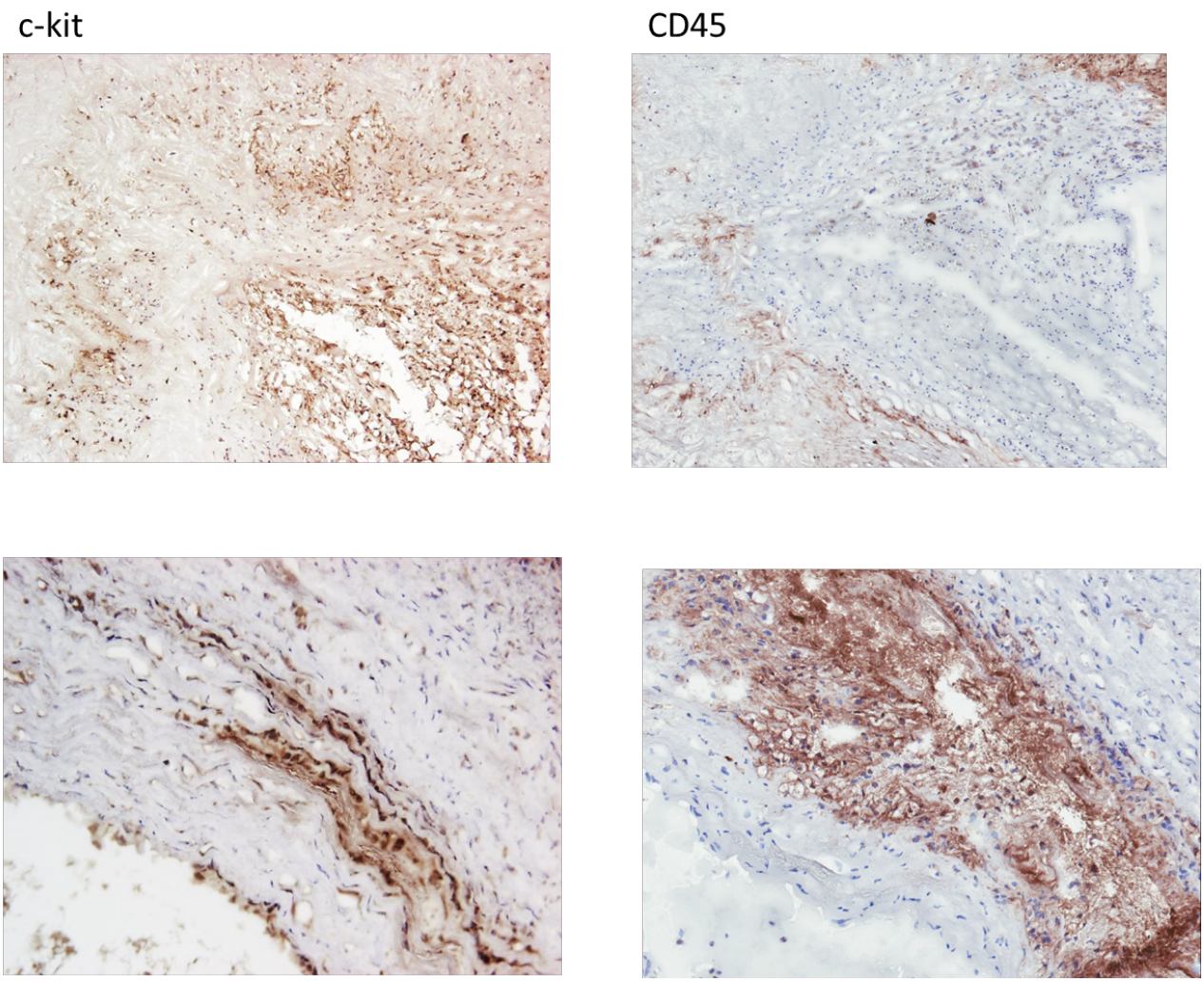

A
B
C
D
E
r=0.38, p<0.000001
Elastin (mg/g)
400
300
200
100
,00
10,00
20,00
30,00
40,00
SCF in plaque homogenate (arbitrary units/g)
F
r=0.21, p=0.002
Collagen (mg/g)
200
150
100
50
,00
10,00
20,00
30,00
40,00
SCF in plaque homogenate (arbitrary units/g)

Figure 3


A
SCF
p<0.000001
Arbitary Units
Current smokers
Never smoked
B
SCF
p<0.000005
Arbitary Units
Previous smokers
Never smoked
C
SCF
p=3.5x10-8
Arbitary Units
Controls
Prevalent Diabetes
D
VEGF-A
p=0.002
Arbitary Units
Controls
Prevalent Diabetes
E
PIGF
p=0.003
Arbitary Units
Controls
Prevalent Diabetes
F
SCF
p=0.02
Arbitary Units
Controls
Incident Diabetes
G
VEGF-A
n.s.
Arbitary Units
Controls
Incident Diabetes
H
PIGF
n.s.
Arbitary Units
Controls
Incident Diabetes

Supplemental Figure



Supplemental figure. c-kit and CD45 staining from consecutive sections from two different endarterectomy samples.